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DEFENSE CHEMISTRY OF *Stenus comma* (COLEOPTERA: STAPHYLINIDAE). LXI.[1,2]

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Abstract—The staphylinid *Stenus comma* secretes from its pygidial defensive glands at least four compounds which could be characterized as 1,8-cineole, 6-methylhept-5-en-2-one, isopiperitenol, and *N*-ethyl-3-(2-methylbutyl)piperidine.

Key Words—*Stenus comma*, staphylinid, pygidial defensive glands, 1,8-cineole, 6-methylhept-5-en-2-one, stenusin, spreading pressure, surface-active agents.

INTRODUCTION

Stenus comma is a small black staphylinid with a tiny yellow spot on each of its wings. The slim beetle which is about 5 mm in length lives at river banks in the immediate neighborhood of water and has adapted itself perfectly to this environment. Not only does it crawl on water, but in case of danger it jets towards the bank and safety with a speed of 45–75 cm/sec and without using its legs (Wigglesworth, 1964). To produce this elegant movement the beetle immerses the tip of its abdomen into the water and emits from two pairs of pygidial defensive glands substances that propel it in the same way as a toy boat is propelled by camphor. A thin film with a faint odor suggesting a mixture of an amine with peppermint essence is left on the water surface.

On dissection of *Stenus comma* and investigation of the pygidial defen-

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[2] Part LX, H. Schildknecht, J. Connert, H. Essenbreis and N. Orfanides, Das Spreitungsalkaloid Stenusin aus dem Kurzflügler *Stenus comma* (Coleoptera: Staphylinidae) *Z. Angew. Chem.* 87:421 (1975).

sive glands it was found that the crushed smaller glands release the peppermint scent and the larger glands the odor of an amine (Schildknecht, 1970). On the basis of the physical phenomenon of the staphylinid's movement one has to assume that at least one of the secretion compounds is a strong surface-active agent.

From the smaller glands we have isolated 1,8-cineole, 6-methylhept-5-en-2-one, and an isopiperitenol. From the larger glands we obtained an aliphatic amine which we named stenusin. Stenusin was characterized as *N*-ethyl-3-(2-methylbutyl)piperidine (X). The compound exhibits high spreading ability and is the actual agent which propels the beetle.

METHODS AND MATERIALS

General

Mass analyses were obtained on CH 4, SM 1A, and 311 mass spectrometers, Varian MAT Bremen, at 70 eV. NMR spectra were recorded using either a Varian CH 100 or HR-SC-220 instrument. IR spectra were recorded on a Perkin-Elmer 621 instrument, either as neat film or in CS_2 solution. Gas chromatographic analyses were carried out on an Aerograph 1520, Varian Aerograph, equipped with a flame ionization detector and a thermal conductivity detector. N_2 or He were used as carrier gas. Specific rotations were obtained on a Perkin-Elmer Polarimeter 141. Surface tension and interfacial tension measurements were carried out with a tensiometer commercially available from Krüss Company, Hamburg. Surface-pressure measurements were carried out on a Langmuir film balance provided by Prof. Dr. H. Kuhn, Göttingen. Spreading-velocity measurements were made with the aid of a Bolex film camera and a Lytax analyzer.

Collection and Storage of Animals

Stenus comma were collected near the village Ketsch on the Rhine. The animals were narcotized with ethyl acetate, frozen in liquid nitrogen, and stored in a deep freezer.

Gas Chromatographic Analyses

A beaker was filled with water, and 35 living insects were put on the surface of the water. They left a film which was extracted with ether. Gas chromatography of the concentrated ether solution on an Emulphor 0 column, 1.8 m × 3.2 mm (steel), loading 5% on Embacel, 100–120 mesh, temperature: 19 min at 80°C then programmed 4°/min to 105°, 30 ml N_2/min,

injector 140°, detector 140°, showed five fractions G1–G5. The glands of 20 insects were excised and the larger glands were separated from the smaller glands. The secretion was sucked out of the glands with fine glass capillaries and subjected to gas chromatographic analysis. The larger glands contained fractions G2 and G3, and the smaller G1, G4, and G5.

Isolation

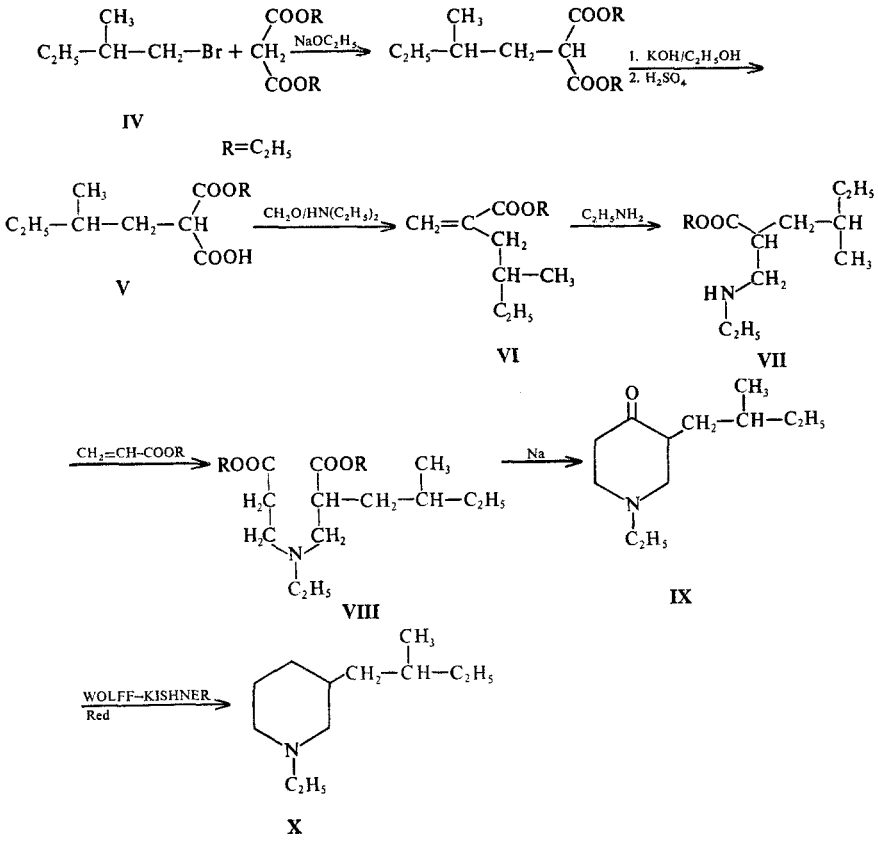
For isolation of the fractions 1000 insects were subjected to distillation in a short still at 0.1 mm and 80°C. The distillate was collected in traps cooled with liquid nitrogen. The secretion was extracted from the distillate with benzene, concentrated by ice zone melting, and fractionated by GC with multiple injections on the same column and under the same conditions as in the GC analysis, using an effluent splitter. The fractions were collected in U tubes cooled with CO_2/acetone.

Synthesis

N-Ethyl-3-(2-methylbutyl)piperidine (X) was synthesized by the following reaction sequence (Scheme I): 1-bromo-2-methylbutane, prepared from (−)-2-methylbutan-1-ol and phosphorus tribromide, reacted with diethyl malonate to give diethyl 2-methylbutylmalonate. The diester was converted to the monoester which gave ethyl 2-(2-methylbutyl)acrylate in a Mannich reaction. Addition of ethylamine produced *N*-ethyl-α-(2-methylbutyl)-β-alanine-ethyl ester which reacted with acrylic acid to give *N*-ethyl-2-(2-methylbutyl)-3,3′-iminodipropionic acid-diethyl ester. Dieckmann cyclization, saponification, and decarboxylation led to *N*-ethyl-3-(2-methylbutyl)-piperidine-4-one which yielded *N*-ethyl-3-(2-methylbutyl)piperidine (X) in a Wolff–Kishner reduction. GC analysis of (X) showed only a single sharp peak. (Column: 4 m × 2 mm, 20% Pennwalt 223, 4% KOH on Gaschrom R. Temperature: column, 190°; injector, 220°; detector, 220°. Carrier gas: 35 ml He/min.

RESULTS AND DISCUSSION

Gas chromatography (Figure 1) of the pygidial gland secretion produced three main components, G1, G2, G3, and two further components in lower concentration, G4 and G5. Comparison with the gas chromatogram of the secretion of the larger glands and the smaller glands revealed that fractions G2 and G3, which smelled like amine, are from the larger bladders and the refreshing smelling fractions G1, G4, and G5 are from the smaller bladders.



SCHEME I. Synthesis of X.

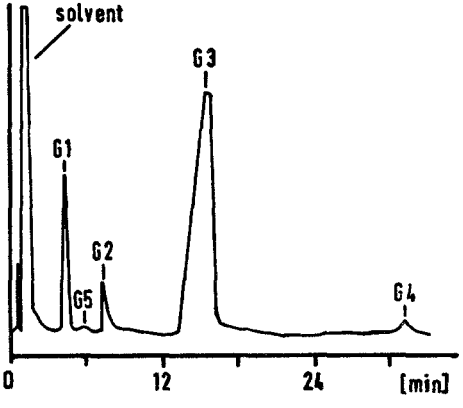
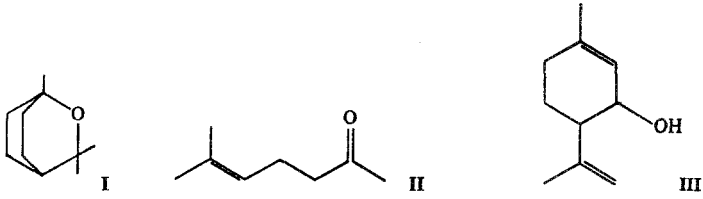


FIG. 1. Components of the secretion of *Stenus comma* separated by gas chromatography.

Collection of fraction G1 yielded 0.8 mg, an amount sufficient to identify the compound by IR, NMR and mass spectroscopy. The IR spectrum shows C—H stretching vibrations at 2980 cm^{-1} and 2870 cm^{-1}; absorptions at 1370 cm^{-1} and 1357 cm^{-1} indicate geminal methyl groups. A not very impressive band at 1073 cm^{-1} makes the presence of an ether possible. The function is confirmed by the mass spectrum which shows a molecular ion at *m/e* 154 and a M—H_2O fragment at *m/e* 136. A key fragment at *m/e* 31 suggests also an ether. From the proportion of the intensity of the molecular ion to the intensity of its ^{13}C isotope peak, the presence of 10 carbon atoms in the molecule can be inferred; this leads to $C_{10}H_{18}O$ as a tentative elemental composition of the compound. The ^1H NMR spectrum is more informative. Integration confirms the presence of 18 protons in agreement with the assumed elemental composition. A singlet corresponding to 6 protons appears at 1.2 ppm indicating equivalent geminal methyl groups at a quaternary carbon atom β to the oxygen function. Another singlet at 1.01 ppm corresponds to three protons and suggests a shielded methyl group. No evidence for double bonds in the molecule can be found in the spectrum, which points to a bicyclic molecule. On consideration of the natural source and the odor of the compound one finds 1,8-cineole (I) (a gift of Dragoco Company, Minden) to be the compound which agrees in its spectral data with the natural product.



I II III

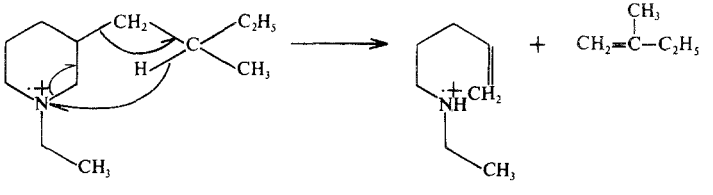
Collection of fraction G5 yielded only 0.03 mg of a colorless highly volatile liquid which could be characterized as *6-methylhept-5-en-2-one* (II) by its chromatographic properties. A thin-layer-chromatographic comparison of the secretion with authentic 6-methylhept-5-en-2-one (a gift of Dragoco Company, Minden) in four different solvent systems gave identical R_f values. A gas-chromatographic comparison on a Carbowax 20 M column and an Emulphor O column showed identical retention times.

Of fraction G4, 0.35 mg could be obtained. Characteristic functionalities can be deduced from the IR spectrum, namely an oxygen–hydrogen stretching absorption at 3580 cm^{-1}, the carbon–hydrogen stretching band of a terminal methylene group at 3070 cm^{-1} with the connected $\alpha{=}C\begin{matrix}H\\H\end{matrix}$ out of plane bending vibration at 886 cm^{-1} and the C=C stretching vibration at 1632 cm^{-1}. A band at 1670 cm^{-1} suggests a further double bond with trisubsti-

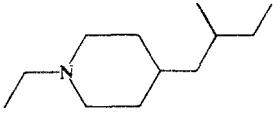
tution. At 1370 cm^{-1} the symmetric CH-bending vibration of a methyl group appears. The integration of the ^1H NMR spectrum gives 16 protons. A proton situated at a trisubstituted double bond absorbs at 5.36 ppm. Two olefinic protons appear at 4.78 ppm and the signal at 3.96 ppm is indicative of a proton in an R_1R_2—CH—O arrangement. Three protons which absorb between 2.2 ppm and 1.9 ppm may be methine or methylene protons situated α to a double bond. The signal between 1.86 ppm and 1.6 ppm corresponds to 8 protons. One can assume that six of these protons belong to methyl groups α to a double bond. The remaining two protons must be methylene or methine protons. The one proton absorption at 1.37 ppm disappears on addition of deuterium oxide and must be due to a hydroxyl proton. In the mass spectrum the peak with the highest mass number appears at *m/e* 134 followed by peaks at *m/e* 119, *m/e* 105, *m/e* 93, *m/e* 91, *m/e* 84, *m/e* 77, *m/e* 65, *m/e* 51, *m/e* 41, and *m/e* 39. No water abstraction can be deduced from this fragment series in spite of the fact that the IR and the NMR spectra indicate a hydroxyl group. Consequently we suspected the *m/e* 134 fragment to be the M^+—H_2O fragment and the molecular weight to be 152. These data make a tentative molecular formula of $C_{10}H_{16}O$ possible. Since the NMR spectrum indicates only two double bonds, the molecule was presumed to be a cyclic dienol which may fragment under electron impact to an aromatic system. The intense smell of G4 and its natural origin suggested a *p*-menthane system. This assumption was supported by the fact that the ^1H NMR spectrum of G4 showed a high degree of similarity with the ^1H NMR spectrum of limonene. Carveol and isopiperitenol are the two compounds which can account for all the spectral data. However, the spectroscopic data of the two isomers of carveol were different from the data of G4. Unfortunately only a very small amount of impure isopiperitenol was available to us. The purification of the compound and the identification of the *cis* and *trans* isomers proved to be difficult. Gas chromatography on an Emulphor O column produced a sample of (most probably) a mixture of the two isomers of isopiperitenol. The IR and NMR data of this sample were nearly identical with those of G4; this makes us assume that G4 is one of the isomers of isopiperitenol (III). The small deviations in the data may be due to steric differences of the compared samples.

In spite of the fact that G3 was the component which could be obtained in the largest quantity (1000 beetles yielded about 6 mg), it was difficult to prove its structure. We assigned the name stenusin to this compound. Stenusin exhibited the strong odor of an aliphatic amine. This odor, together with the fact that the IR spectrum of stenusin showed no N—H vibration, made us presume the compound to be a tertiary amine. In agreement with this assumption the mass spectrum indicated an odd molecular ion at *m/e* 183.1993, corresponding to the elemental composition $C_{12}H_{25}N$. Loss of a

hydrogen atom leads to a M-1 peak with about equal intensity. The base peak at *m/e* 168 is derived through loss of a methyl radical. In accordance with the concept of charge localization at the heteroatom, all intense fragments with lower mass numbers contain nitrogen; they arise either through loss of alkyl radicals or through loss of neutral olefinic units. The single unsaturation, deduced from the molecular formula and the rather intensive M-1 peak, suggest a heterocyclic ring for stenusin. The NMR spectrum gives no evidence for a double bond in the spectrum; this supports the assumed ring structure. Integration corresponds to 25 hydrogen atoms. A quartet at 2.3 ppm shows a coupling constant of 7 Hz. The same coupling constant can be found in a triplet at 1.05 ppm which is suggestive for an ethyl group. Confirmation is given by a double resonance experiment. On irradiation of the methylene protons at 2.3 ppm a singlet appears at 1.05 ppm. Bonding of the ethyl group to nitrogen can be deduced from the chemical shift of the methylene protons. Another key signal is the two-proton absorption at 2.76 ppm for two protons in α position to the nitrogen atom. In a substituted piperidine this could be the signal of the two equatorial protons at 2 and 6 (Booth and Little, 1966). The absorbance between 0.83 ppm and 0.95 ppm may be due to C-bonded methyl groups, one as a neighbor of CH_2 and one as the neighbor of $HC\langle$. The remaining signals lack diagnostic value. Re-investigation of the mass spectrum of stenusin and a study of the metastable transitions in the mass spectrum of stenusin by variation of the acceleration voltage of the double focusing mass spectrometer SM 1 A and by application of the DADI (direct analysis of daughter ions) technique (Beynon and Saunders, 1964; Maurer. et al., 1971) revealed that the odd-numbered ion



m/e 183 *m/e* 113



XI

m/e 113 originates from the molecular ion of stenusin.[3] An unsaturated C_5H_{10} moiety is lost in competition to the loss of an alkyl radical C_5H_{11} which leads to the ion *m/e* 112 with about equal intensity. This fact made probable a disubstituted piperidine with a pentyl substituent in the 2 or 3 position so branched that no loss of a propyl radical was possible. Of the two possible *N*-ethyl-pentyl-piperidines in question X is favored over XI, because in X a hydrogen atom next to the methyl group at the branched position of the side chain can easily migrate to the nitrogen atom; this rearrangement leads with loss of a pentene moiety to the ion at *m/e* 113.

Compound X was synthesized according to the described reaction sequence. The IR, NMR and mass spectra of stenusin and X were practically identical (Figures 2 and 3).

N-ethyl-3-(2-methylbutyl)piperidine possesses two asymmetric C atoms, one is situated at position 3 of the ring and the other one at the branching of the side chain. Therefore four stereoisomers of this compound exist. In the

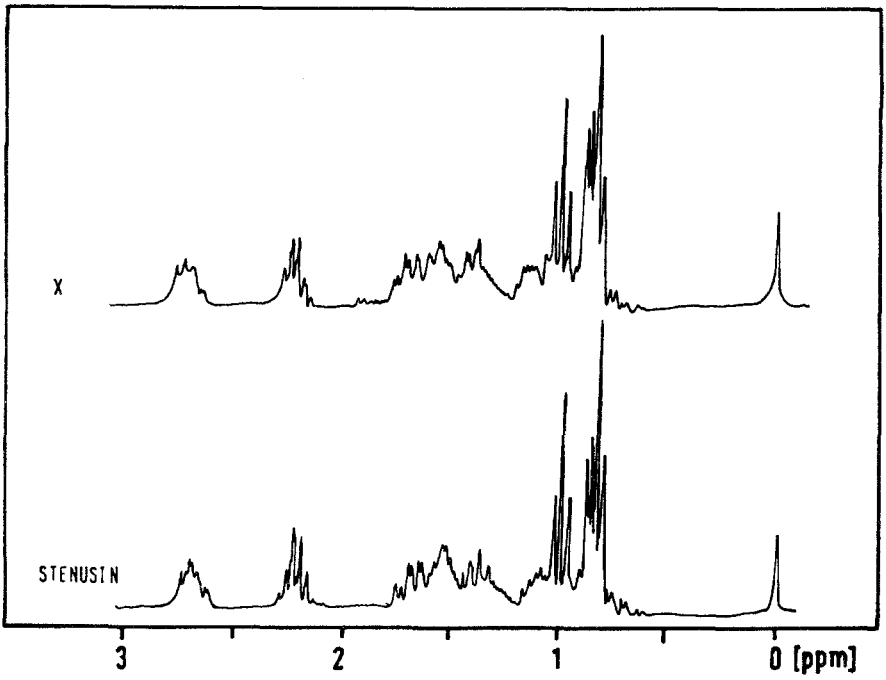


FIG. 2. 220-MHz proton magnetic resonance spectra of stenusin and synthetic *N*-ethyl-3-(2-methylbutyl)piperidine (X) (CCl_4 as solvent, Varian HR-SC-220) (Spectra supplied by Dr. D. Wendisch, c/o Farbenfabrik Bayer AG, Leverkusen).

[3] We thank Varian MAT GmbH, Bremen, for DADI measurements.

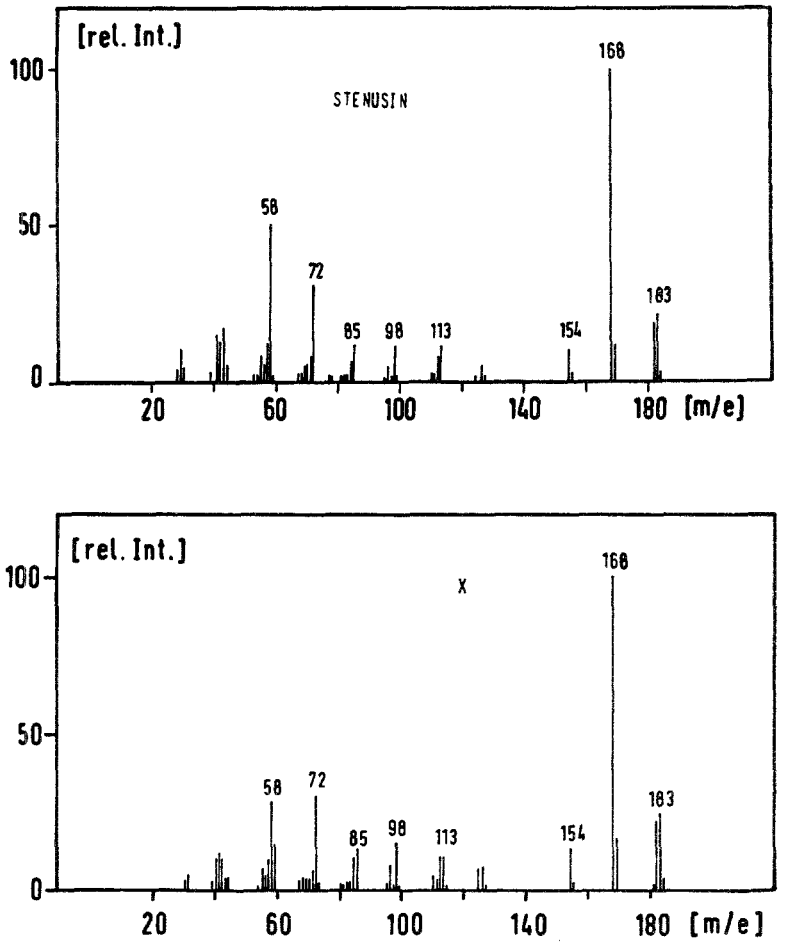


FIG. 3. Mass spectra of stenusin and synthetic *N*-ethyl-3-(2-methylbutyl)-piperidine (X) (Varian MAT SM 1 A).

synthesis of X we used (−)-2-methylbutan-1-ol $[\alpha]_{546}^{20} = -5.55°$ (neat) to introduce the side chain. Since the asymmetric C atom of the side chain was not subjected to a further reaction, we may assume that its configuration was retained. The specific rotation of the synthetic product was $[\alpha]_{365}^{20} = +5.4°$ (c = 3 mg/ml ethanol). The natural product showed $[\alpha]_{365}^{20} = +5.8°$ (c = 1.15 mg/ml ethanol). From these data we may assume that stenusin and X are identical. But owing to the low rotary power of the compounds and the low concentrations which were applied, both optical rotation values may be ambiguous. It would of course be possible to prove the absolute configuration

TABLE 1. SURFACE TENSION, SPREADING PRESSURE, AND SURFACE PRESSURE OF THE THREE COMPOUNDS

Compound	Surface tension $_{19°}$	Spreading pressure ($P_{19°}$)	Surface pressure $_{max\ 22°}$
Stenusin	27.3	36.3	28.4
1,8-Cineol	28.5	29.2	2.3
6-Methylhept-5-en-2-one	28.0	32.4	17.1

of stenusin by an X-ray crystallographic structure determination of a suitable crystalline derivative. This investigation is in progress.

It is interesting that all staphylinids which produce their defensive substances in pygidial glands seem to prefer terpenes or terpene-like substances (Brand et al., 1973). *Staphilinus olens* excretes iridodial (Abou-Donia et al., 1971) as a main component and in the pygidial gland exudates of *Bledius mandibularis* and *Bledius spectabilis* monoterpene aldehydes can be found, but the main component is dodecalactone (Wheeler et al., 1972) and smaller amounts of 1,4-benzoquinone and 1-undecene. The above mentioned staphylinids are typical land beetles. *Stenus comma* is also a land beetle; but it lives in the immediate neighborhood of water and has to protect itself not only against microorganisms and other animals, but also against the danger of drowning. We therefore thought it would be interesting to find out how many of the compounds secreted by *Stenus comma* are spreading reagents. Experiments to measure the surface pressure of 1,8-cineole, 6-methylhept-5-en-2-one, and stenusin showed that all three substances exhibit high spreading ability. With an interfacial tensiometer, we measured the surface tension, σ, of the three compounds against air and the interfacial tension, γ, against water. We calculated the spreading pressure, P, according to the following definition (Wolf, 1957)

$$P = \sigma_{water} - (\sigma_{substance} + \gamma_{substance/water})$$

All three compounds show a low surface tension and therefore a high spreading pressure. Direct measurement of the spreading pressure with a Langmuir film balance (Kuhn et al., 1972) was not possible because the films of the compounds were not stable enough. To estimate the spreading pressure of the unstable films, a surface pressure vs. time diagram was plotted and the pressure maxima of the three compounds were compared (Table 1).

Stenusin exerts the highest surface pressure because it is only barely soluble in water (solubility in water$_{20°}$: stenusin, 0.03 wt.%; 1,8-cineole, 0.3 wt. %; 6-methylhept-5-en-2-one, 0.2 wt.%;). Stenusin spreads not only

over the surface of water, but also over solid materials such as wood, plastic, and glass.

Its spreading velocity was deduced from velocity-displacement curves which were determined with the aid of a film camera. The spreading velocity of stenusin (32.5 cm/sec) is much higher than the spreading velocity of 1,8-cineole (18 cm/sec). We deduce from these data that stenusin is the main spreading agent which propels the beetle.

Stenusin may also have a protective function for *Stenus comma*; it is weakly toxic to mice, 100 mg/kg mouse intraperitoneally caused death after 3–4 min from respiratory failure. It is known that 1,8-cineole has antiseptic properties (Rœmpp, 1966), and it is used as an insect repellent (Radimsky, 1952). Hence a triple function of the secretion can be considered. The first and main function is the spreading pressure to effect the driving of the beetle. The liquid also serves to defend the beetle against microorganisms and other animals.

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A COMPARISON OF THE EFFECTIVENESS OF THE ODORS OF RABBITS, *Oryctolagus cuniculus*, IN ENHANCING TERRITORIAL CONFIDENCE

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Abstract—In 14 series, each of 100 tests, involving 350 individual animals it has been shown that odors derived from the anal, inguinal and chin glands and urine strengthen to different degrees the confidence of European wild rabbits, *Oryctolagus cuniculus*. In the tests, two rabbits were put together on otherwise neutral ground previously treated with the odor from one of the contestants. The confidence of the rabbits was determined from an assessment of the record of the time to first physical contact, the frequencies of approaches and aggression, the number of attempts to escape, and also by making a subjective assessment of their general deportment. The results confirm earlier conclusions on the territorial functions of the anal and chin glands, the role of inguinal glands in individual identification, and the behavioral role of urine. On otherwise neutral ground the rabbits' own chin secretions were found to be the most effective in stimulating the confidence of males and that of their female partners. The odor of the anal gland secretions of males stimulated a similar but slightly weaker effect. The anal gland secretions of females influenced their own confidence but not that of males. Urine seemed to play a more important role in affecting the confidence of females than of males. Inguinal gland secretions had least effect. Odor from this source generally did not influence the confidence of the experimental animals; secretion from the inguinal glands of males had a slight effect on the behavior of their female partners.

Key Words—rabbit, *Oryctolagus cuniculus*, odors, anal, inguinal, chin glands, urine, territorial confidence.

INTRODUCTION

It has been emphasized in earlier reports that rabbits as well as other animals permeate the places in which they live with their own odor and that only within such places do they behave confidently and compete successfully with conspecifics in vitally important activities.

It has been found that the anal and chin gland secretions of the European wild rabbit, *Oryctolagus cuniculus*, function specifically for space marking. The size and the secretory activity of these glands are related to the territorial status of the individuals (Mykytowycz, 1968). In two earlier papers the confidence-giving effect of these secretions has also been demonstrated. It was reported then that on neutral ground one individual dominates another when accompanied by its own or familiar secretions from anal or chin glands. Thus the odors from these glands turn a neutral ground into "home" (Mykytowycz, 1973, 1975).

The secretions from these two glands are deposited by rabbits in the course of specific marking behavior. Other sources of odor in the European rabbit are the inguinal glands and urine. Odor from urine functions during aggressive and courtship displays, but the secretion from the inguinal gland is not involved in any specific marking activities. It has been suggested that this gland plays a role in individual recognition (Mykytowycz, 1966).

The present paper, based on experimental data, compares the effects on the territorial confidence of the European wild rabbit of odors from its own or a familiar animal's anal, chin, and inguinal gland secretions and urine.

The concept of "territorial confidence" used in this paper is an abstract one determined subjectively by taking into account some measurable forms of behavior and our familiarity with the details of the behavior of rabbits.

Although reference to the confidence-giving effect of familiar odors will be made throughout the text, there is no doubt that inhibition of certain forms of behavior in animals confronted with strange odors also took place, this was regarded as an indication of lack of confidence.

METHODS AND ANIMALS

The methods employed were identical with those described earlier (Mykytowycz, 1975), but for the completeness of the present report they are briefly outlined here.

Animals

Fully grown wild-type rabbits, 175 males and 175 females born in the

TABLE 1. SCHEDULE OF THE COMBINATIONS OF SEX OF RABBITS, *Oryctolagus cuniculus*, AND TYPES OF ODOR STIMULI TESTED

	Source of odor			
	Chin gland	Anal gland	Inguinal gland	Urine
With own odor				
♂ against ♂	+	+	+	+
♀ against ♀	Not tested	+	+	+
With odor from mate of opposite sex				
♂ against ♂	Not tested	+	+	+
♀ against ♀	+	+	+	+

laboratory were used. These were normally housed in separate cages in breeding pairs.

Experimental Design

Only 14 of the 16 possible combinations of odor stimulus and sex of the animals were tested. As chin secretions from the females could not be obtained in sufficient quantities for the experiments, neither females nor their sexual partners could be tested in the presence of this particular stimulus. A schedule of the combinations of sex of animals and sources of odor stimulus used in the experiments are shown in Table 1.

25 animals were used for each of the 14 series of tests and were randomly assigned into 50 test pairings. Hence different animals were used for each of the 14 sex–odor combinations providing the necessary conditions of independence for stimuli comparisons. Each specific pair was tested twice, and twice only: once in the presence of the odor familiar to animal A and again in the presence of the odor stimulus familiar to animal B. Out of these two tests each animal obtained a score: "better," "equal," or "worse." Better was scored for an animal when its performance in the presence of its own odor was superior to that in the reversed situation; similarly for equal and worse. These assessments were made irrespective of the other animal's performance. Thus the results provided nonparametric scores which were independent of the natural differences in performance between animals in the presence of their own odor and of the general levels of performance of the two tested animals.

Testing Procedure.

The tests, which lasted 10 min each, were carried out in an indoor pen

5 m^2 in area. It was of the same size and shape as those in which the animals were normally kept.

Before each test the floor of the pen was covered with a sheet of clean paper on which fecal pellets or eight filter pads impregnated with the given odor stimulus were scattered randomly. The two rabbits were introduced into the pen simultaneously. When a rabbit was tested in the presence of its own or a familiar odor, it was referred to as being in the "home" situation or as the "home" or "donor" animal, and when in the opposite situation as being the "away" animal or in the "away" situation.

The following forms of behavior which reflected the confidence of animals were recorded—time to first physical contact between the animals, frequency and duration of approaches, and aggressive behavior. Since fighting, which is the best indicator of the dominance of one individual over the other, often did not occur, the general performance of the two animals was also assessed by using records of attempts to escape from the pen, the amount of movement, and general deportment. From these observations, the performance of the "home" animal was graded as "more confident," "equally," or "less confident" than the "away" rabbit.

For practical reasons it was impossible to standardize the quantity of the odor stimuli. Each one was used in amounts which it was thought would resemble the usual quantities deposited by a rabbit.

Chin Gland. Glands of individual rabbits were massaged to cause drops of secretion to appear on the surface of the skin through the secretory pores. The secretion was collected into glass capillaries, and shortly prior to the test was diluted 1:1000 with distilled water, and two drops placed on each of the eight filter papers. Approximately 0.5 mg of secretion was used for each test.

Anal Gland. Fecal pellets become coated with anal gland secretion when passing through the end part of the rectum and hence are the natural vehicle for its dispersion. 20 fecal pellets, freshly deposited by the donor animal, were scattered on the floor of the pen.

Urine. Urine was collected overnight from beneath the rabbits' cages, precautions being taken to avoid contamination with fecal pellets. Four drops of urine were placed on each of the eight filter pads.

Inguinal Gland. Small quantities of sebum from the inguinal pouches were transferred to the eight filter pads using a clean cotton-wool swab.

Statistical Procedure

A nonparametric test (Siegel, 1956, p. 105) was used for all comparisons. As there were 100 tests in each series, frequencies and percentages are equivalent in all tables.

RESULTS

Level of Confidence of Rabbits in the Presence of Their Own Odors

In a high proportion of tests ($P < 0.001$) males displayed more confidence when accompanied by their own chin and anal gland secretions. The presence of urine had a much weaker effect ($P < 0.05$), while the presence of inguinal secretion had no significant influence on their confidence.

The female's own anal secretion and urine induced confidence in a highly significant number of tests ($P < 0.001$), but their own inguinal secretion had no statistically significant effect.

The sexual partners' anal secretion and urine had only a slight influence on the confidence of males ($P < 0.05$), and the presence of their females' inguinal secretion did not produce a statistically significant effect.

All odor stimuli derived from male partners strengthened significantly the confidence of females, although inguinal secretion was again somewhat less effective than the other sources of odor (see tests between "more" against "less" in Table 2).

The overall results indicate convincingly the territorial significance of chin and anal secretions for both sexes, and of urine for females. The odor from inguinal glands does not seem to be of any importance to males in the context of these tests, although the same source of odor derived from their male partners may possibly play some territorial role in females (see tests between stimuli in Table 2).

Effects of the Different Odor Stimuli on Behavior

More detailed analyses of the confidence-giving effects of the different odors are presented in Tables 3, 4, 5, and 6 which indicate the effects of the various odors on selected forms of behavior. Each table summarizes the data from, respectively, males and then females, in the presence of their own, and also their mate's odor. Results of χ^2 tests are included to show whether the observed frequencies of each of the three behavioral responses reflect significant differences between odor stimuli.

Males in the Presence of Their Own Odors

Significant differences between stimuli for all behavioral parameters, i.e., first contact, approaches, and aggression, are evident from Table 3. This table also shows the greater effect of their own chin and anal secretions and the generally less pronounced effect of inguinal secretions on the males.

TABLE 2. LEVELS OF CONFIDENCE SHOWN BY RABBITS, *Oryctolagus cuniculus*, IN ENCOUNTERS IN AN OTHERWISE NEUTRAL ENVIRONMENT CONTAINING THEIR OWN OR THEIR MATES' ODORS (FREQUENCIES OF OCCURRENCE)

		Level of confidence	Source of odor[a]				Tests between stimuli	
			Chin gland	Anal gland	Inguinal gland	Urine	$\chi^2_{(4)}$ without chin	$\chi^2_{(6)}$ with chin
Own odor stimuli	♂ against ♂	More	77	69	52	56		
		Equal	8[b]	10[b]	9(N.S.)	7[c]	9.24[c]	21.90[b]
		Less	15	21	39	37		
	♀ against ♀	More		66	51	65		
		Equal		9[b]	14(N.S.)	13[b]	6.87(N.S.)	
		Less		25	35	22		
Odor stimuli from mate of opposite sex	♂ against ♂	More		57	53	55		
		Equal		5[c]	10(N.S.)	11[c]	2.77(N.S.)	
		Less		38	37	34		
	♀ against ♀	More	69	64	52	44		
		Equal	17[b]	8[b]	20[d]	38[d]	27.23[b]	36.54[b]
		Less	14	28	28	18		

[a] Significance tests apply to "more" and "less" only, e.g., 77 vs. 15.
[b] $P < 0.001$.
[c] $P < 0.05$.
[d] $P < 0.01$.

TABLE 3. PERFORMANCE OF MALE RABBITS, *Oryctolagus cuniculus*, WHEN CONFRONTED WITH OTHER MALES IN THE PRESENCE OF THEIR OWN ODORS[a]

Form of behavior	Level of performance	Source of odor				Tests between stimuli	
		Chin gland	Anal gland	Inguinal gland	Urine	$\chi^2_{(4)}$	$\chi^2_{(6)}$
First contact	Earlier	85	75	62	51		
	Equal	5	3	3	29	42.40[b]	72.67[b]
	Later	10	22	35	20		
Approaches	More	81	80	61	62		
	Less or equal	18	16	39	29	22.27[b]	32.47[b]
	None	1	4	0	9		
Aggression	More	36	46	13	26		
	Less or equal	18	12	11	18	31.67[b]	35.20[b]
	None	46	42	76	56		

[a] $\chi^2_{(6)}$ indicates whether the observed frequencies of responses to four odors are different. $\chi^2_{(4)}$ as above excluding chin gland secretions (frequencies of occurrence).
[b] $P < 0.001$.

TABLE 4. PERFORMANCE OF FEMALE RABBITS, *Oryctolagus cuniculus*, WHEN CONFRONTED WITH OTHER FEMALES IN THE PRESENCE OF THEIR OWN ODORS[a]

Form of behavior	Level of performance	Source of odor				Tests between stimuli, $\chi^2_{(4)}$
		Chin gland	Anal gland	Inguinal gland	Urine	
First contact	Earlier	Not tested	71	66	75	
	Equal		3	2	10	14.28[b]
	Later		26	32	15	
Approaches	More		62	51	65	
	Less or equal		29	36	28	4.99 (N.S.)
	None		9	13	7	
Aggression	More		24	19	25	
	Less or equal		16	12	3	10.66[c]
	None		60	69	72	

[a] $\chi^2_{(4)}$ indicates whether the observed frequencies of responses to three odors are different (frequencies of occurrence).
[b] $P < 0.01$.
[c] $P < 0.05$.

TABLE 5. PERFORMANCE OF MALE RABBITS, *Oryctolagus cuniculus*, WHEN CONFRONTED WITH OTHER MALES IN THE PRESENCE OF THE ODORS FROM THEIR FEMALE PARTNERS[a]

Form of behavior	Level of performance	Source of odor: Chin gland	Anal gland	Inguinal gland	Urine	Tests between stimuli, $\chi^2_{(4)}$
First contact	Earlier	Not tested	63	57	54	
	Equal		2	20	18	17.86[b]
	Later		35	23	28	
Approaches	More		56	51	54	
	Less or equal		35	43	43	4.29 (N.S.)
	None		9	6	3	
Aggression	More		29	17	19	
	Less or equal		14	18	17	4.96 (N.S.)
	None		57	65	64	

[a] $\chi^2_{(4)}$ indicates whether the observed frequencies of responses to three odors are different (frequencies of occurrence).
[b] $P < 0.01$.

Females in the Presence of Their Own Odors

In the series involving females in the presence of their own odors, incidences of aggression and first contact show some significant variation between stimuli ($P < 0.01$ and $P < 0.05$, respectively). It is interesting that their own urine was most effective in inducing confident behavior. As in males, their own inguinal secretions were weakest (see Table 4).

Males in the Presence of Their Female Partners' Odors

Odors from the females produced a relatively uniform distribution of results when used as stimuli for their male partners (Table 5). Variations were significant only for first contact ($P < 0.01$), and this was due to the distribution of results in tests involving anal gland secretion. Unlike tests with females, the anal secretion, rather than urine, released the more pronounced responses.

Females in the Presence of Their Male Partners' Odors

Significant variations ($P < 0.001$) between the stimuli were recorded in all behavioral measures when females were tested in the presence of their male partners' odors (Table 6). The large amount of variation between stimuli (see $\chi^2_{(6)}$ tests) is mainly due to the effect of chin-gland secretion. In cases of

TABLE 6. PERFORMANCE OF FEMALE RABBITS, *Oryctolagus cuniculus*, WHEN CONFRONTED WITH OTHER FEMALES IN THE PRESENCE OF ODORS FROM THEIR MALE PARTNERS[a]

Form of behavior	Level of performance	Source of odor				Tests between stimuli	
		Chin gland	Anal gland	Inguinal gland	Urine	$\chi^2_{(4)}$	$\chi^2_{(6)}$
First contact	Earlier	70	71	60	75		
	Equal	28	2	16	3	19.71[b]	59.35[b]
	Later	2	27	24	22		
Approaches	More	78	58	51	61		
	Less or equal	22	23	37	34	12.56[c]	35.24[b]
	None	0	19	12	5		
Aggression	More	52	26	8	16		
	Less or equal	12	10	11	9	11.99[c]	62.23[b]
	None	36	64	81	75		

[a] $\chi^2_{(6)}$ indicates whether the observed frequencies of responses to four odors are different. $\chi^2_{(4)}$ as above excluding chin gland secretion (frequencies of occurrence).
[b] $P < 0.001$.
[c] $P < 0.05$.

aggression and number of approaches when the differences between only the anal and inguinal secretions and urine are compared, the significance levels are reduced ($P < 0.05$, $\chi^2_{(4)}$).

DISCUSSION

Olfactory marking by animals of the space they occupy has always been and will probably remain the subject of controversy because of the many ways in which the space is used and even more so because of the diverse views held by biologists about territory and its function.

The repelling effect of the markings of strange territory is frequently emphasized. The confidence-giving effect of familiar odor is referred to less often. Biologists have paid little attention to the attractant property of space markings made by mammals, whereas for birds it is an important attribute of an individual's territory. Olfactory space marking has been discussed more fully elsewhere (Mykytowycz, 1975). An important reason for lack of agreement concerning the role of territoriality and the function of olfactory marking is that very few researchers have been in a position to study the problem fully by experimentation under entirely natural conditions.

Earlier observations have established beyond doubt the exceptionally strong territoriality of the European rabbit and the existence of special odor-producing organs which function for the differential marking of the various types of space utilized by them (Mykytowycz, 1974).

Our earlier observations also showed clearly that when two strange rabbits meet, the first matter to be resolved is that of space ownership, i.e., unhindered use of the space. The fact that individuals of opposite sexes become engaged in fights, even in the absence of competition for food, shelter, and other essential commodities, is convincing proof that competition for the space is involved.

The experiments described here were conducted in the same way as those aimed at demonstrating the superiority or dominance of an animal in its own territory over an intruder (Mykytowycz and Hesterman, 1975). Basically identical results were obtained, and this supports our view that it was the level of territorial confidence that was tested in the present experiment.

It is true that aggression was more prevalent in the experiments in which animals were confronted with intruders in their own home cages (Mykytowycz and Hesterman, 1975), but this does not affect the overall results and final conclusions. Aggression was not the subject of the present study, it was only one of the types of behavior which were used as indicators of confidence or the lack of it.

It is a common error of many workers who experiment with odor to regard it as a specific releaser of aggression. In fact odor is only a means of communication, carrying messages which will be interpreted differently at different times by the same individuals, and only sometimes releasing actual fighting depending on the social and physiological states of the contestants.

Our aim in the present study was to determine to what extent the various odors with which a rabbit saturates its own environment contribute towards the "homeyness" of the occupied space.

From the data presented above it is obvious that the presence of its own or a familiar odor in an otherwise neutral environment gives a rabbit the confidence which allows it to dominate a conspecific. However, not all the rabbit odors which are in the environment are equally effective in influencing territorial behavior, and not all sources of smell are of the same importance to both sexes in a given situation.

In the present experiment the males' chin gland secretion most consistently produced territorial confidence in both sexes.

Although it was not possible to obtain chin gland secretion in sufficient quantities for experimentation from the females born in the laboratory, the results of an earlier study in which field-caught rabbits were used showed that females also respond very strongly to their own chin gland secretion (Mykytowycz, 1975).

Thus the territorial function of the chin gland suggested in numerous earlier reports which were based on general observations of free-living rabbits and on studies of laboratory-bred animals whose social status was known (Mykytowycz, 1968) has been confirmed.

Secretion from the anal glands, transported on the surface of hard fecal pellets, particularly that of the males, was the second most effective odor in inducing territorial confidence. The females' anal gland secretion although strongly influencing the donors' confidence, had a much weaker effect on males. The present report confirms earlier statements on the territorial function of the anal gland and the special role of "buck hills" in space marking (Mykytowycz and Hesterman, 1970).

The space-marking function of urine is well established in many species of mammals and in mice particularly (Eibl-Eibesfeldt, 1950; Desjardins et al., 1973; Johnson, 1973). It is known that the rabbit uses enurination in the course of fighting as well as during mating behavior (Southern, 1948; Myers and Poole, 1961), but what role this source of odor plays in space marking was not clear from earlier observations. The evidence emerging from this experiment suggests that for males urine may be of less importance in a territorial context than the secretions of the anal and chin glands.

In females, however, both their own and their male partners' urine stimulated behavior which was indicative of confidence. It has been noted previously that female rabbits often use urine to mark the sealed entrances to their breeding stops (Mykytowycz, 1974).

In comparison with the other three odors used in the study, the inguinal secretion seemed to be of least importance in shaping the territorial activities of the rabbits. This odor had no significant influence on the overall results of the tests except in the series involving females with their male partners' odor. Only with regard to the time spent on sniffing the other animal did the inguinal secretion match or surpass the other stimuli in effectiveness. This supports the idea expressed earlier that the inguinal glands function for individual identification (Mykytowycz, 1966).

Since each animal was used at least twice for experimentation, it is possible that learning may have taken place which would influence the results of subsequent tests. It was, however, not so. If this did occur it would tend to reduce the effect of the animals' own odors. In fact in 19 out of the 50 pairs of tests involving males in the presence of their own anal gland secretions, the donor individuals in the second phase were the more confident despite being submissive during the first phase of the test. In the chin gland series this reversal of dominance occurred in 28 pairs of tests. No reversals of dominance could be expected in the absence of some advantage arising from the presence of an animal's own odor and in the presence of learning.

The results presented above indicate again the reality of communication

by odors in the European wild rabbit as well as its complexity and the need for further studies to fully understand the behavior of the species.

Together with two earlier papers (Mykytowycz, 1973, 1975), these results also give experimental support to the suggestion made by other authors (Johnson, 1973) that an animal's own marking odors, apart from repelling intruders, boost the confidence of the occupant of a given area.

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BEHAVIORAL AND CARDIAC RESPONSES OF THE RABBIT, *Oryctolagus cuniculus*, TO CHEMICAL FRACTIONS FROM ANAL GLAND

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Abstract—Unrestrained rabbits, 2–3 months old and nestlings, were exposed to the odors of the acid-neutral, acid, neutral, and basic fractions of the anal gland of male rabbits. The acid-neutral, acid, and neutral fractions elicited the strongest response in the form of avoidance. The heart rates of the nestlings were monitored using a radio telemetry technique. Statistically significant lowering of the heart rate and an increase in variability occurred on exposure to the fractions. The extent of the changes varied consistently in relation to a given odor, being greatest for the acid and neutral fractions. The results indicate the usefulness of heart-rate monitoring as a tool in the study of the perception of odors by animals.

Key Words—behavior, cardiac responses, rabbits, *Oryctolagus cuniculus*, chemical fractions, anal glands.

INTRODUCTION

During our studies of the behavior of the European wild rabbit, *Oryctolagus cuniculus*, the behavioral functions of the odor-producing glands—anal, inguinal, and chin—have been studied and their chemical compositions examined (Goodrich and Mykytowycz, 1972).

During the chemical investigations the need arose to test the behavioral importance of numerous chemical fractions extracted from the glands. The methods commonly employed for this purpose involve recording the overt behavior of animals in relation to the various substances presented to them in manipulated social or physical environments. While the final criterion for the

behavioral potency of a substance must be its effect on freely acting, unrestricted individuals in natural situations, the screening of large numbers of odors in such situations is extremely time consuming, and the maintenance of standardized conditions over a lengthy period is difficult.

Physiological variables such as galvanic skin response, breathing rate, and cardiovascular changes are commonly used by experimental psychologists as objective measures of the response of subjects to various stimuli.

There has been particular interest in the use of heart-rate measurements in behavioral research in the past few years, and their value in this field has been suggested by a number of workers (Lacey and Lacey, 1970). Cardiac activity was also found to be a useful indicator of response to external stimuli (Frisch, 1965), including olfactory ones (Wenzel and Sieck, 1972).

In this study the three products of an initial chemical fractionation of the anal glands of male rabbits were tested for their effects on the overt behavior and heart rates of young rabbits.

METHODS AND MATERIALS

Extraction and Fractionation of Anal Gland Materials

All solvents used were ACS reagent grade. Solvent extracts were dried over anhydrous magnesium sulfate prior to evaporation under reduced pressure at 30–35°C in a rotary evaporator.

Anal Gland Extract. The whole anal glands from 50 adult male wild rabbits shot in the field were used in the initial extraction. The glands were collected from the dead animals within 1 hr and stored at −27°C. After removal of as much nonglandular tissue as possible from the samples, the total weight of the material used for extraction was 65 g.

The glands were suspended in 200 ml ether, cooled in ice, and homogenized in an Ultra-Turrax high-speed homogenizer (Janke and Kunkel Kg) for 5 min. The mixture was then centrifuged in the cold and the supernatant ether layer decanted off. The residue was similarly extracted twice more with 200-ml portions of ether. The combined ether extracts after drying and evaporation yielded 1.89 g of a light brown oil.

Acid-Neutral Fraction. To remove basic organic material, 1.09 g of the anal gland extract was dissolved in 150 ml ether and extracted five times with 20 ml portions of pH 1.0 water (100 g NaCl and 6 ml conc. HCl/liter water). The ether phase was washed with water, and after drying and evaporation gave 1.02 g of a pale yellow oil.

Basic Fraction. The aqueous extracts from the preparation of the acid-neutral fraction were pooled and washed twice with 40-ml portions of ether to remove nonbasic organic material. After adjustment to pH 8.5 with 1 N

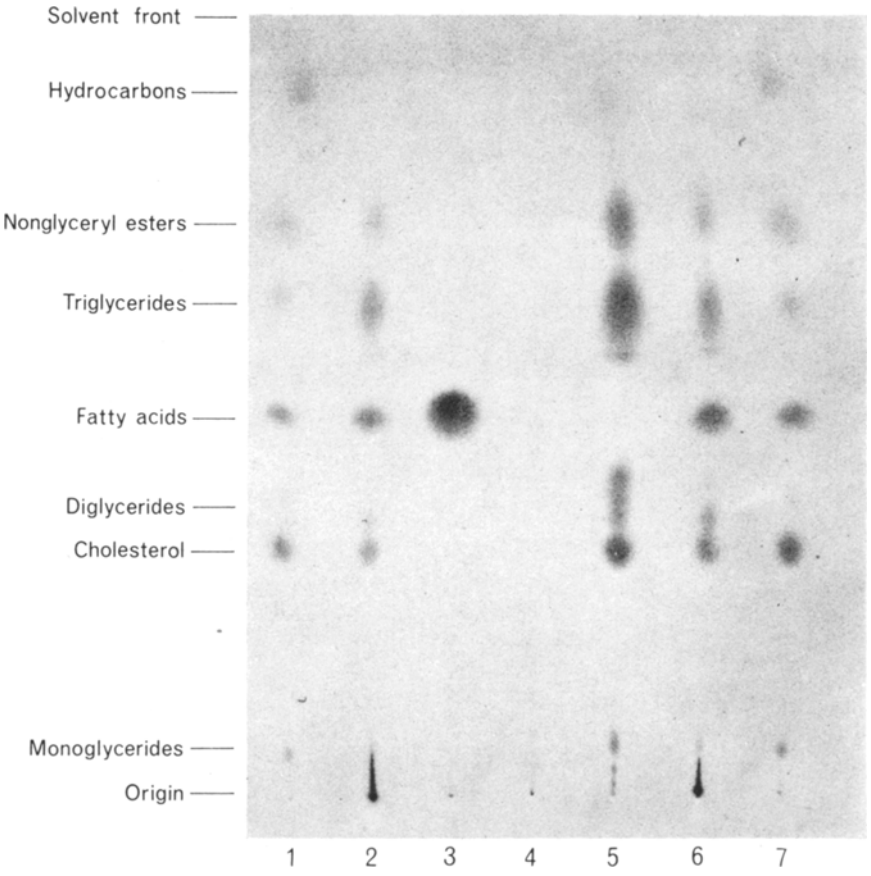

FIG. 1. Thin-layer-chromatographic analysis of the extract and fractions of pooled male rabbit anal glands. 1 and 7, standard lipid mixture; 2, anal gland extract; 3, acid fraction; 4, basic fraction; 5, neutral fraction; 6, acid-neutral fraction.

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KOH, the aqueous phase was extracted five times with 20-ml portions of ether. Evaporation of the ether yielded 2.0 mg of a dark brown solid.

Acid Fraction. A solution of 0.43 g of the acid-neutral fraction in 40 ml ether was extracted three times with 20-ml portions of 0.1 N NaOH. The combined alkaline extracts were washed twice with 20 ml portions of ether to remove any residual neutral material and these washings combined with the organic phase. The alkaline extract was cooled in ice, acidified to pH 3 with 1 N HCl, saturated with NaCl, and extracted five times with 20-ml portions of ether. The combined ether extracts were washed with water, dried, and evaporated and yielded 0.13 g of a pale yellow oil.

Neutral Fraction. The organic phase remaining after removal of the acid fraction was washed twice with 10 ml water. After drying and evaporation, 0.27 g of a colourless wax was obtained.

Chemical Composition of Anal Gland Extract and Fractions

The result of thin layer chromatographic examination of the anal gland extract and fractions is shown in Figure 1. Standard lipids obtained from commercial sources and used without further purification are included for comparative purposes. The chromatography was carried out on Silica Gel G (E. Merck A.G., Darmstadt, West Germany). The plates were activated at 120°C for 1 hr, spotted, and developed to a height of 16 cm with light petroleum (bp 40–60°C)–ether–acetic acid (80:20:1). After air drying the plates were developed in the same dimension to a height of 6.5 cm using light petroleum (bp 40–60°C)–ether–acetic acid (50:50:1). Compounds were made visible by spraying the plates with 50% sulfuric acid and charring in an oven at 170°C.

Test Procedures

The four fractions obtained from the anal gland extract were presented as odour stimuli to two age groups of rabbits under two different conditions. To facilitate the measurement and application of the fractions each was made up into a standard ether solution containing 1 mg of fraction to 1 ml of ether. For the study of the behavior of penned rabbits in relation to the odor stimuli, eight different litter groups of wild-type rabbits totalling 45 animals aged 2–3 months were used. All groups were tested in their home pens which were of a standard design with a floor area of 5 m^2.

Two series of tests were conducted. For comparison, in each series two different chemical fractions were presented to the rabbits at the same time. In the first involving 39 animals in 8 groups, the reactions of the rabbits to the acid-neutral and basic fractions of the anal gland extract were measured

while in the second series the effects of the acid fraction and neutral fraction on the behavior of 33 animals in 6 groups was observed.

As fecal pellets are the natural vehicle for the distribution of the anal gland secretion these were used in presenting the fractions. Fresh, undamaged fecal pellets obtained from the group of animals involved in the test were divided into two separate, 20-pellet collections. Each collection was treated with one of the two fractions to be compared by placing one drop of the standard ether solution of the given fraction onto each pellet. The treated pellets were exposed in air until no smell of ether could be detected by the experimenter and were then placed in two prescribed areas on the floor of the pen, the positions being alternated between tests. The floors of the pens were not cleaned prior to testing.

The rabbits were tested as a group and were allowed to emerge at will from their normal resting position in a shelter box attached to the open pen. The behavior of all individuals in relation to the treated fecal pellets was recorded for 30 min starting from the first contact of any one of the group with either of the stimuli.

To test overt responses and heart-rate changes of nestling rabbits in relation to odor stimuli, 40 wild-type nestling rabbits between 3 and 12 days of age and comprising 8 different litters born in the laboratory were used.

Clean cotton-wool swabs attached to wooden applicator sticks were each impregnated with 1 drop of one of the standard ether solutions of the four anal gland fractions. A fifth swab was treated with one drop of ether. All swabs were exposed in air until no smell of ether could be detected by the experimenter. For comparative purposes fresh fecal pellets from the kitten's mother and from an adult male rabbit were individually attached to wooden applicator sticks and were also presented to the kittens. All odor stimuli were kept in sealed glass containers.

A miniature ECG radio transmitter was used to record heart beats. Two small pin electrodes connected to the transmitter by flexible leads were inserted into the skin approximately 1 cm apart in the sternal region of the animals. The signal was received and recorded on magnetic tape by a high quality FM radio/cassette recorder. By suitably demodulating and filtering the signals the tape recordings were later transformed to a strip chart trace. Chart recordings were made at a chart speed of 50 cm/min from which the number of heart beats in each 1.2-sec interval were scored manually.

The general testing procedure followed was similar to that described earlier by Mykytowycz and Ward (1971). Kittens were removed from their nest and the electrodes attached to their skin. They were wrapped in towelling for comfort and heat control and held in the hand by an assistant. When the kitten had settled down, heart-beat recording was commenced and after 30 sec an odor stimulus was presented by holding it approximately 5 mm from

the kitten's nostrils for 15 sec. Care was taken to avoid touching the animal's vibrissae. Heartbeat recordings were continued for a further 15 sec after withdrawing the odor stimulus.

Apart from heart rate, the sniffing movements of the kitten's nostrils were counted for the two 15-sec periods prior to and during the presentation of the stimuli, and the overt reactions of the kittens in the form of grimacing, pulling away from, or following the stimulus and closing nostrils (see Mykytowycz and Ward, 1971) were also recorded.

Subsequent stimuli were presented at approximately 3-min intervals until the series was completed. The order of presentation of the different odors was randomized for each kitten.

RESULTS

Overt Responses of Unrestrained Rabbits to the Chemical Fractions of the Anal Gland

Table 1 summarizes the responses of unrestrained 2–3 month old rabbits to the fecal pellets treated with the four chemical fractions of the male anal gland.

In the first series of tests, the pellets treated with the acid-neutral fraction were sniffed at on a significantly greater number of occasions ($P < 0.05$) than were those impregnated with the basic fraction. Chinning was also more intensive in relation to the pellets treated with the acid-neutral fraction, but because of the low overall incidence of this form of activity tests for significance would be meaningless. The most obvious differences in response to the basic and acid-neutral fractions were in the forms of behavior, termed cautious approach and avoidance. In the first, the rabbit faced the object and approached very slowly with the body close to the ground and head and ears stretched forward. This is characteristic of the exploratory movements of a rabbit introduced into the territory occupied by another social group. Both the incidence and duration of cautious approaches were much greater in relation to the acid-neutral than the basic fraction ($P < 0.001$).

Some individuals, after sniffing at the stimulus pellets hastily withdrew from them, often jumping or running from their vicinity. This reaction, termed an avoidance, was elicited in 12 animals on 25 occasions by the acid-neutral fraction but only once in one rabbit by the basic fraction ($P < 0.001$).

The responses of rabbits in the separate groups did not vary significantly, hence the results could not be biased due to the characteristics of individual animals.

In the second series of tests no significant differences were seen in reactions to pellets treated with the acid and the neutral fractions of the anal

TABLE 1. INCIDENCE AND DURATION OF OVERT RESPONSES OF YOUNG RABBITS TO THE BASIC, ACID–NEUTRAL, ACID, AND NEUTRAL FRACTIONS OF MALE ANAL GLAND EXTRACTS

	Sniffing			Chinning			Cautious approach			Avoidance	
	Individuals (*N*)	Occasions (*N*)	Total duration (sec)	Individuals (*N*)	Occasions (*N*)	Total duration (sec)	Individuals (*N*)	Occasions (*N*)	Total duration (sec)	Individuals (*N*)	Occasions (*N*)
Number of rabbits tested = 39											
Basic fraction	38	87	577	2	5	4	2	2	4	1	1
Acid-neutral fraction	35	117[a]	516(N.S.)	3	8	15	20	41[b]	73[b]	12	25[b]
Number of rabbits tested = 33											
Neutral fraction	24	55	190	1	1	1	10	11	20	11	16
Acid fraction	27	55 (N.S.)	208 (N.S.)	0	—	—	13	16	27 (N.S.)	6	7

[a] $P < 0.01$
[b] $P < 0.001$

TABLE 2. NUMBER OF NOSTRIL MOVEMENTS MADE BY NESTLING RABBITS DURING 15-SECOND PRESENTATIONS OF DIFFERENT ODOR STIMULI

Stimulus	Number of nostril movements	Significance of difference from ether control
Neutral fraction	25.7	$P < 0.001$
Acid-neutral fraction	22.5	$P < 0.001$
Acid fraction	21.7	$P < 0.001$
Stranger's feces	16.5	$P < 0.001$
Basic fraction	15.5	$P < 0.01$
Familiar feces	11.2	N.S.
Ether control	7.9	—

gland. Both stimuli elicited a somewhat lower level of response than that produced by the acid-neutral fraction in the first series.

Reactions of Nestlings to Different Odor Stimuli

Frequency of Sniffing. The mean frequencies of sniffing indicated by the nostril movements of nestlings during the 15 sec of presentation of the olfactory stimuli are shown in Table 2. Frequencies were highest during stimulation by the neutral, acid-neutral, and acid fractions of the anal gland (25.7,

TABLE 3. PERCENTAGES OF RABBIT NESTLINGS SHOWING DIFFERENT OVERT RESPONSES TO VARIOUS ODOR STIMULI

Stimulus	Grimace	Avoidance	Suspension of sniffing	Attraction
Neutral fraction	16.1	32.2	9.7	6.4
Acid-neutral fraction	16.1	41.9	19.4	3.2
Acid fraction	25.8	29.0	16.1	0
Stranger's feces	6.4	22.6	16.1	12.9
Basic fraction	9.7	12.9	19.4	3.2
Familiar feces	0	12.9	0	3.2
Ether control	0	3.2	6.5	3.2
	$Q^a = 20.38^b$	$Q = 20.19^b$	$Q = 11.44$ (N.S.)	$Q = 4.89$ (N.S.)

[a] Q represents test for significance between proportions in matched samples (Cochran, 1950).
[b] $P < 0.01$.

22.5, and 21.7 movements, respectively) and lowest in response to the ether control swab (7.9 sniffs). All odors except the mother's fecal pellets produced significantly higher sniffing rates than the control ($P < 0.01$ or less).

Overt Responses. The percentages of nestlings showing overt responses in the form of grimacing, avoidance, suspension of sniffing, and attraction to the different odors are summarized in Table 3.

Grimacing was elicited most frequently by the acid (25.8%), acid-neutral (16.1%), and neutral (16.1%) fractions of the male anal gland but never occurred in response to the mothers' fecal pellets or the ether control swab. The overall differences were statistically significant ($P < 0.01$), but this test does not refer to differences between individual odor stimuli.

Variations in suspension of sniffing and attraction in relation to the different odors were not statistically significant.

Heart-Rate Changes

For the statistical analysis of the heart rate changes the data for each of the three 15-sec periods before, during, and after presentation were subdivided into two 7.5-sec intervals.

Figure 2 shows the changes in the mean heart rates of the nestlings in relation to the 7 different olfactory stimuli. There were no significant differences in mean rates before presentation of the stimuli (intervals B_1 and B_2). A decrease in heart rate is evident in response to most of the odors during the first 7.5 sec of presentation (D_1), and further depression of heart rate occurred during the second half of the presentation period (D_2), at which point the overall differences in mean heart rates are highly statistically significant ($P < 0.001$).

The two stimuli in the form of fecal pellets did not significantly affect heart rate. Some deceleration occurred in response to the control swab and the basic and neutral fractions of the male anal gland, but the decrease in heart rate was most marked during exposure to the acid and acid-neutral fractions.

For the five odors which produced deceleratory changes a progressive return towards the prestimulus rates occurred after their withdrawal, although none recovered fully within the 15-sec period and overall differences between the mean heart rates remained statistically significant ($P < 0.001$ and $P < 0.05$ at A_1 and A_2, respectively). Differentiation between three groups of stimuli is evident from the statistical tests carried out on mean rates in the second half of the presentation period (D_2) and first half of the postpresentation period (A_1). The rates for the basic and neutral fractions and ether control differed significantly ($P < 0.001$) from those for the 2 fecal pellet stimuli as well as for the acid and acid-neutral fractions of the anal gland.

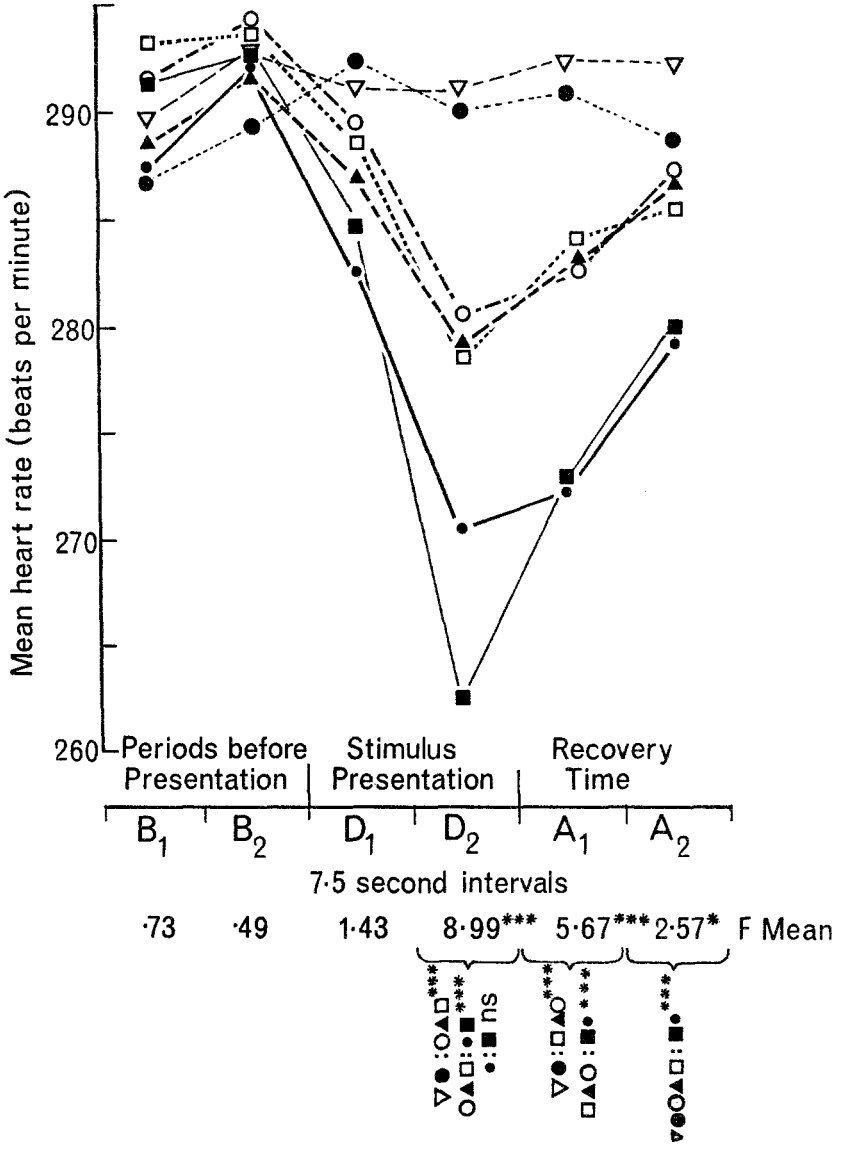

FIG. 2. Changes in mean heart rates of nestling rabbits in response to different odor stimuli. ●, acid-neutral fraction; ▲, basic fraction; ■, acid fraction; □, neutral fraction; ⬤, stranger's feces; ▽, familiar feces; ○, ether control. ns, not significant; *, $P < 0.05$; ***, $P < 0\ 001$

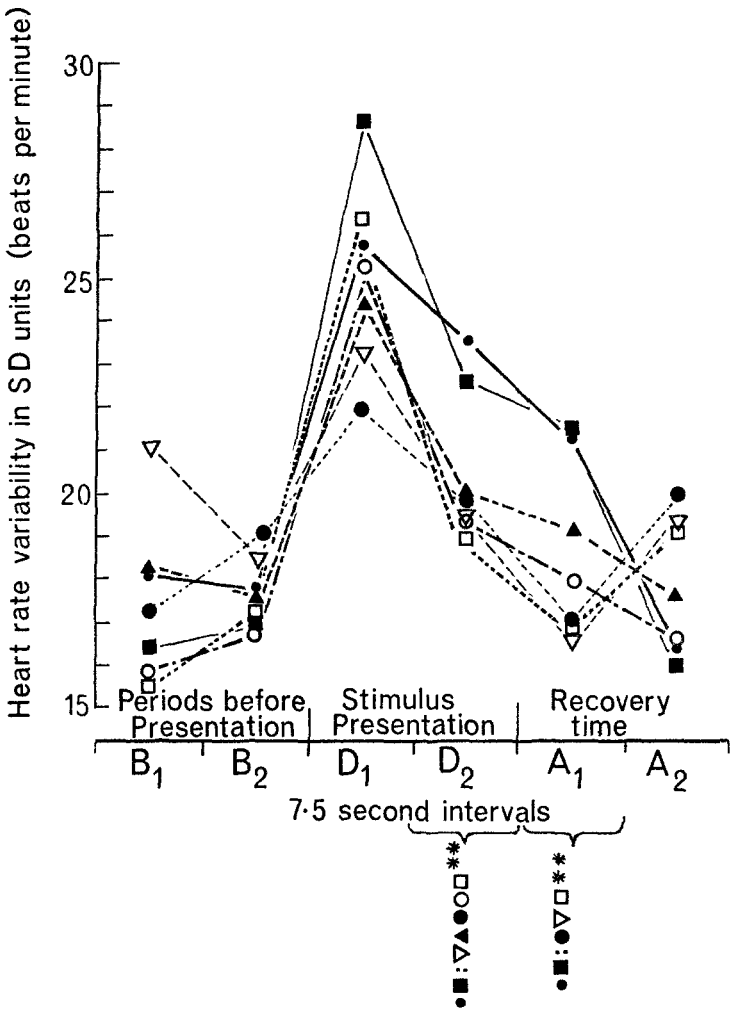

FIG. 3. Changes in mean variabilities of heart rates of nestling rabbits in response to different odor stimuli. ●, acid-neutral fraction; ▲, basic fraction; ■, acid fraction; □, neutral fraction; ⬤, stranger's feces; ▽, familiar feces; ○, ether control. **, $P < 0.01$.

Changes in the mean individual variabilities of the kittens' heart rates are shown in Figure 3. All stimuli produced some increase in heart-rate variability and in every case this reached its maximum level in the first 7.5-sec interval of the presentation period (D_1). The greatest variabilities were shown in this interval in response to the acid, neutral, and acid-neutral fractions of

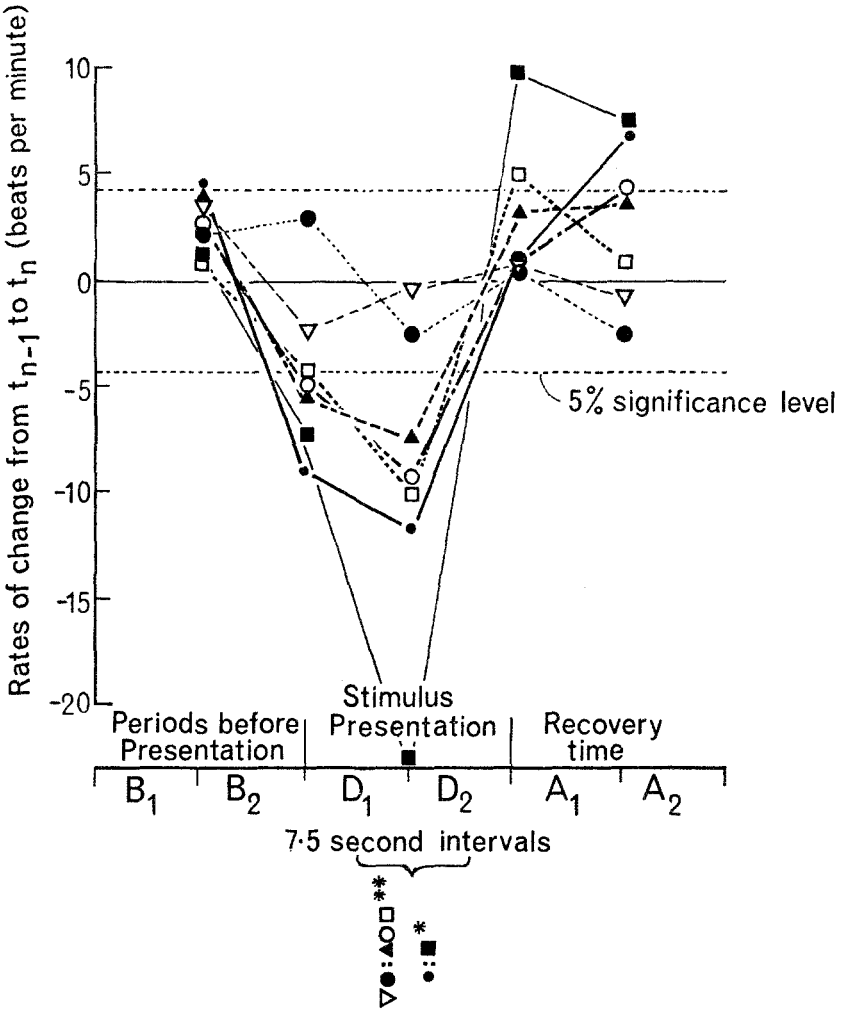

FIG. 4. The rates of change in mean heart rates of nestling rabbits in response to different odor stimuli. ●, acid-neutral fraction; ▲, basic fraction; ■, acid fraction; □, neutral fraction; ⬤, stranger's feces; ▽, familiar feces; ○, ether control. *, $P<0.05$; **, $P<0.01$.

the male anal gland. The increase in heart-rate variabilities obtained during tests with the acid and acid-neutral fractions persisted for the longest period and were significantly greater ($P<0.01$) than for the other stimuli during the D_2 intervals.

In Figure 4 the rates of change of heart rate between the successive 7.5-sec intervals are presented graphically. This again illustrates the marked

deceleration in heart rate produced by all stimuli except the 2 fecal pellet samples. The greater rate of change and the longer recovery period after stimulation by the acid, acid-neutral, and neutral fractions are evident.

DISCUSSION

This study is a part of a detailed investigation into the behavioral role of the anal gland secretion of rabbits in which attention is also being paid to its chemical composition.

The nature of the chemical components of the secretion will be reported elsewhere. The results presented here indicate that behaviorally important components are contained in both the acid and neutral soluble fractions of the gland secretion. Aliphatic acids have been identified as the behaviorally potent components in the odoriferous secretions of a number of mammals including the red fox (Albone and Fox, 1971), the macaque (Michael et al., 1971), pronghorn antelope (Müller-Schwarze et al., 1974), and Mongolian gerbil (Thiessen et al., 1974).

The overt responses of the rabbits towards the acid and neutral fractions were mainly of an avoidance type and are in line with the information on the repelling effects of strange territory (Mykytowycz, 1968). More specific reactions could not be expected considering the artificiality of the experimental situation. The results indicate only in a general way whether the rabbits were indifferent or attentive to particular odors, thus suggesting which fractions would merit further investigation. The strength of the reactions elicited by the acid-neutral fraction suggests the possibility of a synergistic effect which may have to be considered in attempts to pinpoint the behaviorally most important compounds.

Another object of this study was to test the usefulness of heart-rate measures as an indicator of the rabbits' response to olfactory stimuli. In the past, experimental psychologists have frequently used this technique to measure the responses of man (Lacey and Lacey, 1970) as well as animals (Frisch, 1965) to visual, acoustic, and tactile stimuli. This method has also been used in the course of olfactory studies in birds (Wenzel and Sieck, 1972). While there is uncertainty concerning the underlying mechanisms (Kagan and Lewis, 1965) and the most suitable methods of analysis (Firth, 1973), there is general agreement that the cardiovascular system is a delicate response mechanism capable of revealing individual variations in reaction to stimuli.

In the results presented above there is a clear correlation between the changes in heart rate and the overt responses of the rabbits to the same stimuli. Although there are some unresolved problems associated with the use of the technique in studies with rabbits, such as why acceleration in heart rate

was never observed, or what is the most suitable method of analysis of the data, the usefulness of the technique in studies of the behavioral role of odors is obvious.

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SEXUAL COMMUNICATION AND ASSOCIATIVE LEARNING IN THE PARASITIC WASP *Itoplectis conquisitor* (SAY)

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Abstract—Extracts of newly emerged virgin females and newly emerged virgin males of the parasitic wasp *Itoplectis conquisitor* were highly active in eliciting sexual behavior in older males. Newly emerged males did not respond to the extracts. A reliable biological assay was devised in which the responses of males to extract could be evaluated under varying conditions. Optimum male responses to female extract were obtained at 27°C and at 1.0 female equivalents (the amount of pheromone extractable from the body surface of a female). A decrease in sexual response was observed when males were repeatedly exposed to pheromone. Males also learned to associate bioassay conditions with the presence of pheromone.

Key Words—sex pheromone, learning, *Itoplectis conquisitor* (Say).

INTRODUCTION

Recent years have witnessed the development of a great amount of interest in the field of chemical communication among insects. Of particular concern has been the use of sex pheromones as a possible means of controlling or monitoring populations of undesirable insect species. Relatively few sex pheromones, however, have been studied in beneficial insects, e.g., the parasitic Hymenoptera. Among the Hymenoptera reported to utilize sex pheromones are the braconids *Opius alloeus* (Boush and Baerwald, 1967) and *Bracon hebetor* (Grosch, 1948) and the ichneumonids *Apanteles medicagenis* (Cole, 1969) and *Campoletis sonorensis* (Vinson, 1972). Studies of chemical

signals used by insect parasites may provide ways to monitor beneficial insect populations and subsequently aid in the development of management programs which rely on entomophagous insects for pest control.

During laboratory rearing of the parasitic wasp *Itoplectis conquisitor* (Say) (Hymenoptera: Ichneumonidae), observations of mating behavior suggested the presence of chemical communicating agents. Newly emerged females elicited orientation, rapid wing fluttering, upward bending of the abdomen, and copulatory attempts from *Itoplectis* males. Moreover, newly emerged males[1] elicited identical sexual behavior from older males.[2] Mated females and older males failed to elicit equally intense sexual behavior from males. These observations led to a more controlled study of sexual communication in *I. conquisitor*.

METHODS AND MATERIALS

Laboratory Rearing

A laboratory culture of *I. conquisitor* was maintained on pupae of the lepidopteran host, *Trichoplusia ni* (Hübner) (the cabbage looper). Adult parasites were fed pupae of *T. ni* and a 20% aqueous solution of honey from the time of emergence. The culture was maintained at a temperature of $26 \pm 2°C$ and a light regime of 15 hr L/9 D using fluorescent and Gro-lux® lights.

Preparation of Extracts

Extracts of females were prepared by washing newly emerged virgin females with approximately 0.1 ml of spectrograde methanol and filtering through a plug of glass wool in a pipet. Extracts were stored at 0° C.

Extracts of newly emerged virgin males were prepared in a similar manner. Males used for extraction emerged from pupae which were isolated from other insects from the time they were stung. This precaution was taken to prevent contamination by female wasps.

The amount of pheromone which could be washed from one wasp by the method described was termed an equivalent. Washings from female wasps were thus termed female equivalents (FEQ); washings from male wasps were termed male equivalents (MEQ).

Bioassay

Bioassay chambers (Figure 1) were constructed from inverted, clear

[1] Males under 24 hr old.
[2] Males 1–10 days old.

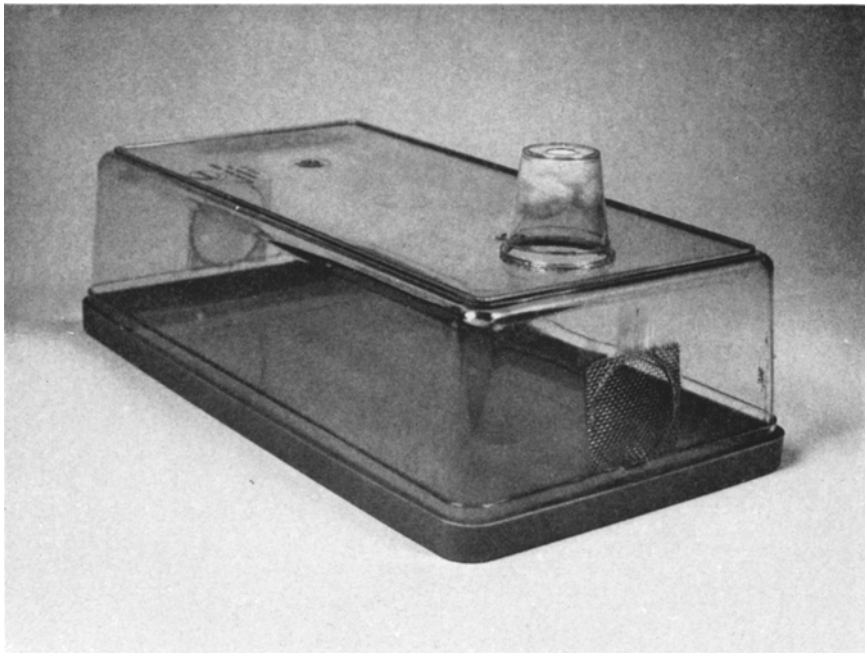

FIG. 1. Laboratory bioassay chamber used to test responses of male *I. conquisitor* to sex pheromone. The sample was placed on cotton in the sample cup on the chamber.

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plastic shoe boxes (Hugh H. Wilson Company, Wilson Manufacturing Corporation) measuring approximately $30 \times 15 \times 9.5$ cm. Three circular openings, each 4 cm in diameter, were made in each box. Two openings were made on opposite ends of the box and covered with wire mesh to allow free air flow through the chamber. The third opening, for introducing samples, was made in the top of the chamber with its center about 4.5 cm from one end. A small jelly cup, 4.5 cm in diameter at its open end but tapering to 3 cm in diameter at its closed end, fit into this opening to prevent insects from escaping. A 20% aqueous solution of honey was kept inside each chamber near the end opposite the sample opening. Five virgin males were released inside each chamber; a test using one chamber was defined as one replicate.

Unless stated otherwise, the following conditions were constant for each experiment. Laboratory temperature was maintained at $26 \pm 2°C$ with lighting furnished by overhead cool white fluorescent and Gro-lux® lights. During bioassays, each chamber was oriented with the sample opening away from the highest light intensity in the room. The positive phototropic response of the insects kept them away from the sample opening. An air flow of 0.1 m/sec was created inside the chamber by a fan located about 2 m from the sample opening. Samples were introduced in 10μl of methanol spotted on a ball of cotton (approximately 4 cm in diameter) pressed into a jelly cup of the previously described dimensions. The cup was then inverted and placed over the sample opening and the wasps' activity was observed for 3 min. Depending on the experiment, responses were recorded as follows:

1. Attractions—the number of wasps to contact the cotton in the cup;
2. Time of first attraction—the time recorded for the first wasp to be attracted to the cotton;
3. Upward bending of the abdomen—the number of wasps to bend the posterior segments of the abdomen upward;
4. Copulations—the number of wasps to attempt copulation with the cotton.

Behavioral Tests

Male and Female Responses to Male and Female Extracts. One equivalent of female extract and one equivalent of male extract were bioassayed on 2–10-day-old males and females in separate experiments. Two replicates of each of the 4 combinations were tested. Female extract (0.1 FEQ) was also bioassayed on 5 replicates of newly emerged males and 5 replicates of 1-day-old males.

Male Responses to Varying Amounts of Female Extract. Female extracts were diluted with methanol to 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} FEQ and con-

centrated to 10 FEQ. All concentrations including 1 FEQ were tested on 2–7-day-old male wasps that were previously unexposed to pheromone testing. Ten replicates were performed at each concentration except 10 FEQ for which 8 replicates were tested.

Effect of Temperature on Male Sexual Responsiveness. Males (2–7 days old) previously unexposed to pheromone testing, were maintained at each of 7 temperatures in a Sherer Controlled Environment Lab (Model CEL 257 HL, Sherer-Gilbert Co.). The temperatures $\pm 0.5°$, were 16, 21, 24, 27, 29, 32, and 39°C. The wasps remained at their respective temperatures for 3 hr before 0.1 FEQ was applied to each box. 12 replicates were tested at each temperature.

Tests for Possible Desensitization Due to Pheromone Exposure. Males (2–7 days old) were tested with 0.1 FEQ of pheromone for 3-min periods, each separated by 1 hr, for 4 consecutive hr and then at 24 hr after the first dosage. Males used in this test were previously unexposed to pheromone testing; 20 replicates were conducted.

Test for Associative Learning of Males. Males (2–7 days old) were given 3-min exposures to sample cups containing only cotton for each of 2 consecutive hr. The following 2 hr, the same wasps were given 3-min exposures to 0.1 FEQ in identical cups. During the next 2 hr, the wasps were again given blank cups. 20 replicates were conducted. A control experiment was carried out giving males a blank cup for 3-min exposures every hour for 6 consecutive hr. 10 replicates were performed. A second control experiment in which 0.1 FEQ was placed on a piece of filter paper located on the upwind screen during hours 2 and 3 was also conducted. Blank cups were used each hour; 10 replicates were performed.

RESULTS AND DISCUSSION

Males whose ages range from 1–10 days responded to extracts of virgin females with typical sexual behavior. The usual pattern of events was orientation toward the source of extract and rapid wing fluttering concomitant with bending of the posterior segments of the abdomen into the air as the source was approached. Copulatory responses were elicited when the source was contacted. Responses of males (1–10 days old) to extract of newly emerged males were identical to the responses to female extract. Newly emerged males did not respond in this manner and seemed to be nearly insensitive to the pheromone (Table 1). Females did not respond to either male or female extract.

Apparently, pheromone is present on the body surfaces of both newly emerged males and females; however, the mechanisms involved in this

TABLE 1. MALE AND FEMALE RESPONSES TO NEWLY EMERGED, VIRGIN MALE AND FEMALE EXTRACTS AND RESPONSES OF NEWLY EMERGED AND 1-DAY-OLD MALES TO NEWLY EMERGED, VIRGIN FEMALE EXTRACT IN *I. conquisitor*

Responses of:	Attractions	Upward abdomen bending	Copulation
2–10-day-old males to 1.0 FEQ[a]	9	5	8
2–10-day-old males to 1.0 MEQ[a]	9	6	8
2–10-day-old females to 1.0 FEQ[a]	0	0	0
2–10-day-old females to 1.0 MEQ[a]	0	0	0
Newly emerged males to 0.1 FEQ[b]	4	0	1
1-day-old males to 0.1 FEQ[b]	23	18	21

[a] Based on 2 replicates.
[b] Based on 5 replicates.

phenomenon are not clear. One possibility is that the parasitic larvae of both sexes sequester the pheromone from the nutrients in their host pupae (similar mechanisms have been described for Lepidoptera; Hendry et al., 1975). Another possibility is that both males and females have the ability to biosynthesize pheromone at some time during growth or maturation. Biosynthesis of insect pheromones has been reported (Kasang et al., 1974; Hughes, 1974; Mitlin and Hedin, 1974).

As the amount of pheromone was increased from 10^{-4} to 10^{0} FEQ, a corresponding increase in attractions and copulations was observed (Figure 2). While a further increase in the amount of pheromone to 10 FEQ had little effect on the amount of sexual activity at the pheromone source, it actually increased the general sexual activity of the wasps in the chamber (similar results were reported by Hendry et al., 1973). Attempted copulation with the sides of the chamber and with each other was common. The observation that at high levels of pheromone sexual communication disruption seems to occur is consistent with studies of other insects (Shorey et al., 1972; Hendry et al., 1973). The mean time for the first attraction per replicate also reaches an optimum at the higher concentrations of pheromone.[3]

The effect of varying temperatures on the sexual responsiveness of male wasps is summarized in Figure 3. Both the number of wasps attracted and the number of copulations appear to peak at 27°C. The optimum temperature range is probably much broader, as is indicated by the confidence intervals. It is possible that the optimum temperature observed in this experiment could be related to the temperature at which the wasps were reared ($26 \pm 2°$). In this regard, before mass-release programs for *I. conquisitor* are undertaken, this parameter should be further explored.

[3] If no wasps were attracted, 180 sec was scored.

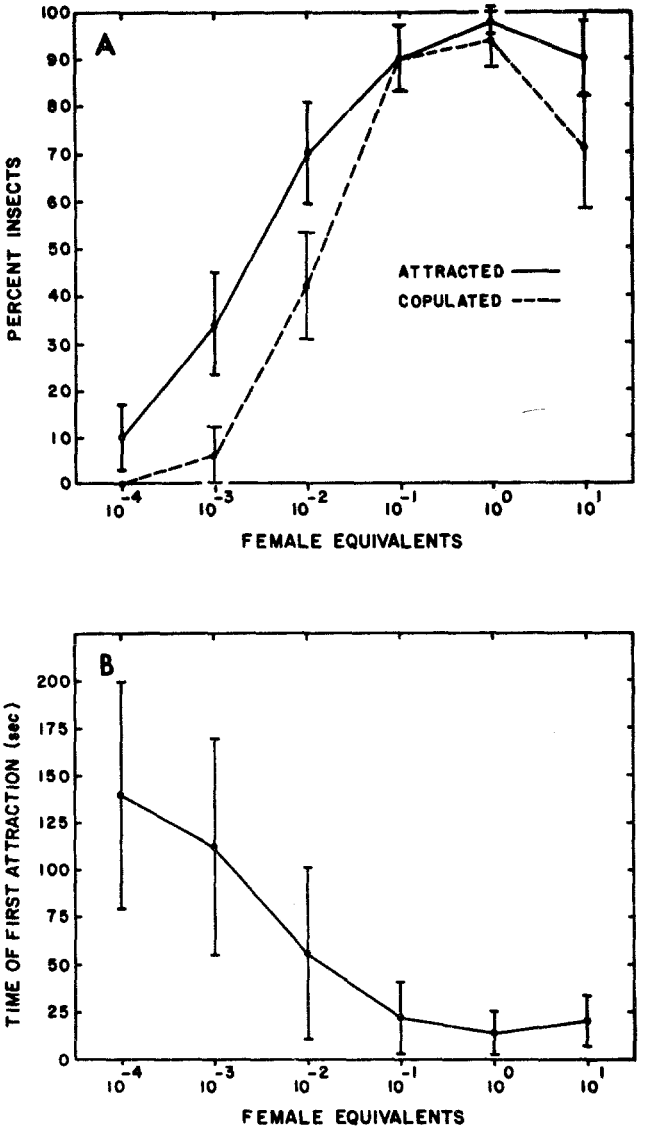

FIG. 2. Responses of *I. conquisitor* males in the bioassay chamber to varying amounts of pheromone in 10 μl of methanol. 10 replicates were conducted for each concentration except 10 FEQ for which 8 replicates were tested. (A, top) Percentage of males which were attracted to or copulated with the pheromone source. Error bars represent 90% confidence intervals. (B, bottom) Average time required for 1st male (of 5) to be attracted to the pheromone source. Error bars represent standard deviations.

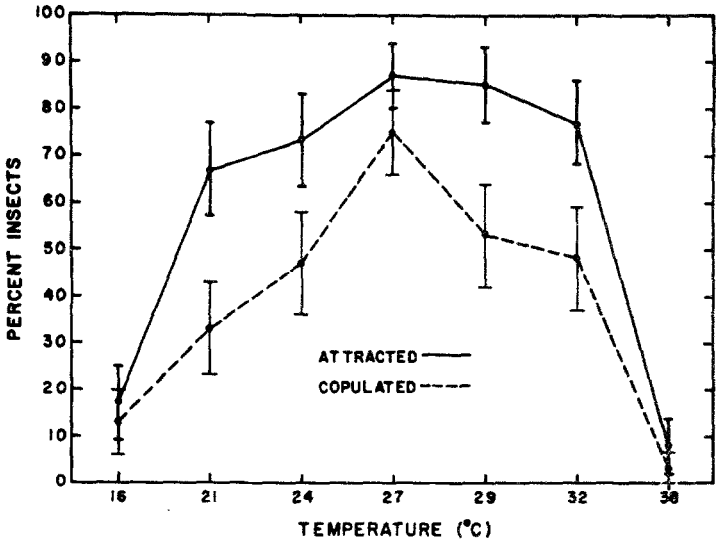

FIG. 3. Percent response of *I. conquisitor* males to 0.1 FEQ at various temperatures. 12 replicates were tested at each temperature; error bars represent 90% confidence intervals.

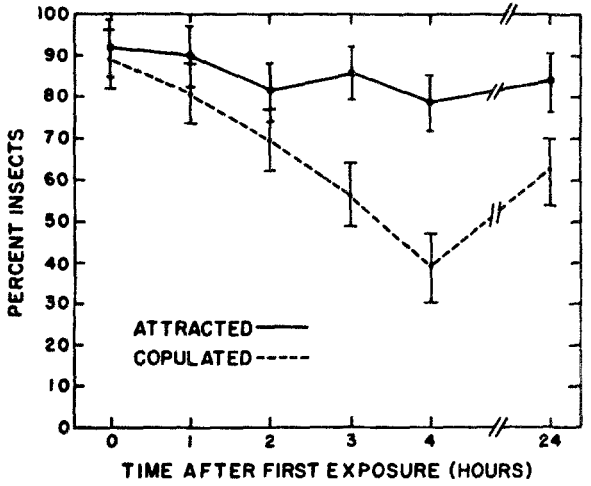

FIG. 4. Percent response of *I. conquisitor* males to pheromone after repeated exposure. 20 replicates were performed; error bars represent 90% confidence intervals.

TABLE 2. LEARNING STUDIES—RESPONSES OF *I. conquisitor* MALES TO BLANK SAMPLE CUPS AFTER EXPOSURE TO PHEROMONE IN IDENTICAL CUPS (20 REPLICATES)

Time	Sample	Attractions	Upward abdomen bending	Copulation
Hour 0	Blank	5 (1)[a] (3)[b]	0	0
Hour 1	Blank	8 (1)[a] (5)[b]	0	0
Hour 2	0.1 FEQ	94 (3)[a] (6)[b]	45	84
Hour 3	0.1 FEQ	88 (2)[a] (2)[b]	43	81
Hour 4	Blank	41 (1)[a] (1)[b]	5	0
Hour 5	Blank	25 (5)[a] (0)[b]	1	0

[a] Control test. In a separate experiment, a blank cup was used as the sample for every test; the number of attractions are in parentheses. No upward abdomen bending or copulation was observed in 10 replicates.

[b] Control test. A blank cup was used as the sample for every test. During hours 2 and 3, 0.1 FEQ was placed on a piece of filter paper located on the upwind screen of the chambers. The attractions are shown in parentheses. No upward adomen bending was observed during hours 0, 1, 4, or 5 in 10 replicates.

The results of the desensitization experiments are shown in Figure 4. The number of wasps attracted to the pheromone source remained nearly constant throughout the experiment, possibly due to the wasps learning where the source was located. The number of wasps to attempt copulation showed a definite decrease over the first 4 hr, then increased slightly after 24 hr. The mechanism for this desensitization process is unclear, but might be critical to mass release of insect parasites. One artifact which cannot be ruled out is that the males could have learned that no females were present at the pheromone source.

Experiments to test possible learning by *I. conquisitor* males are summarized in Table 2. The large number of attractions to the blank cups after exposure to pheromone demonstrates the ability of the wasps to learn. In addition to the increase in attraction, a few wasps bent their abdomens up while approaching the blank cup. However, no wasps attempted to copulate with the cotton. The number of attractions to the blank cups used in the control tests did not change significantly throughout the experiments. Furthermore, no wasps bent their abdomens up or attempted copulation with the control cups.

Arthur (1966, 1967) demonstrated that females of *I. conquisitor* learn to discern host shelter differences. The finding that males also learn quickly

may be the expected result. Exactly what factors *Itoplectis* learns to associate with the presence of pheromone are not known. Most likely, they learn to associate both the location of the cup and the air flow through the chamber with the presence of pheromone in the cup. Such use of landmarks for insect navigation has long been known in honeybees (Wolf, 1926) and in other insects such as the hymenopteran *Ammophila campestris* (Baerends, 1941).

It is clear that *I. conquisitor* utilizes an intriguing chemical communication system for sexual activity. To our knowledge, this is the first report which suggests that insects may learn to associate external stimuli with olfactory perception of pheromone. Also of interest is the observation that newly emerged males may contain detectable amounts of pheromone. The exact mechanisms consistent with these and other observations of the sexual behavior of *I. conquisitor* remain to be elucidated but clearly merit further investigation.

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SPECIFIC ANOSMIA TO TRIMETHYLAMINE: THE FISHY PRIMARY ODOR

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Abstract—About 7% of human subjects are specifically anosmic to the odor of trimethylamine. Odor threshold measurements on 16 aliphatic amines were made with panels of specific anosmics and normal observers. The anosmia is most pronounced with low-molecular-weight tertiary amines, but is also observed in lesser degree with primary and secondary amines. It is suggested that this specific anosmia corresponds with the absence of a new olfactory primary, the "fishy" odor. Trimethylamine has been reported to occur in human menstrual blood and in the anal gland of the red fox, implying some pheromonal function.

Key Words—amines, anosmia, fishy odor, odor thresholds, primary odor, specific anosmia, tertiary amines, trimethylamine.

INTRODUCTION

We are continuing a program of research aimed at identifying the primary odors of the human sense of smell. Our approach consists in mapping out the chemical extent of the corresponding specific anosmias, which occur quite commonly in the population (Amoore, 1967, 1969, 1970, 1974). As an incidental observation in the preceding paper of this series (Amoore et al., 1975a), we noted that 3 of our 70 panelists were about 1000 times less sensitive than the average observer to the odors of trimethylamine and *N*-methylpyrrolidine. The following experiments were designed to measure the extent and severity of this newly described variety of specific anosmia.

METHODS AND MATERIALS

Our general experimental procedures have been described in detail elsewhere (Amoore et al., 1968, 1975*a*). The odorants were commercially available reagents. Seven bases (methyl-, *n*-pentyl-, di-*n*-propyl-, trimethyl-, dimethylethyl- and methyldiethyl-amine, and ammonia) were purified by extracting a solution of the sulfate or hydrochloride 10 times with mineral oil. The other nine bases were purified by recrystallizing their hydrochlorides twice from ethanol. Tri-*n*-propylamine is somewhat unstable in alkaline solution.

For olfactory testing up to 13 binary steps above the average normal threshold, we employed plain 125-ml Erlenmeyer flasks capped with inverted polypropylene medicine cups (Amoore et al., 1975b). This reduces odorous contamination of the flasks by the subjects' fingers. At still higher concentrations, the use of the more air-tight ground-glass-stoppered flasks was continued (Amoore et al., 1968). Test solutions containing odorants at 10 steps or more above the normal threshold (i.e., more than 1000 times threshold) were presented to specific anosmics in a separate room. This avoided contaminating the atmosphere for the normal observers.

RESULTS

The average odor detection threshold (μ) for trimethylamine determined with normal observers was at dilution step 29.71, which corresponds with 0.000467 ppm free base (Figure 1). The standard deviation (σ) was ± 1.63 steps. Next, using concentration step 26 (0.0061 ppm, approximately 2σ above the normal threshold), we screened 286 laboratory personnel and found 21 specific anosmics (about 7% of our population). The detection thresholds for the anosmics were determined with a higher concentration series. The mean threshold for the specific anosmics to trimethylamine was at step 18.54 (1.07 ppm).

The difference in threshold (Δ) between the normals and the anosmics was 11.17 steps. On the average, the anosmics were 2290 times less sensitive than the normals to the odor of trimethylamine. One of our anosmics has a personal threshold of 400 ppm for this compound, or about 1 million times higher than the norm. For all subsequent tests on various odorants in this series, we used a panel of 14 trimethylamine anosmics and compared their average threshold with that obtained from a control panel of 51 normal observers not affected by this anosmia.

We have noticed a strong association between the incidences of anosmia to trimethylamine and anosmia to isovaleric acid. Of the 13 trimethylamine

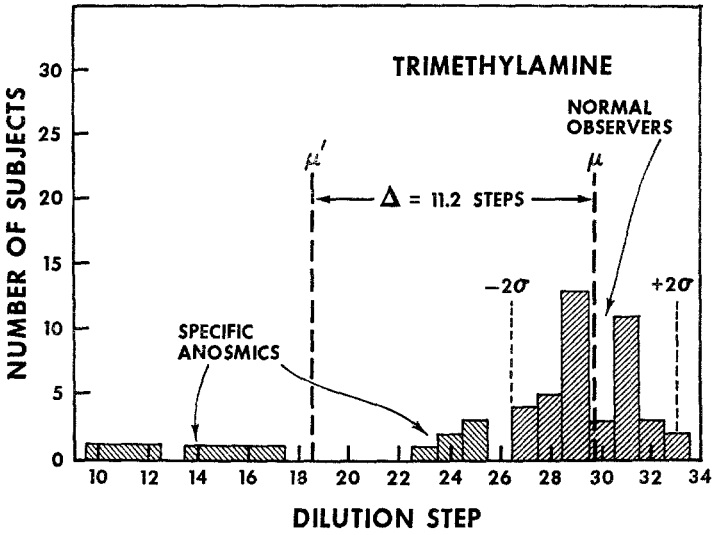

FIG. 1. Olfactory thresholds to trimethylamine. Each binary dilution step represents a halving of the concentration, starting from "0" for the saturated solution of the base in water (410,000 ppm at 19°).

anosmics who had previously been screened, there were 6 individuals who had qualified as specific anosmics to isovaleric acid. That is 46%, far higher than the 2% expected in the general population (Amoore, 1967). The converse is also true; of the 8 known isovaleric acid anosmics who were later screened with trimethylamine, the same 6 qualified for the present panel. This makes 75%, contrasting with the 7% in our population sample. Nevertheless, neither among our isovaleric acid anosmics, nor among our trimethylamine anosmics, were there any significant departures from the expected incidences of 1-pyrroline anosmia (20%; Amoore et al., 1975*a*) or pentadecalactone anosmia (9%; Whissell-Buechy and Amoore, 1973). We have no way at present of choosing among various hypotheses which might account for this remarkable and selective association between the isovaleric acid and trimethylamine anosmias.

In order to map out the chemical extent of the trimethylamine anosmia, we tested a total of 16 adequately purified odorants on our two panels of normal observers and specific anosmics to trimethylamine. The detailed results are given in Table 1. The compounds are all aliphatic amines or close relatives, selected by a trial-and-error procedure in order to determine what molecular features are associated with this variety of anosmia.

The lowest threshold was observed with trimethylamine itself, which is detectable by the average normal observer at 0.000467 ppm (less than 1 part

TABLE 1. SPECIFIC ANOSMIA TO TRIMETHYLAMINE AND OTHER ALIPHATIC AMINES

Odorous compound	Solubility in water[a] (ppm)	pK_a	Thresholds in water[b] Normal (ppm)	Thresholds in water[b] Anosmic (ppm)	Anosmics' defect[c] (steps)
Primary amines					
Methylamine (g)[d]	549,000	10.65	182	1,010	2.47
n-Propylamine	∞	10.58	62.4	378	2.60
n-Pentylamine	∞	10.64	9.21	122	3.73
n-Hexylamine	14,300	10.64	4.84	37.9	2.97
Secondary amines					
Dimethylamine (g)[d]	552,000	10.72	34.4	253	2.88
Diethylamine	∞	10.98	21.2	210	3.31
Di-*n*-propylamine	54,000	10.91	24.8	211	3.09
Piperidine	∞	11.00	70.6	569	3.01
Tertiary amines					
Trimethylamine (g)[d]	410,000(19°)	9.74	0.000467	1.07	11.17
Dimethylethylamine	∞	10.16	0.00762	4.01	9.04
Methyldiethylamine	∞	10.43	0.198	18.4	6.54
Triethylamine	101,000	10.87	2.21	36.4	4.04
Tri-*n*-propylamine	760	10.65	5.14	33.6	2.71
N-Methylpyrrolidine	∞	10.53	0.0167	12.4	9.54
N-Methylpiperidine	∞	10.19	1.17	41.7	5.16
Miscellaneous					
Ammonia (g)[d]	277,000	9.25	23.9	81.6	1.77
Isobutyl isobutyrate	670	—	2.67	7.41	1.47

[a] At 25° except where otherwise indicated.
[b] Concentration of free base in 0.1 M carbonate buffer, pH adjusted to the pK_a of the base.
[c] Anosmics' defect is the logarithm on base 2 of the ratio between the anosmic and normal thresholds. Average standard error 0.91 step (range 0.49–1.80).
[d] (g) = gas, concentration as w/v. All other compounds are liquids at 25°, and their concentrations are v/v.

by weight in 10^9 of water). This compound also demonstrated the greatest anosmic defect, 11.17 binary steps, with this group of anosmics. Hence, trimethylamine is the best chemical example of which we are aware for demonstrating this variety of specific anosmia.

Trimethylamine is the simplest tertiary amine, and it is noticeable that the tertiary amines as a class, and especially the lower members, exhibit much lower thresholds than their isomeric and/or homologous primary and secondary amines. They also (except for tri-*n*-propylamine) reveal substantially higher anosmic defects of 4–11 steps. These relationships are brought out in Figure 2, which shows the results of six successive additions of

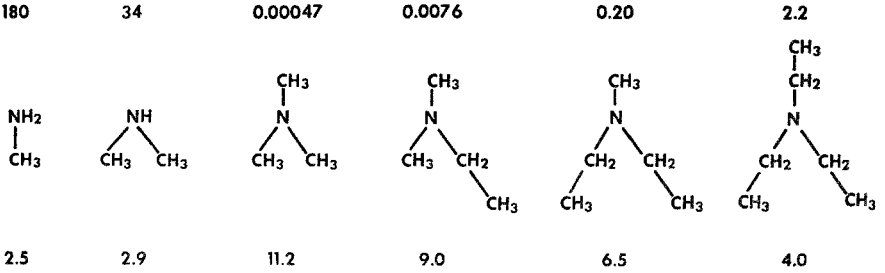

FIG. 2. Homologs and close chemical relatives of trimethylamine. The upper numbers represent their normal odor thresholds in ppm, and the lower numbers show the measured anosmic defect.

methylene groups to the parent molecule, ammonia. The odor detection threshold drops precipitously, by a factor of about 10^{-5}, on passing from dimethylamine to trimethylamine. This suggests that there is some extraordinary affinity of the normal olfactory organ for the simplest tertiary amine, presumably depending upon some special property of the tertiary nitrogen atom. Further additions of methylene groups seem to mollify this effect, perhaps by steric hindrance, until there is nothing very remarkable about the olfactory threshold of triethylamine. The specific anosmics, as a class, seem to be relatively oblivious to the presence or accessibility of the tertiary nitrogen atom. Accordingly, they exhibit anosmic defects which tend to vary inversely with the normal thresholds.

Remarkable as the properties of the tertiary amines are, it should not be overlooked that the specific anosmics systematically exhibited a rather general defect of about 3 steps towards all the primary and secondary amines tested. Nevertheless, their sensitivity approached more closely to normal when they were tested with isobutyl isobutyrate, an ester having a fruity odor utterly distinct from the amines ($\Delta = 1.47$ steps). They were only slightly deficient in detecting the pungent smell of ammonia.

From the available evidence we conclude that the key molecular feature in this type of specific anosmia is the $-N\langle$ functional group (amino), with a strong predilection for the tertiary form. These relationships remain much the same even if the nitrogen atom forms part of a heterocyclic system, as in piperidine which is a secondary amine, and *N*-methylpyrrolidine and *N*-methylpiperidine which are tertiary.

DISCUSSION

The experimental evidence presented in this paper demonstrates a previously unrecognized, but quite common, defect in the human olfactory mech-

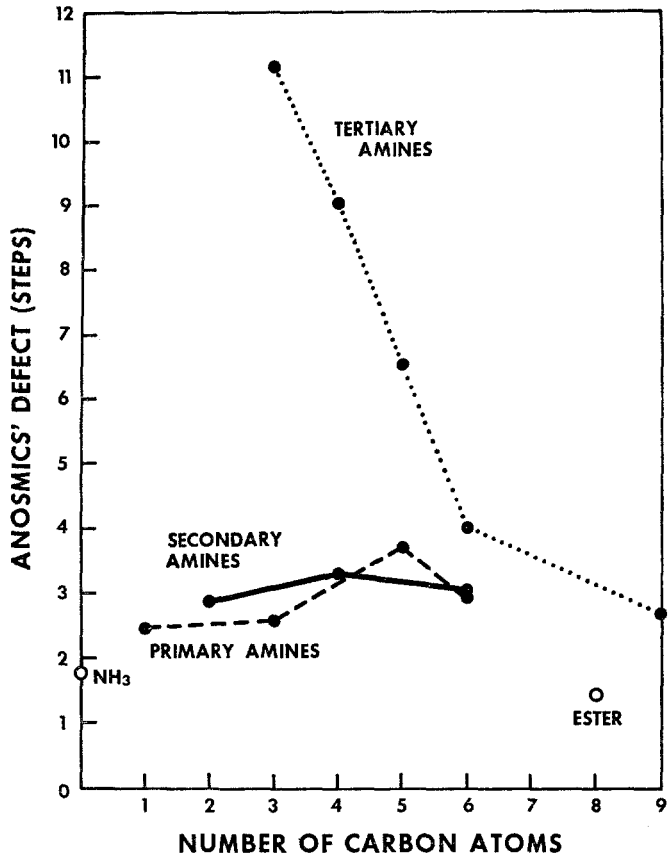

FIG. 3. Specific anosmia to aliphatic amines.

anism—specific anosmia to certain aliphatic amines, especially tertiary amines. The extent and severity of the defect is illustrated in Figure 3, which shows the anosmic defect as a function of number of carbon atoms in three homologous series of amines. The primary and secondary amines can be lumped together in the same range of 2.5–3.7 binary steps deficiency, with little effect of molecular size. The reality of this defect can be appreciated when it is compared with the distinctly lower values for ammonia and isobutyl isobutyrate. The tertiary amines are outstanding in the degree of anosmic defect they reveal, starting at the high point in the 3-carbon compound, and falling steeply and nearly linearly to the 6-carbon homolog.

Following the principle enunciated by Guillot (1948) and the rationale previously advanced (Amoore, 1967, 1970), we believe that the trimethylamine anosmia represents lack of the ability to perceive the corresponding

primary odor. The odor of trimethylamine is well known to organic chemists, who usually describe it unhesitatingly as fishy (e.g., Beilstein, 1922). The reason is clear: marine teleosts, such as the herring, retain high concentrations of trimethylamine oxide in the blood, probably to help counter the osmotic pressure of sea water. On the death of the fish, if it is not refrigerated, bacterial reduction sets in, freeing trimethylamine and generating the characteristic smell of (dead) fish. Indeed, the concentration of free trimethylamine is a widely used index for the freshness of commercial fish (Jones, 1967). In these circumstances it seems reasonable for us to adopt the name "fishy" for the new primary odor epitomized by trimethylamine.

A search through *Chemical Abstracts* reveals the occurrence of trimethylamine in many biological materials, not just in sea food. Another possible metabolic pathway leading to trimethylamine is from choline, which is a part of the lecithin molecule. It is also readily formed by bacterial action on betaine, which can cause taint in the milk of cows fed beet tops. Trimethylamine is probably the worst offender in the effluvia of cattle feedlots (Stephens, 1971).

There is a good deal of indirect evidence that trimethylamine may be an important mammalian pheromone. Albone and Fox (1971) found trimethylamine, in very variable amounts, in the anal gland of a female red fox. They did not report any behavioral research on this species. Nevertheless, their observation calls to mind the idiom about "dragging a red herring across the trail" to confuse the scent, both literally and figuratively. Even a fresh herring analyses at 200–800 ppm of trimethylamine (Okoloff, 1932). It was noted long ago by Linnaeus (1756) that the domestic dog is excessively interested in the odor of stinking goosefoot *Chenopodium vulvaria* L., whose leaves happen to contain about 400 ppm of trimethylamine (Cromwell and Richardson, 1966).

Trimethylamine was isolated from human menstrual blood by Klaus (1927), and it is probably responsible for the characteristic fishy odor of the discharge and of stale menstrual sweat. Evidently Linneaus was exhibiting his customary perceptive acumen when he named the above species of *Chenopodium, vulvaria.* Klaus also emphasized the quite well-known phenomenon that the odor of a menstruating woman brings many male animals into a state of sexual excitation. This suggests that trimethylamine might be a common estrus-signaling pheromone for several mammalian species. Nevertheless, the value of trimethylamine remains rather obscure for the human female, where ovulation (fertility) occurs some 10 days after menstruation ends, coinciding instead with an increased concentration of certain lower fatty acids, such as isobutyric and isovaleric, in the vaginal secretion (Michael et al., 1974).

The "sweaty" odor epitomized by isovaleric acid and its congeners was, in fact, the first primary to be elucidated by our specific anosmia method

(Amoore, 1967). This was followed by the "spermous" primary odor of 1-pyrroline (Amoore et al., 1975*a*), which may have some pheromonal significance. The successful identification of the third primary odor, the "fishy" smell of trimethylamine, increases our confidence in this experimental approach. The probable existence of many more primary odors is conjectured (Amoore, 1969), although only a few can be expected to convey true pheromonal information (Amoore, 1975). The remainder are likely to provide sensory input about foods, localities, and predators, to mention just a few possibilities.

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ODOR CORRESPONDENCE BETWEEN *Melitta* FEMALES AND MALES OF THEIR NEST PARASITE *Nomada flavopicta* K. (HYMENOPTERA: APOIDEA)[1]

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Abstract—Analyses have been performed with the help of combined capillary gas chromatography and mass spectrometry on volatile secretions emanating from the Dufour glands of female *Melitta haemorrhoidalis* and *M. leporina* and heads of male *Nomada flavopicta*, a nest parasite of the *Melitta* species. It was found that the cephalic secretions of *N. flavopicta* males have definite similarities with the Dufour gland secretions of the two species of *Melitta*. They all contain octadecyl butyrate as a major component. Monounsaturated alcohols of different chain lengths are also present in the secretions. Beside these compounds, *Melitta* has some other esters, notably C_{12}-, C_{14}-, C_{16}-, and C_{20}-butyrates as well as C_{12}- and C_{16}-acetates.

Key Words—*Melitta haemorrhoidalis*, *Melitta leporina*, *Nomada flavopicta*, Dufour gland, octadecyl butyrate.

INTRODUCTION

In an earlier publication (Tengö and Bergström, 1975) we reported on the relationship between volatile secretions of bees of the genus *Andrena* Fabr. and their nest parasites of the genus *Nomada* Scop. We found that the same

[1] This report forms part XIX of the series "Studies on Natural Odoriferous Compounds."

farnesyl or geranyl esters occurred in the cephalic secretion of the male *Nomada* as in the Dufour gland secretion of the female hosts, genus *Andrena*, We have now studied this type of relationship further. The present study concerns similarities between the volatile secretions of *N. flavopicta* K. and its hosts *Melitta leporina* Panz. and *M. haemorrhoidalis* Fabr.

METHODS AND MATERIALS

The biological material used in this study was collected in Öland, southern Sweden, in the neighborhood of the Ecological Station. Taxonomical determination of *Melitta* has been done according to Schmiedeknecht (1930) and for *Nomada* according to Stöckert in Schmiedeknecht (1930). The volatile secretions were extracted from heads or from Dufour glands with hexane. The further analyses of the material will be evident from Table 1.

The main analytical tools in the chemical analyses have been separate capillary gas chromatography and capillary gas chromatography coupled to mass spectrometry. The apparatus and equipment is the same as that used in earlier studies (Ställberg-Stenhagen, 1972; Tengö and Bergström, 1975).

TABLE 1. ANALYSES BY GAS CHROMATOGRAPHY AND MASS SPECTROMETRY OF VOLATILE SECRETIONS FROM *Melitta haemorrhoidalis*, *M. leporina*, AND *Nomada flavopicta*

Species	Year		Method of analysis
	Collection	Analysis	
M. haemorrhoidalis	1971	1971[a]	GC-MS[b]
♀, Dufour gland	1973	1974	GC-MS
	1974	1974	GC-MS
M. leporina	1971	1971[a]	GC-MS
♀, Dufour gland	1973	1973	GC[c]
N. flavopicta	1973	1974	GC[d] and GC-MS
♂, head			
N. flavopicta	1973	1974	GC
♀, head			

[a] Whole dissected gland used. In remaining analyses extracts in hexane were used.

[b, c, d] See Figures 1, 2, and 3, respectively, for actual analyses.

The specimens being used in the present study are deposited at the Ecological Station in Öland, together with gas chromatograms and mass spectra.

RESULTS

Dufour Gland Secretion of Melitta haemorrhoidalis *and* Melitta leporina

The chemical analyses were carried out by capillary gas chromatography and mass spectrometry. They showed that the volatile compounds, which emanate from the Dufour gland of *M. haemorrhoidalis*, are dominated by a series of straight-chain saturated butyrates, octadecyl butyrate being the most prominent one. Other straight-chain compounds present in smaller amounts are two saturated acetates and two monounsaturated alcohols. The mass spectra of the straight-chain saturated butyrates are characterized foremost by peaks at $m/e = 89$ and $m/e = 144$.

Figure 1 shows a typical capillary gas chromatogram obtained from an extract in hexane of a single Dufour gland of *M. haemorrhoidalis*. This is a recording from a capillary column coupled to the mass spectrometer. The vertical lines in the lower part of the chromatogram are from mass spectral recordings. The following substances have been identified. Butyrates: 5 = dodecyl butyrate, 9 = tetradecyl butyrate, 13 = hexadecyl butyrate, and 14 = octadecyl butyrate. Eicosyl butyrate is not shown in this chromatogram, but its presence has been established by other analyses (see Table 1). Acetates: 3 = dodecyl acetate and 10 = hexadecyl acetate. Monounsaturated alcohols: 6 = a hexadecenol and 11 = an octadecenol. Peak number 2 is due to a contamination.

A capillary gas chromatogram of an extract in hexane of one Dufour gland from *M. leporina* is shown in Figure 2. There are considerable similarities between this secretion and that of *M. haemorrhoidalis*. In this chromatogram peak number 1 corresponds to tetradecyl butyrate and peak number 2 is an octadecenol. Peaks number 3 and 4 correspond to hexadecyl butyrate and octadecyl butyrate, respectively. Eicosyl butyrate is also present, although it is not shown in the chromatogram. As shown in Table 1 these results have been obtained from some analyses by capillary gas chromatography alone and some in combination with mass spectrometry. Four *M. haemorrhoidalis* analyses were made by coupled gas chromatography and mass spectrometry in 1971 on material collected that year and in 1974 on material collected in 1973 and 1974. There is a very good agreement between these analyses. *M. leporina* has been analyzed in 1971 and 1973 on material collected in those years. Again the results from separate analyses are in good agreement, showing the constancy in the composition of the secretion.

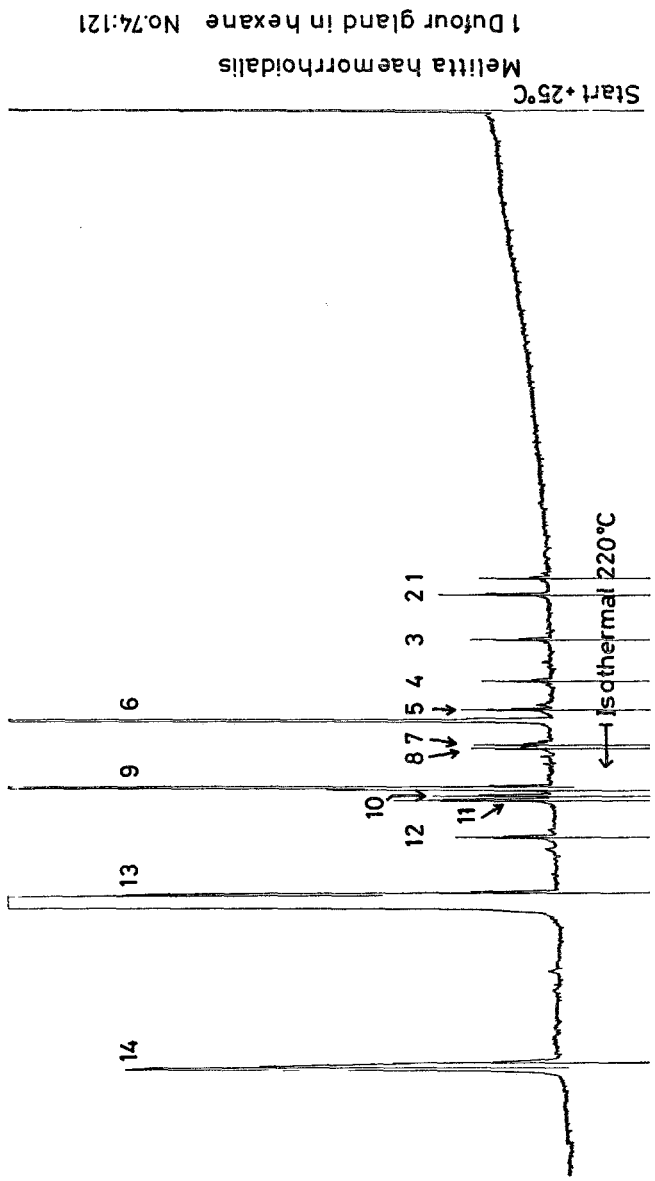

FIG. 1. Capillary gas chromatogram of an extract in hexane of a single Dufour gland from *Melitta haemorrhoidalis*.

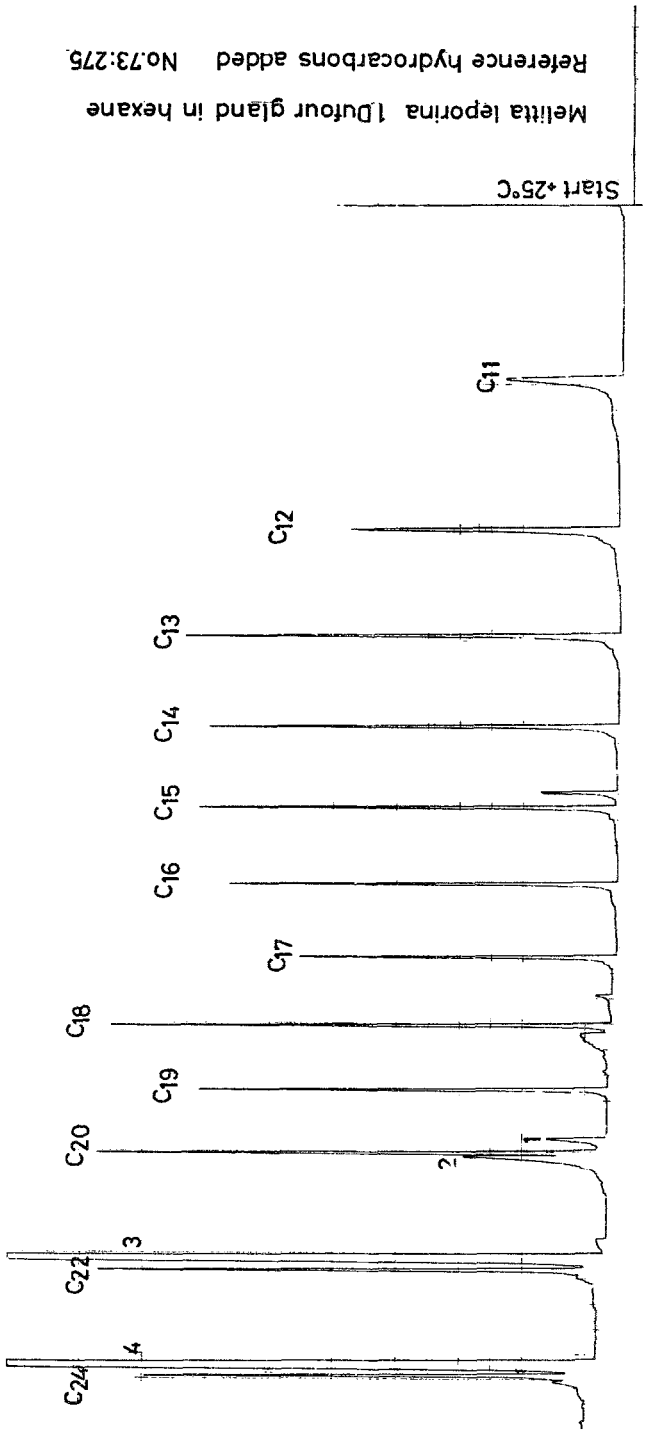

FIG. 2. Capillary gas chromatogram of an extract in hexane of a single Dufour gland from *Melitta leporina* with reference hydrocarbons added.

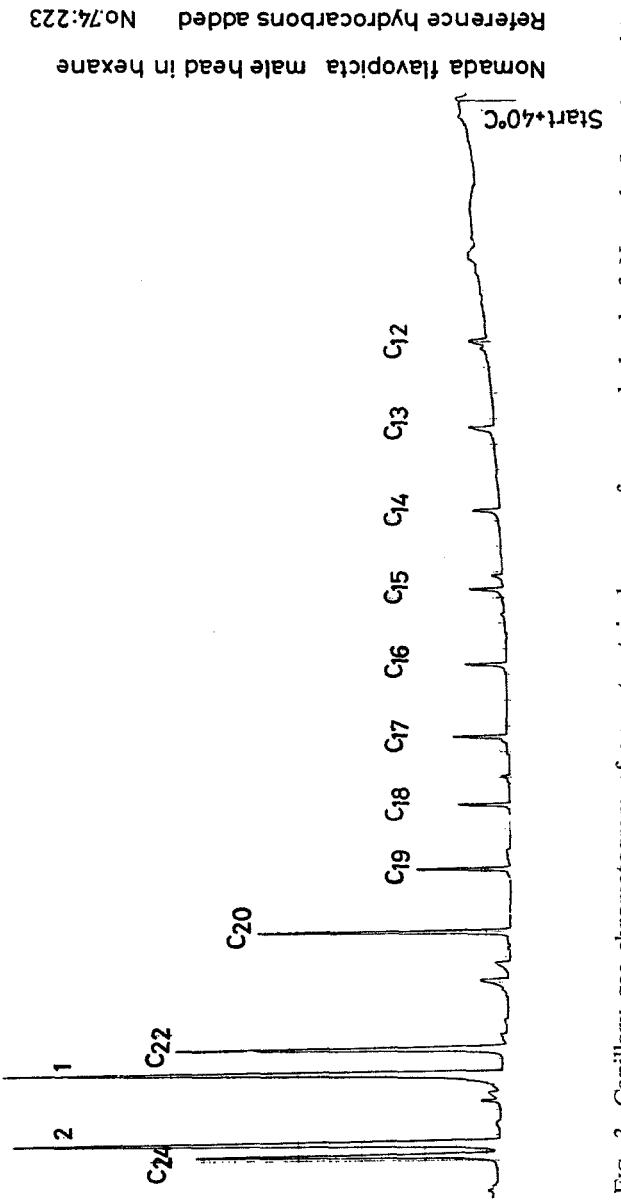

FIG. 3. Capillary gas chromatogram of an extract in hexane of one male head of *Nomada flavopicta* with reference hydrocarbons added.

Cephalic Secretion of Nomada flavopicta

A typical capillary gas chromatogram of the volatile compounds present in the secretion from heads of male *N. flavopicta* is given in Figure 3. The two dominating compounds in this secretion, components number 1 and 2, have been identified as an eicosenol and as octadecyl butyrate, respectively. The separate analyses performed are given in Table 1. All the material of *N. flavopicta* which has been analyzed in this work was collected in 1973. The separate capillary gas chromatographic analysis of *N. flavopicta* male head and that by combined capillary gas chromatography and mass spectrometry were made on different collections (different male individuals) of that year. There is good agreement between these analyses.

In the cephalic secretion of *N. flavopicta* there is evidently about equal amounts of the unsaturated C_{20}-alcohol and the C_{18}-butyrate. Analyses of the volatile compounds from heads of females of this species show that these substances are not present in the females. The females instead contain fairly large amounts of straight-chain hydrocarbons, viz., tricosane and pentacosane, as well as tricosenes and pentacosenes.

DISCUSSION

Comparison Between the Secretions of the Dufour Gland of Melitta haemorrhoidalis *and* M. leporina *and the Head of* Nomada flavopicta *male*

The intriguing finding of these analyses is that there are considerable similarities between host and nest-parasite secretions. For one thing, both contain octadecyl butyrate in relatively large amounts. Secondly, both contain unsaturated straight-chain alcohols, albeit of different chain lengths. *Melitta* possesses a C_{16} and a C_{18} unsaturated alcohol, whereas *Nomada* contains a C_{20} unsaturated alcohol.

It is interesting to compare these similarities with those between *Andrena* and *Nomada*. Even here, as reported recently (Tengö and Bergström, 1975), similarities were found between the secretions of the Dufour gland of the host female and the head of the parasite male. A discussion of this host–nest-parasite relationship with hypotheses on the functional significance will be given in a forthcoming paper.

Most species of *Nomada* are nest parasites of a certain species or a group of species of *Andrena*. The genera *Melitta* and *Andrena* are morphologically and ecologically alike, but in evolutionary relationship they are considered to belong to different families, Melittidae and Andrenidae (Michener, 1974). The genus *Nomada* is a member of a third bee family, Anthophoridae, tribe Nomadini, and is not closely related to its ordinary hosts.

TABLE 2. RESULTS OF CHEMICAL ANALYSES

Compounds identified	*M. haemorrhoidalis* ♀ Dufour gland	*M. leporina* ♀ Dufour gland	*N. flavopicta* ♂ head
Dodecyl butyrate	x[a]		
Tetradecyl butyrate	xx	xx	
Hexadecyl butyrate	xxx	xxx	
Octadecyl butyrate	xx	xxx	xxx
Eicosyl butyrate	x	xx	
Dodecyl acetate	x		
Hexadecyl acetate	x		
Hexadecenol[b]	xx		
Octadecenol[b]	x	xx	
Eicosenol[b]			xxx

[a] xxx, xx, and x indicate the relative amounts of respective compounds, xxx being the dominant one.
[b] Double-bond isomerism has not been determined.

Another host–parasite relationship encountered in our studies of biologically active, volatile compounds is the one between bumblebees of the genus *Bombus*, which are nest-parasitized by *Psithyrus*, another bumblebee genus. Many *Psithyrus* species are bound to specific *Bombus* species. In our studies about 25 species of bumblebees have so far been investigated. We have not found any evident paired host–parasite similarities in the composition of their male marking secretions (Kullenberg et al., 1970).

In the literature there are a number of reports on hosts of the genus *Andrena* for *N. flavopicta* (Alfken, 1914; Bischoff, 1927; Friese, 1923; Möschler, 1938; Stöckert, 1930). Wolf (1950) is of the opinion that *Melitta leporina* is the host of *N. flavopicta*. He finds good correlation in phenology, choice of biotope, and distribution between the two species. For the same reason, Wolf considers *Nomada emarginata* Mor. to be a nest parasite of *Melitta haemorrhoidalis*. The latter *Nomada* species has not been found in our study area on the island of Öland where *M. haemorrhoidalis* is rather abundant. As shown in Table 2 the secretion of *N. flavopicta* is more similar to that of *M. leporina* than to that of *M. haemorrhoidalis*.

Acknowledgments—Most of this work has been performed at the Ecological Station of Uppsala University in Öland. We are most grateful for the financial help obtained from the Carl Trygger foundation and from the Ekhaga foundation.

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KAIROMONES AND THEIR USE FOR MANAGEMENT OF ENTOMOPHAGOUS INSECTS. IV. EFFECT OF KAIROMONES ON PRODUCTIVITY AND LONGEVITY OF *Trichogramma pretiosum* RILEY (HYMENOPTERA: TRICHOGRAMMATIDAE)[1,2]

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Abstract—Kairomones in the scales of *Heliothis zea* (Boddie) increased the percent parasitization, the number of progeny produced, and the longevity of female *Trichogramma pretiosum* Riley.

Key Words—kairomone, parasites, productivity, longevity, *Trichogramma pretiosum*, biological control.

INTRODUCTION

There have been numerous studies of kairomones as defined by Brown et al. (1970), partly because they offer considerable potential in the management of insect pests. Lewis et al. (1972) demonstrated that scales left by ovipositing female *Heliothis zea* (Boddie) were the source of a kairomone that elicited a host-seeking response from *Trichogramma evanescens* Westwood females. Jones et al. (1973) determined that the most active component of the moth scales for *T. evanescens* was tricosane. Studies by Lewis et al. (1975*a*), using

[1] In cooperation with the University of Georgia College of Agriculture Experiment Station, Coastal Plain Station, Tifton, Georgia 31794.

[2] Mention of a commercial or proprietary product in this paper does not constitute endorsement by the USDA.

tricosane and a hexane elute of the moth scale extract (Jones et al., 1973) with released *Trichogramma achaeae* Nagaraja and Nagarkatti and naturally occurring *Trichogramma* spp., demonstrated that the rate of parasitization of both natural and applied host eggs was improved by a complete coverage spray of the kairomones. Lewis et al. (1975*b*) demonstrated that kairomones function to increase parasitization by *Trichogramma* spp. by releasing and continuously reinforcing an intensified searching behavior. They also demonstrated that parasites in this intensified search state distributed their eggs more efficiently among the host eggs than did control parasites.

The above cited studies had shown that rates of parasitization could be increased in periods up to 3 days, but the effect of continuous exposure to the kairomone over a long period of time was not considered. Ashley et al. (1974) found that female *Trichogramma pretiosum* Riley produced more progeny, parasitized more host eggs, and lived longer when they were provided naturally deposited *H. zea* eggs as hosts than when they were provided naturally deposited *Trichoplusia ni* (Hübner) eggs as hosts. They suggested that these differences may have resulted from differences in stimuli received by the adult parasitoids, i.e., kairomones.

The present study was an attempt to determine what effect continuous exposure to the kairomones would have on the longevity of female *T. pretiosum* and whether that exposure increases the total production of the parasitoid.

METHODS AND MATERIALS

Pairs of freshly emerged *T. pretiosum* were placed in 25 × 200-mm test tubes (1 pair/tube), and the tubes were plugged with cotton. Eggs of *H. zea* irradiated with 25 Krad, ^{60}Co source (to prevent eclosion; dosage based on unpublished data) were placed on ca. 20 × 160-mm strips of paper (20 eggs/strip) using a solution of Plantguard® and H_2O (1:2) (Nordlund et al., 1974). Treated strips were sprayed with a 1/1000 dilution of a standard moth scale hexane extract elute (Jones et al., 1973), using an aerosol spray device (Lewis et al., 1972), at the rate of ca. 1 ml/strip. Control strips received no treatment. A drop of honey-water (1:1 by volume) was placed on each strip to provide food for the adult parasitoids. One egged strip was placed in each tube, and the tubes were held for 24 hr at 26°C and 70% relative humidity. The strips were then removed, the condition of the adults was checked, and a new strip put into each tube. This process was continued until the female died. The exposed strips were placed in clean tubes and held at 26°C and 70% relative humidity. Percent parasitization was determined by black-egg counts. Counts of progeny were made after eclosion was complete. 9–10 replications

of this test were conducted on four different occasions for a total of 38 replications.

The *T. pretiosum* used in these studies were reared in the laboratory in *H. zea* eggs irradiated with 25 Krad (^{60}Co source) at 26°C and 70% relative humidity.

Statistical analysis was done by paired *t* test. Means in the text are followed by the standard errors in parenthesis.

RESULTS AND DISCUSSION

The data shown in Figure 1 demonstrate that constant exposure to the kairomone(s) found in the moth scales of *H. zea* increased the longevity of *T. pretiosum* females slightly. The mean number of days of life was 10.6 ($\pm$1.6) for the control and 12.2 ($\pm$1.6) (significant at $P = 0.01$) for the treated females.

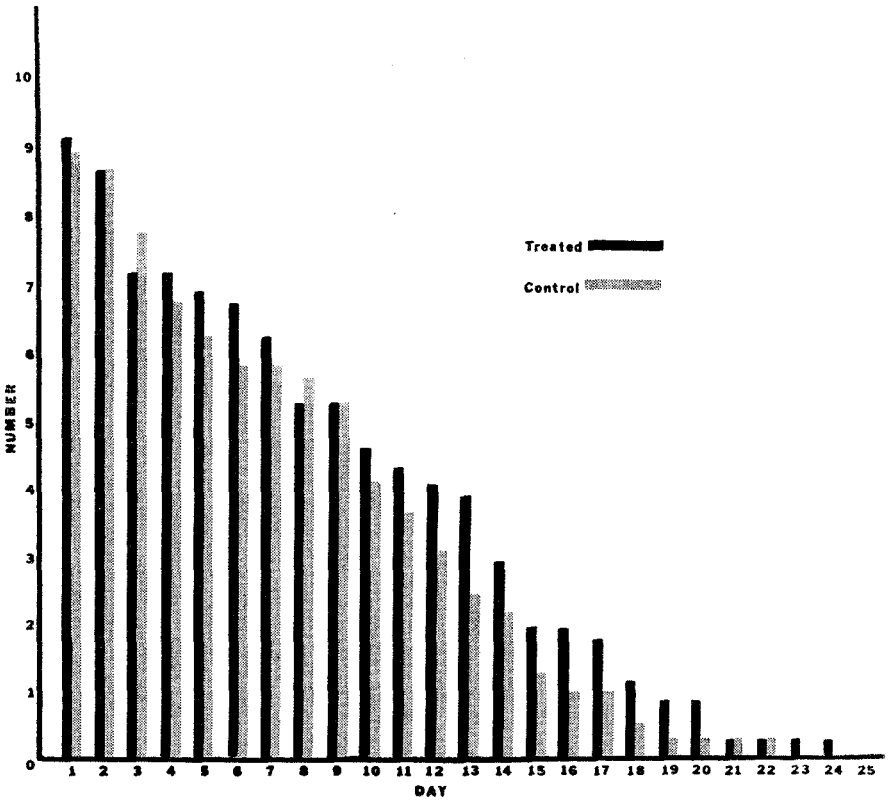

FIG. 1. The mean number of female *T. pretiosum* surviving in tubes.

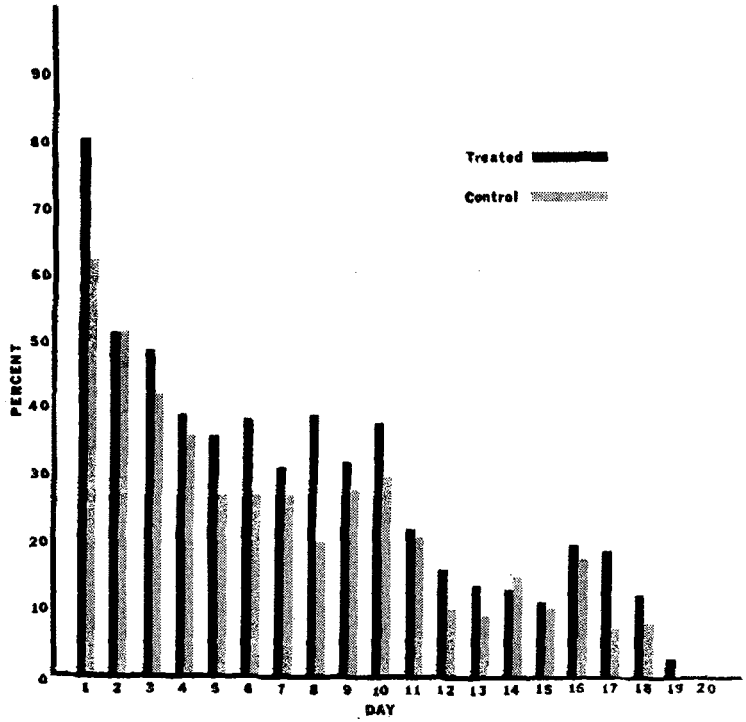

FIG. 2. The mean percent parasitization of *H. zea* eggs by female *T. pretiosum*.

The positive response of the *T. pretiosum* females to the kairomone(s) is indicated in Figure 2; the mean percent parasitization by treated females was greater than that by control females on almost every day of the test. By considering only parasitization through those days when both treated and control females are living, the mean percent for the treated females was 50.4 ($\pm$2.9) and 43.0 ($\pm$2.5) (significant at $P = 0.01$) for control females. This procedure is necessary to reduce the dilution of the percent parasitization by long life-span.

The mean number of progeny produced per female during the test was 110.8 in the treated tubes and 80.6 ($\pm$7.7) (significant at $P = 0.01$) in the control tubes. The total number of progeny produced during the entire test was 4253 (2053 males, 2200 females) in the treated tubes and 2981 (1411 males, 1570 females) in the control tubes. The data shown in Figure 3 indicate that female *T. pretiosum* were responsive to the kairomone during their entire life because the mean number of progeny produced per living female on each day was generally greater for the treated females. These

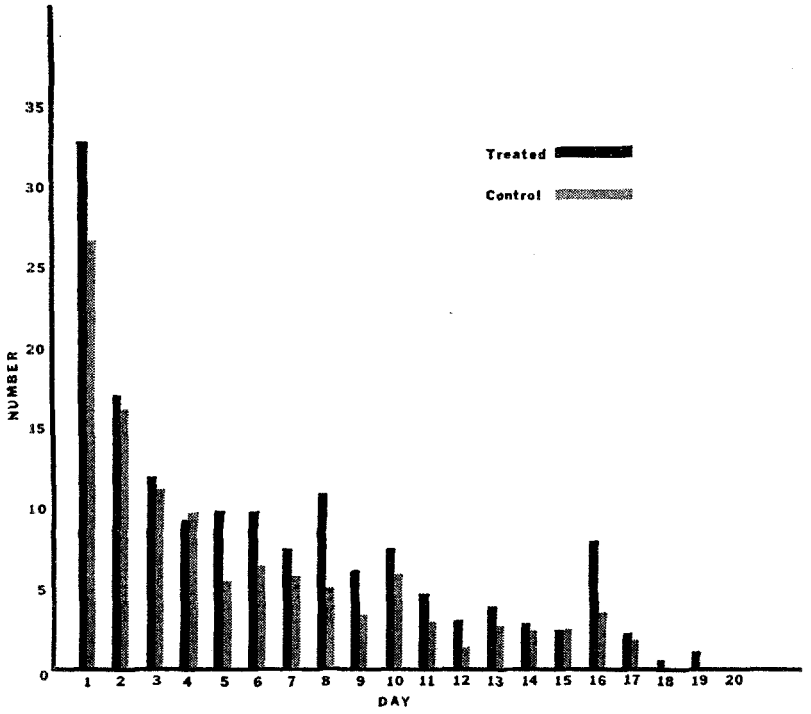

FIG. 3. The mean number of progeny per living female.

increases did not significantly alter the sex ratio (male/female) which was 0.93 in the treated and 0.90 in the controls.

The results of the study indicate that the results reported by Ashley et al. (1974) were caused by kairomones produced by adult female *H. zea* and not by *T. ni* females. In preliminary studies by the authors, no response was obtained from *T. pretiosum* females to the moth scales of *T. ni* (unpublished data).

CONCLUSIONS

Kairomones, therefore, can improve the total production of progeny by female *T. pretiosum* because increased rates of parasitization caused by kairomones result from the production and/or deposition of more parasite eggs and not just from the more efficient distribution of the parasite eggs demonstrated by Lewis et al. (1975*b*). Constant exposure to kairomones tends to increase the longevity of *T. pretiosum* females. These findings have great significance for the use of kairomones in pest control. Kairomones not only

increase pest mortality, they also increase the parasite population, which will produce additional increases in pest mortality.

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RESPONSE TO PHEROMONE TRAPS AND DISRUPTION OF PHEROMONE COMMUNICATION IN THE LESSER PEACHTREE BORER AND THE PEACHTREE BORER (LEPIDOPTERA: SESIIDAE)[1]

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Abstract—The sex pheromone communication of the lesser peachtree borer, *Synanthedon pictipes* (Grote and Robinson), and the peachtree borer, *Sanninoidea exitiosa* (Say), can be disrupted by permeation of the atmosphere with their respective sex pheromones, (E,Z)- and (Z,Z)-3,13-octadecadien-1-ol acetate. The two isomers seemed equally effective against both species. Disruption was greatest when the pheromone was evaporated from the tops of the peach trees; also, pheromone traps placed in the tree tops captured significantly more males than did traps placed lower in the trees. Neither the color nor the directional placement in a tree (NE, NW, SE, SW) of pheromone-baited traps influenced captures of male lesser peachtree borers.

Key Words—*Synanthedon pictipes*, *Sanninoidea exitiosa*, Sesiidae, pheromone communication disruption, insect pheromone trapping, peachtree borer, lesser peachtree borer.

INTRODUCTION

The sex pheromones of two economically important orchard pests, the lesser peachtree borer (LPTB), *Synanthedon pictipes* (Grote & Robinson), and the

[1] Mention of a commercial or proprietary product in this paper does not constitute a recommendation or an endorsement of that product by the USDA.

peachtree borer (PTB), *Sanninoidea exitiosa* (Say), were identified as the (E,Z) and (Z,Z) isomers of 3,13-octadecadien-1-ol acetate (ODDA), respectively (Tumlinson et al., 1974). In field bioassays, Tumlinson et al. (1974) found that male PTB were strongly attracted to synthetic (Z,Z)-ODDA, even when it was contaminated with as much as 5% of the (E,Z) isomer; however, the presence of as little as 1% of the (Z,Z) isomer significantly inhibited the response of male LPTB to (E,Z)-ODDA. The (Z,E) isomer was not attractive to either species, and binary mixtures of this isomer with the (E,Z) isomer did not affect its attractiveness to LPTB males. In view of these findings, it is time to consider how best to make use of the materials to control the two pest species. Three principal approaches have emerged for management of insect populations with synthetic aggregation pheromones (Birch, 1974). Two involve luring the receptive insects to a location to obtain evidence of their presence and/or abundance or to remove a portion of the reproductive potential. The third approach involves the inhibition of the normal behavior of the responding insects so as to disrupt some vital function such as mating. Both male annihilation and disruption appear possible with LPTB and PTB. Wong et al. (1972) used traps baited with virgin female LPTB to capture 7900 of the estimated 8200 males that emerged in 1970 from cherry trees on Washington Island, Wisconsin. However, continuous disruption of communication by volatizing a high concentration of sex pheromone into the environment might be more effective and practical than trapping, and this procedure appears promising for the control of several lepidopteran species (see Mitchell, 1975, and references therein).

We therefore studied the influence of height, color, and cardinal orientation on the response of male LPTB to traps baited with synthetic sex pheromone. We also attempted disruption of the female–male communication of both LPTB and PTB via atmospheric permeation with the synthetic sex pheromones.

METHODS AND MATERIALS

The pheromones and their isomers were synthesized and purified by Doolittle and Tumlinson. The (Z,Z)-ODDA contained <0.5% impurities and ca. 1% contamination with other isomers. The (E,Z)-ODDA contained <0.5% impurities, <0.5% of the (Z,Z) isomer, and ca. 2% (Z,E)-ODDA. The (Z,E) isomer contained ca. 20% (E,Z)-ODDA, <0.5% other isomers, and <0.5% of other impurities. In atmospheric permeation trials against the PTB, the traps were baited with (Z,Z)-ODDA (ca. 92% pure) synthesized by Farchan Division, Story Chemical Co., Willoughby, Ohio, that contained

ca. 8% of other isomers, principally (E,Z) and (Z,E). This mixture, for unknown reasons, causes more male PTB to be captured in sticky traps than the highly pure material (Nielsen et al., 1975).

For ease in handling, the chemicals were diluted to 2 mg/ml or to 40 μg/ml in 99 mol % hexane. An antiozonant, UOP 688 (Universal Oil Products Co., East Rutherford, New Jersey), was added at a rate of 15 mg/ml.

Pherocon 1C sticky traps (Zoecon Corp., Palo Alto, California) were used in all experiments. These traps were baited with either three virgin LPTB females or with the synthetic pheromone of LPTB or PTB. When the virgin LPTB females were used, an arrangement similar to that of Buriff and Davis (1974) was used to bait the traps, and the moths were provided with plain water from a reservoir-fed dental roll wick. These females had been reared on an artificial diet (Antonio et al., 1975), held as pupae in a greenhouse where they were exposed to the prevailing natural photoperiod, and placed in the traps the morning of emergence. They were replaced with new females every 3rd day. When the synthetic pheromones and their isomers were used as bait, they evaporated from the surface of circular, 2.5-cm-diam × 0.7-cm-deep, stainless-steel planchets. These metal dishes were glued to laboratory corks and suspended upside down from the center of a trap or from the limbs of peach trees by a wire attached to the opposite end of the cork.

All experiments were conducted in peach orchards near Hawthorne, Florida, or Byron, Georgia.

RESULTS

Directional Orientation, Color, and Height of Trap

In the experiment to determine optimum orientation, traps baited with 10 μg of (E,Z)-ODDA were placed at mid-tree height (ca. 1.3 m above ground) in either the NE, NW, SE, or SW side of peach trees, and the rate of male capture was determined. The test was designed as a randomized complete block with traps placed on every other tree in a row (7–8 m apart) in each block; 4 blocks, each separated by 4 tree rows (ca. 20 m), were used. The traps were examined 12 times in 15 days. Each time a trap was examined, the captured males were removed and it was moved to the next trapping position in that block. The mean (±SE) number of male LPTB captured by traps in the four quadrants of the trees ranged from 2.1±0.4 to 2.9±0.5/trap/day. There was no significant difference among the treatments ($P = 0.05$).

The possible effect of color on the response of male LPTB to the Pherocon 1C trap was examined by painting all areas of the trap except the sticky surface, which remained white. Colors included blue, yellow, green, red,

or gold (spray cans of Ace Hardware, Chicago, Illinois, numbers 108, 113, S-6, S-23, S-34); with purple, orange, or silver (spray cans of Rust-Oleum Corp. Evanston, Illinois, numbers 924, 1151, 470); or with a blacklight-fluorescent pigment (Ultra Violet Products, Inc., San Gabriel, California). An unpainted (white) trap was included. Each trap was baited with 10 μg of (E,Z)-ODDA. Two complete randomized blocks (separated by ca. 300 m) were established in an orchard. Each block consisted of a line of the colored traps hung on every other tree (7–8 m apart) along a tree row. During 14 days of the test, the traps were checked 7 times (1- to 3-day intervals) and moved each time to the next trapping position in the block.

The mean ($\pm$SE) of male LPTB captured per day by the various colored traps ranged from 1.4 ± 0.6 to 2.1 ± 0.7. There was no statistical difference in the capture rate among the various colored traps ($P = 0.05$). Therefore, in all other tests the white Pherocon 1C trap was used.

A similarly designed test was conducted concurrently in another portion of the orchard (300 m from the cardinal orientation experiment) to determine the effect of trap height. Treatments consisted of traps hung either from the top of peach trees (ca. 3 m above ground), at mid-tree, or from the lowest branches (ca. 0.7 m). The traps placed at the top, middle, and bottom of the tree canopy captured means ($\pm$SE) of 4.4 ± 0.7 (a), 2.6 ± 0.6 (b), and 2.3 ± 0.5 (b) LPTB males/day. The means followed by uncommon letters differ significantly ($P = 0.05$) in Duncan's multiple range test.

Atmospheric Permeation

The objective of the second series of tests was to determine the feasibility of disrupting the female–male chemical communication system of LPTB and PTB by surrounding pheromone-releasing females or a synthetic pheromone source with evaporators that continually released one or more of the isomers. The traps and pheromone evaporators were hung from the outer portion of the limbs of peach trees. In all test plots, a central trap was surrounded by 36 pheromone evaporators placed in a 6×6 array to form a checkerboard-like grid. The degree of disruption of communication was assessed relative to the rate of capture of males in traps placed in nearby untreated areas.

The first five experiments involved disruption of communication in the LPTB. In the first test, pheromone evaporators each containing 10 μg of (E,Z)-ODDA were spaced so as to form a plot ca. 18×23 m surrounding a female-baited trap. A second female-baited trap was placed 300 m distant in the same orchard. The two traps were examined 23 times (at 1- to 3-day intervals) for 44 days, and each time the traps were checked the treatment was moved to surround the other (previous control) trap. The evaporators were recharged the 8th and 13th days of the test.

TABLE 1. DISRUPTION OF SEX PHEROMONE COMMUNICATION IN LPTB (REDUCTION IN CAPTURES OF MALES) BY PERMEATION OF THE ATMOSPHERE SURROUNDING FEMALE-BAITED TRAPS WITH ISOMERS OF 3,13-ODDA

Test number[a]	Isomer and amount/evaporator (μg)	$\bar{X}$ ♂/day captured in untreated area ± SE	Disruption (%)[b]
1	(E,Z)-10	6 ± 1	71 ± 8
2	(E,Z)-10	9 ± 1	9 ± 6
3	(E,Z)-200	13 ± 2	99 ± 0.6
4	(Z,Z)-200	11 ± 2	99 ± 0.9
5	(E,Z):(Z,Z) 200	11 ± 3	99 ± 0.7

[a] Each test had 36 pheromone evaporators in a 6 × 6 array around a female-baited trap; plots were 18 × 23 m except in test 2. In test 2 the plot was 36 × 45 m.
[b] Mean daily reduction relative to control ± SE.

The second test had the same design but was conducted for only 12 days (inspected 10 times at 1- to 2-day intervals). Again, the evaporators were charged with 10 μg of (E,Z)-ODDA, but they were arranged on an every-other-tree spacing to give a 36 × 46-m plot.

Tests 3, 4, and 5 were conducted concurrently using 18 × 23-m plots separated by not less than 300 m. Traps baited with virgin females were surrounded by 36 evaporators each containing 200 μg of (E,Z)-ODDA (test 3), (Z,Z)-ODDA (test 4), or a binary mixture of (E,Z)- and (Z,Z)-ODDA (test 5). These tests were conducted for 13 days, and the traps were examined 11 times with alternation of each treatment between two trap sites.

Table 1 shows that pheromone communication in the LPTB was disrupted when either (E,Z)- or (Z,Z)-ODDA permeated the atmosphere. In test 2, the effectiveness of the treatment was almost eliminated by moving evaporators charged with 10 μg of LPTB pheromone further apart so as to reduce the amount of pheromone volatilized per unit area of land to 0.25 that volatilized in test 1. Nearly complete disruption was achieved by increasing the concentration in the small plot ca. 20-fold (Table 1, test 3). The two isomers appear to be equally effective disruptants when used alone or in combination (Table 1, tests 3, 4, 5).

The sixth test involved disruption of communication in PTB. Since no females were available to bait the traps, they were baited with 100 μg of the synthetic sex pheromone synthesized by Farchan Division (Z,Z/F). Six 31 × 31-m areas in a 0.8-hectare peach orchard were established with 2 rows

TABLE 2. DISRUPTION OF SEX PHEROMONE COMMUNICATION IN PTB BY PERMEATION OF THE ATMOSPHERE SURROUNDING A TRAP BAITED WITH 100 μg OF THE FARCHAN SYNTHESIS OF (Z,Z)-ODDA[a] USING VARIOUS 3,13-ODDA ISOMERS[b]

Permeating isomer	$\bar{X}$ ♂/day captured in traps ± SE[c]	$\bar{X}$[d] disruption (%)
(E,Z)	0 (a)	100 ± 0 (a)
(Z,Z)[a]	0.50 ± 0.27 (a)	88 ± 8 (b)
Farchan (Z,Z)[a]	0.50 ± 0.27 (a)	87 ± 7 (b)
(Z,E)[e]	1.25 ± 0.45 (a)	71 ± 9 (b)
Control	4.50 ± 0.95 (b)	—

[a] The Farchan formulation of the PTB pheromone: 92% pure; 8% other isomers.
[b] All plots had 6 × 6 arrays of evaporators each containing 100 μg of the test material. Evaporator spacing 6.1 × 6.1 m. Test conducted for 11 consecutive days and checked 8 times at 1- to 2-day intervals, i.e., 8 replicates.
[c] Values followed by an uncommon letter differ significantly in Duncan's new multiple range test ($P = 0.05$).
[d] Daily mean reduction relative to control. Values followed by an uncommon letter differ significantly in Duncan's new multiple range test ($P = 0.05$).
[e] Contained 20% (E,Z)-ODDA.

of 3 plots each; all plots were separated by 43 m. At the beginning of the test, the center plot in each row was left untreated, and the 4 treatments were assigned at random to the remaining plots. Treatments consisted of 100 μg/evaporator of (E,Z), (Z,Z), (Z,E), or (Z,Z/F). The test was conducted for 11 days, and the traps were inspected 8 times at 1- to 2-day intervals. At each inspection, the treatments were rotated to the next plot location.

The results (Table 2) demonstrated that pheromone communication in the PTB, like communication in the LPTB, was disrupted by permeation of the atmosphere with either (E,Z)- or (Z,Z)-ODDA. Moreover, the relatively pure (Z,Z) isomer was as effective as the less isomerically pure Farchan material. The effect of the (Z,E)-ODDA is not clear in these tests because of the high level of (E,Z)-ODDA contamination.

Since captures of male LPTB had been influenced by the vertical location of the pheromone traps, an experiment was conducted to determine the efficiency of the permeation method against the LPTB at various levels in the peach trees. The 6 × 6 arrays of evaporators were established in four 18 × 23-m plots (70–200 m between plots) in a block of peach trees. Each evaporator was charged with 100 μg of the LPTB pheromone, and the central

TABLE 3. EFFECT OF EVAPORATOR HEIGHT ON DISRUPTION OF SEX PHEROMONE COMMUNICATION IN LPTB BY PERMEATION OF THE ATMOSPHERE SURROUNDING TRAPS BAITED WITH THE SYNTHETIC SEX PHEROMONE[a]

Trap elevation	$\bar{X}$ ♂ captured/day ± SE with evaporators at		$\bar{X}$ ♂ captured/day ± SE with no evaporators[b]
	Treetop	Mid-tree	
Treetop	0.1 ± 0.1 (d)	3.6 ± 0.6 (b)	9.3 ± 1.2 (a)
Mid-tree	0.6 ± 0.2 (cd)	0.1 ± 0.1 (d)	4.2 ± 0.6 (b)
Lower tree	0.2 ± 0.1 (d)	0 (d)	1.1 ± 0.2 (c)

[a] 6 × 6 array of 36 evaporators, each containing 100 μg (E,Z)-ODDA, in an 18- × 23-m plot. Traps baited with 100 μg (E,Z)-ODDA.
[b] Values followed by uncommon letters differ significantly ($P = 0.05$) in Duncan's new multiple range test using $n + 1$ conversion.

traps were also baited with 100 μg of this material. However, in this test, 3 (instead of 1) central traps were placed one above the other in the lower portion (ca. 0.7 m above ground), middle portion, and top portion (ca. 3.6 m high) of a peach tree. At the beginning of the test, every other plot was left untreated (no evaporators). In one of the other plots the pheromone evaporators were placed at treetop height; in the other, they were placed at mid-tree. This experiment was conducted for 10 days, and the traps were inspected daily. At each inspection the treatments were rotated from one plot to the next around the 4 plots. The results are shown in Table 3. The effect of the pheromone evaporators was statistically ($P = 0.05$) different depending upon the vertical plane in which they were placed relative to the trap. Location in the treetop reduced the captures of males by 95%; evaporators placed at mid-tree caused a 75% reduction.

DISCUSSION

The efficiency of Pherocon 1C traps baited with synthetic sex pheromone in capturing male LPTB was apparently not influenced by the directional orientation of the trap or by any trap color tested. Since the LPTB is a day-flying species and the males, while on the wing, strike at and couple with stationary females, visual cues—perhaps form rather than color—seem to be important to the males. Perhaps such cues can be exploited in trapping once they are understood.

The LPTB males, like other moths (Saario et al., 1970; Sharma et al.,

1971; Hendricks and Leal, 1973; Mitchell et al., 1974) are captured in greatest numbers with pheromone traps at the top of the plant canopy. This is the area where traps should be placed for monitoring or male annihilation, and this is the area that is most critical in disrupting mating communication by atmospheric permeation with the pheromone (Table 3).

Permeation of the atmosphere with sex pheromones therefore may be a feasible method of controlling the mating of PTB and LPTB (Tables 1 and 2). Moreover, (Z,Z)- and (E,Z)-ODDA appear to be equally effective against either species. This finding is encouraging economically because the (Z,Z) isomer is much less expensive to synthesize, and only a single formulation would be required.

In the tests we did not quantify the amount of synthetic pheromone released per unit area of land, but disruption of mating communication seemed to be very quickly lost below some critical level (Table 1, tests 1, 2, 3). This phenomenon was consistent with quantified permeation tests with the cabbage looper, *Trichoplusia ni* (Hübner), and the pink bollworm, *Pectinophora gossypiella* (Saunders) (Shorey et al., 1972; McLaughlin et al., 1972).

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IMPROVED SYNTHESIS OF *endo*-BREVICOMIN FOR THE CONTROL OF BARK BEETLES (COLEOPTERA: SCOLYTIDAE)

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Abstract—*endo*-Brevicomin for testing as pheromone for the control of bark beetles was synthesized in experimental quantities from *trans*-3-hexen-1-ol. Methods used were adapted from procedures for synthesizing *exo*-brevicomin.

Key Words—pheromone, synthesis, *endo*-brevicomin, *Dendroctonus frontalis*, *D. brevicomis*.

INTRODUCTION

The general unavailability of *endo*-brevicomin (*endo*-7-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]octane) has hampered recent field testing of pheromones to control destructive bark beetles. This compound has been implicated in the response of the southern pine beetle, *Dendroctonus frontalis* Zimmermann (Anon., 1971), and the western pine beetle, *D. brevicomis*[1], to sex attractants. The stereo isomer of the *endo* compound, *exo*-brevicomin (*exo*-7-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]octane), has been established as an active sex attractant of the western pine beetle (Silverstein et al., 1968).

Reported here is a method of readily synthesizing *endo*-brevicomin and a clarification of a number of heretofore conflicting points about its preparation.

[1] Personal communications with Dr. W.D. Bedard, Pacific Southwest Forest and Range Experiment Station, Forest Service, U.S. Department of Agriculture, Berkeley, California 94701, June, 1973.

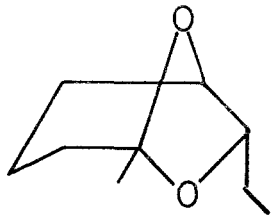

FIG. 1. *endo*-Brevicomin.

endo-Brevicomin (Figure 1) was previously synthesized in milligram amounts by Bellas et al. (1969), Wasserman and Barber (1969), Mundy et al. (1971), and Rodin et al. (1971). However, the product is usually contaminated with the *exo* isomer because of the nonstereospecific nature of the synthetic procedures. The Rodin *exo*-brevicomin synthesis is stereospecific, and *endo*-brevicomin was produced as a byproduct. The *endo* isomer originated from *trans*-3-hexen-1-ol (Figure 2), which is the major impurity (ca. 5%) in the commercial leaf alcohol (*cis*-3-hexen-1-ol) used as the starting material in the synthesis. Therefore, it is possible to start with pure *trans*-3-hexen-1-ol and produce pure *endo*-brevicomin in reasonable yield. The *endo*-brevicomin prepared from this alcohol was free of the *exo* isomer and was identical to that described by Bellas et al. (1969) in physical properties.

METHODS AND MATERIALS

trans-3-Hexen-1-ol was prepared from *trans*-3-hexenoic acid (Research Organic Corp., Sun Valley, California[2]) by the method of Crombie and Harper (1950). However, it was more convenient to use Red-al® (Aldrich Chemical Co., Milwaukee, Wisconsin) instead of lithium aluminum hydride in the reduction. Thus, 40 g of 3-hexenoic acid was added slowly to a refluxing solution (165 g of a 70% benzene solution) of Red-al, followed by 1.5-hr reflux, decomposition with 1:4 sulfuric acid and water, and distillation of the organic phase to give ca. 10 g of the *trans* alcohol (bp 64.5–67°C/22 mm).

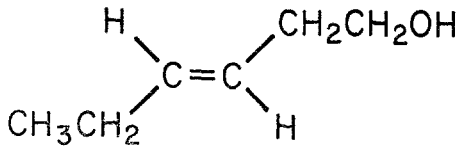

FIG. 2. *trans*-3-Hexen-1-ol.

[2] Trade names and commercial products or enterprises are mentioned solely for information. No endorsement by the U.S. Department of Agriculture is implied.

The *trans* alcohol was tosylated and treated, following the same procedures of Rodin et al. (1971) for the *cis* alcohol, to give *endo*-brevicomin (1.8 g from 13.6 g of alcohol). Thus 13.6 g (0.14 mole) of *trans*-3-hexen-1-ol prepared by the above procedures dissolved in 50 ml pyridine and cooled to 0–5° was treated with 33 g (0.17 mole) of *p*-toluene-sulfonyl chloride added over a period of 1 hr. The mixture was allowed to stand at 0–5°C for 2 days, then was diluted with 100 ml of water and extracted with methylene chloride. Evaporation of solvent after a wash with water and 12 N sulfuric acid and drying of the extract with magnesium sulfate gave 22.3 g (67%) of the tosylate ester: λ^{film} 8.6 μm (—SO_3OR).

trans-6-Nonen-2-one. The tosylate was added in one portion to a solution of sodium ethyl acetoacetate prepared from 2.5 g sodium, 75 ml absolute ethanol, and 13.9 ml distilled ethyl acetoacetate. The solution was heated to start an exothermic reaction, allowed to proceed 1 hr without further heating, then heated another hour at reflux. The mixture was cooled, neutralized with acetic acid, and diluted with 30 ml water. Extraction with three 50-ml portions of methylene chloride, evaporation of solvent after a water wash, and drying of the extract with magnesium sulfate gave 20.5 g of the keto-ester intermediate, which was immediately treated by stirring in 5.8 g sodium hydroxide in 97 ml water at room temperature for 48 hr. The resulting mixture was extracted with two 25-ml portions of ether to remove byproducts and heated under reflux for 5 hr after acidification (pH 2) with 18 N sulfuric acid. Extraction of the cooled solution with two 50-ml portions of ether, followed by a 5% sodium bicarbonate wash, magnesium sulfate drying, and evaporation of the solvent from the extract gave a product that was distilled at 88°/37 mm to give 2.86 g of *trans*-6-nonen-2-one: λ^{film} = 10.3 μm (*trans*—C=C—).

endo-Brevicomin. The ketone was dissolved in 50 ml benzene, cooled to 10–15°C, and treated with 4.2 g of 85% *m*-chloroperoxybenzoic acid added over a period of 20 min. The solution was then heated to reflux for 6 hr. The chlorobenzoic acid then was filtered off from the cooled solution. The benzene solution was washed in turn with aqueous sodium bicarbonate, 5% sodium bisulfite, and aqueous sodium bicarbonate. After the benzene was dried with magnesium sulfate, it was removed through a short Vigreux column at atmospheric pressure followed by the distillation of the product at 75°/25 mm giving 1.8 g of *endo*-brevicomin (10% from alcohol); no *exo* isomer was present.

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STRUCTURE ELUCIDATION OF INSECT PHEROMONES BY MICROANALYTICAL METHODS[1,2]

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Abstract—The isolation and identification of pheromones at the microgram level requires specialized techniques. The pheromones are obtained in the purity required for structural studies by high-speed, high-pressure liquid chromatography using silicic acid- and silver nitrate-treated columns and micropreparative GLC. Then, microchemical reactions, including hydrogenation, ozonolysis, and epoxidation are combined with infrared, nuclear magnetic resonance, and mass spectral studies to elucidate the structure of these compounds. In many cases these techniques are synergistic when combined. Thus, samples of 5–10 μg of most organic compounds under molecular weight 350 are sufficient for complete structural elucidation by using microchemical and spectroscopic techniques. The reduction in sample (2 μg) size necessary for good PMR spectra is significant because the information provided by this technique is often critical to the elucidation of a structure, and sensitivity of PMR has been one of the limiting factors in microspectroscopic analysis.

Key Words—insect pheromones, microspectroscopic techniques.

INTRODUCTION

Insect pheromones, which are highly active chemical messengers produced and used by the insects themselves, are now used as effective insect population

[1] Mention of a pesticide or a commercial or proprietary product in this paper does not constitute an endorsement of the product by the USDA.

[2] Presented at the Symposium on Chemicals Affecting Insect Behavior and Development, Third International Congress of Pesticide Chemistry (IUPAC), Helsinki, Finland, July 3–9, 1974.

survey tools and will soon be used in insect control programs. Since most of these compounds are produced by the insects in only nanogram or picogram amounts their isolation and structure elucidation requires large numbers of insects or special techniques. For example, millions of boll weevils, *Anthonomus grandis*, were reared to obtain enough material to identify their four-component pheromone (Tumlinson et al., 1969). Fortunately, techniques and equipment have improved significantly in the last five years, so we now need only a few micrograms of a compound for identification. To a great extent this has eliminated the tremendous expense of rearing vast numbers of insects for pheromone identification. Recent studies have shown that pheromones are often multicomponent rather than single compounds, and trace amounts of synergizing or modifying compounds can be important. The isolation and identification of the complete pheromone complex, including the trace modifiers, is crucial to complete understanding of the effect of the phermone or insect behavior.

At the Insect Attractants, Behavior, and Basic Biology Research Laboratory we have adapted and improved existing techniques and devised new ones to enable us to isolate and identify compounds with minimal material. These procedures, to be described, are applicable to a wide range of problems in the life sciences.

PROCEDURES, RESULTS, AND DISCUSSION

Purification

The pheromones must be pure before they can be identified and usually purity is essential for biological activity. Trace amounts of isomers and other hard-to-separate compounds may have beneficial or deleterious effects on the activity of naturally derived and synthesized pheromones (Tumlinson et al., 1972, 1974*b*; Beroza et al., 1973).

High-speed, high-resolution liquid chromatography is one of our most important methods of pheromone purification. Recent developments, including high-pressure pumps and small particle, high-resolution columns, have placed this technique on a par with gas–liquid chromatography (GLC) in terms of speed, resolution, and convenience of operation. The major disadvantage of this type of liquid chromatography is the low sensitivity of the available detectors. Usually there is insufficient pheromone to be detected, but most natural extracts contain other compounds in sufficient quantities to serve as markers. We have used three types of columns with considerable success in isolating natural pheromones and purifying synthesized compounds.

Usually, our first step in the separation of the pheromone from the crude

extract is gel permeation chromatography. A glass column, 1.27 cm (ID) is packed to a height of about 90 cm with a hexane slurry of Poragel 60A, 37–75 μm (Waters Associates). The column is eluted with hexane at a flow rate of 300 ml/hr and a column inlet pressure of 40 psi. A 0.5-ml sample of concentrated crude extract is loaded on this column and the entire separation requires only about 1 hr. In this system, compounds with a molecular weight over 2000 will not permeate the pores of the packing and will be eluted virtually without holdup. Smaller molecules, which fall within the working range of the packing, are separated in reverse order by molecular size. With this column we have obtained a 20-ml fraction that contained all the active pheromone from the crude extract of 2500 *Heliothis virescens* females; it was pure enough to proceed directly to gas chromatographic separations.

The second liquid chromatographic column that we have used extensively is a 0.64 (OD) × 50-cm stainless-steel column packed with 10 μm LiChrosorb (E. Merck) by the technique of Majors (1972). We normally use hexane and ether mixtures as solvent both in isocratic and gradient systems. The particular solvent system depends on the compounds to be separated. This column is useful in separating synthetic mixtures as well as natural extracts. Thus we found that we could separate (Z,E)-3,5-tetradecadien-1-ol acetate, a sex attractant for *Prionoxystus robiniae* (Doolittle et al., 1973), from its (E,E) isomer with 3% ether in hexane.

Our most useful liquid chromatographic column has been a 1.27 (OD) × 50-cm stainless-steel column dry-packed with Adsorbosil-2-ADN (Applied Science Laboratories), a 20% $AgNO_3$-coated silica gel used in thin-layer chromatography; benzene is used exclusively as the solvent. It is capable of eluting a wide range of organic compounds in a reasonable time, and the column can be reused indefinitely. The particle size of the Adsorbosil varies from about 2–11 μm, a wide range for high-performance liquid chromatography. Nonetheless, we have achieved complete separation of the (Z) and (E) isomers of at least two classes of compounds using this column. For example, the synthesized pheromone of *Synanthedon pictipes*, (E,Z)-3,13-octadecadien-1-ol acetate, contained about 3–5% of the (Z,Z)-isomer, rendering it almost totally unattractive to *S. pictipes* males (Tumlinson et al., 1974*b*). When this mixture was chromatographed on this column at a benzene flow of 6.5 ml/min and a column inlet pressure of 4700 psi, the (E,Z)-isomer eluted between 10 and 14 min after injection and the (Z,Z) isomer between 21 and 27 min. Similarly, the synthesized (Z) and (E) isomers of the aldehydic *H. virescens* pheromone were separated in 3 min at a flow of 4.0 ml/min.

Once a pheromone has been purified as much as possible by liquid chromatography we find micropreparative GLC our most reliable method of obtaining microgram quantities of naturally derived pheromones in a highly purified state. When 2–5% of the column effluent is directed to a flame

ionization detector and the remainder diverted to an efficient thermal gradient collector (Brownlee and Silverstein, 1968), we have achieved recoveries of the target compounds in excess of 90%. The components are collected and sealed in glass capillaries for subsequent purification or identification. Purity of the isolated pheromones is established by GLC on open tubular capillary columns coated by the method of Mon (1971). Using Carbowax 20M, Dexsil, and OV-101 phases in 0.76-mm × 60-m columns with over 60,000 theoretical plates, we are usually able to show that a compound is at least 99% pure.

Microchemical Techniques

Two microdegradative techniques that we find most useful in determining the number and position of olefinic bonds are hydrogenation over neutral palladium catalyst and ozonolysis. These techniques were developed on a microscale by Beroza and co-workers and the experimental techniques are adequately described elsewhere (Beroza and Sarmiento, 1966; Beroza and Bierl, 1966, 1967). The advantages of these procedures are that they can be performed with less than a microgram of material, and the products are available for analysis in a few minutes at most. The hydrogenation is carried out in the inlet of the gas chromatograph and the product is analysed without further handling as it is swept from the catalyst bed onto the GLC column by using hydrogen as a carrier gas. If the gas chromatograph is connected to a mass spectrometer the mass spectrum of the product is recorded as it is eluted from the GLC column. Under the proper conditions, this procedure only hydrogenates olefinic bonds; thus a vast amount of information is gained very quickly. For example, the mass spectrum of an unknown indicated that it was a straight-chain molecule with a molecular weight of 210. Thus, it could either be a 14-carbon alcohol with two olefinic bonds or 14-carbon aldehyde with one. Insufficient material was available for an infrared spectrum. Reduction of the compound in the inlet of the gas chromatograph and subsequent mass spectroscopy showed that the product had a molecular weight of 212. Thus, the unknown was a monounsaturated 14-carbon aldehyde. Similarly, the products of ozonolysis can be analyzed by GLC and mass spectroscopy with equal facility.

Another reaction that we have adapted for microanalysis is epoxidation of olefinic bonds. There are several instances when ozonolysis is not sufficient to locate with certainty all olefinic positions in a molecule. This is often the case when there are two or more olefinic bonds in a chain and the ozonolysis products are very small volatile molecules that are difficult to analyze.

Epoxidation is easily carried out by adding the olefin to chloroform that

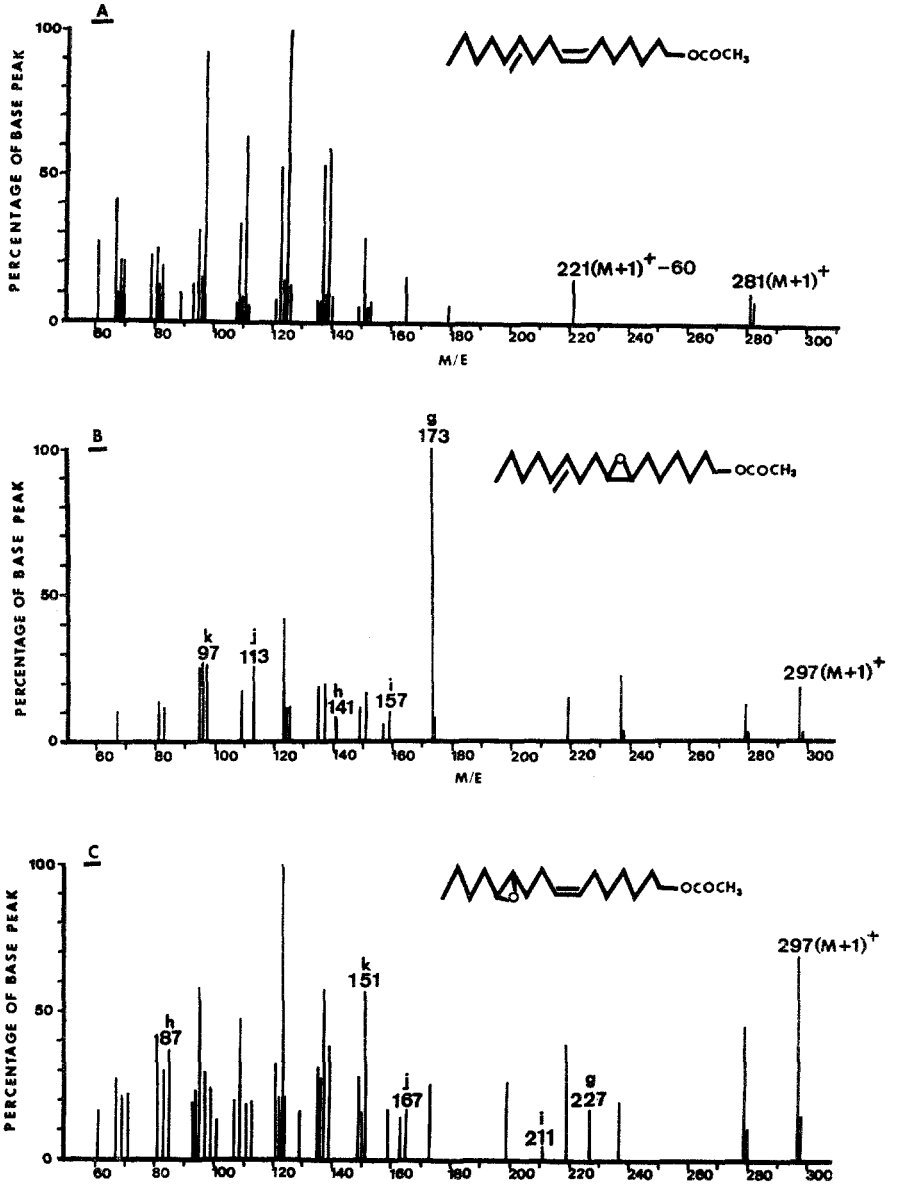

FIG. 1. Isobutane ionization mass spectra of: A, (Z,E)-7,11-hexadecadien-1-ol acetate; B, 7,8-epoxy-11-hexadecen-1-ol acetate; C, 11,12-epoxy-7-hexadecen-1-ol acetate. Spectra were obtained with 0.5 μg or less material. Direct comparison of the olefin and epoxide spectra facilitates location of the epoxide ring. A fragmentation scheme is shown in Figure 2.

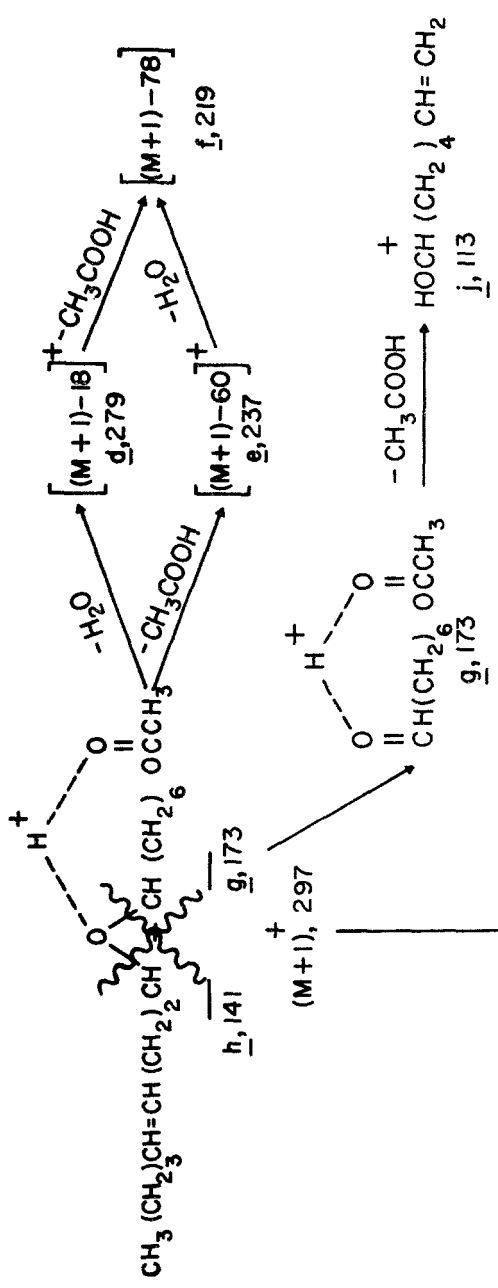

FIG. 2. Suggested isobutane ionization fragmentation scheme for epoxyhexadecen-1-ol acetates. The relative abundance of each ion is shown in Figure 1B.

contains a molar excess of 99+% *m*-chloroperbenzoic acid (Schwartz and Blumberg 1964) and allowing the mixture to stand at room temperature. When compounds containing two ethylenic bonds are epoxidized the reaction can be monitored by gas chromatography with a column containing 3% OV-210 on 100/120 mesh Varaport 30 and then stopped at the monoepoxide stage. The position of the olefinic bonds in a chain is then located by analysis of the monoepoxides by chemical ionization mass spectroscopy with isobutane as a reagent gas (Tumlinson et al. 1974*a*). Furthermore, the configuration of the olefinic bonds can be determined by infrared analysis of the monoolefins.

We used this technique in the analysis of a synthetic isomeric mixture of the Angoumois grain moth, *Sitotroga cerealella*, pheromone (Z,E)-7,11-hexadecadien-1-ol acetate (Vick et al., 1974). Although the mixture was separated into four isomers by chromatography, we did not know which component was the desired (Z,E)-7,11-hexadecadien-1-ol acetate. The most

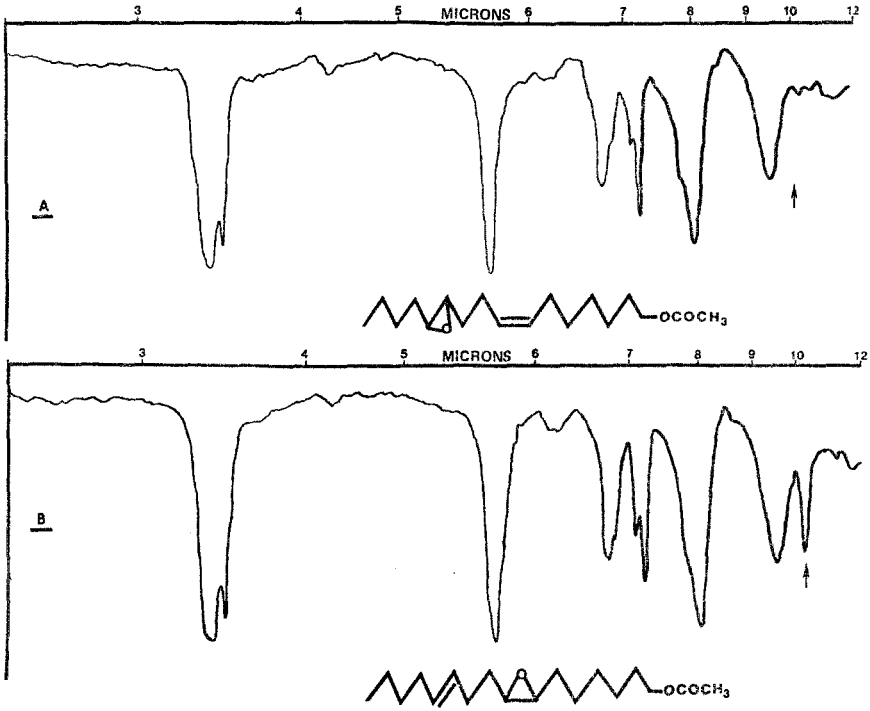

FIG. 3. Infrared spectra of: A, 11,12-epoxy-(Z)-7-hexadecen-1-ol acetate, and B, 7,8-epoxy-(E)-11-hexadecen-1-ol acetate. The arrow in each spectrum indicates the characteristic frequency of the trans olefinic absorption (10.2 μm, 980 cm^{-1}). The absence (3A) or presence (3B) of this band reveals whether the nonepoxidized olefinic band is cis or trans, respectively.

likely component was epoxidized, and the monoepoxides were analyzed by isobutane ionization mass spectroscopy. Figure 1 shows the mass spectra of the original component and the two monoepoxides. Figure 2 shows the suggested fragmentation pattern for 7,8-epoxy-11-hexadecen-1-ol acetate, which is similar to that observed for 11,12-epoxy-7-hexadecen-1-ol acetate (Figure 1) and is typical for acetates of straight chain epoxy alcohols. Thus, when the spectra of the original olefin and the two monoepoxides are compared, the location of the olefinic bonds is easily established. Spectra of this type can be easily obtained with 0.5 μg of sample.

Figure 3 shows the infrared spectra of these two olefinic epoxides. The trans band at about 970 cm^{-1} that appears in one spectrum and is absent in the other (see arrows) establishes the configuration of the double bond in each compound. Thus, we have conclusive proof that the component we selected was (Z,E)-7,11-hexadecadien-1-ol acetate.

Mass Spectroscopy

Recently developed, highly sensitive spectroscopic equipment has greatly facilitated analysis of microsamples. Only five years ago at least 100 μg of sample was required for complete spectroscopic analysis by infrared, proton magnetic resonance, and mass spectroscopy. With the most sophisticated equipment and techniques available today, it is possible to obtain all three spectra with less than 5 μg of a compound, and with 10 μg of sample the analyses are routine. In fact, the most difficult task involves handling and transferring these small samples without considerable loss.

Usually the first spectrum obtained of a sample is the mass spectrum because so little material is required and the information derived is very helpful in maximizing the effectiveness of the other spectral analyses. The advent of chemical ionization mass spectroscopy has increased the analytical capability of this technique even more. Since the reagent gas reacts with the sample molecule in the ion source of the mass spectrometer, the reagent gas may be varied considerably (Hunt and Ryan, 1971, 1972*a*,*b*; Hunt et al., 1971) so a particularly suitable reactant for a class of compounds may be chosen. Alternatively, a compound may be subjected to various ionizing gases to yield more structural information. As an example, Hunt et al. (1972) found that when D_2O was used as the reagent gas, the number of active hydrogens in a sample could be determined because they were all replaced with deuterium.

An additional advantage of chemical ionization mass spectroscopy is that the reagent gas can often be used as the carrier gas for the interfaced gas chromatograph. Thus, the total effluent from the GLC column is directed into the ion source, and no sample is lost.

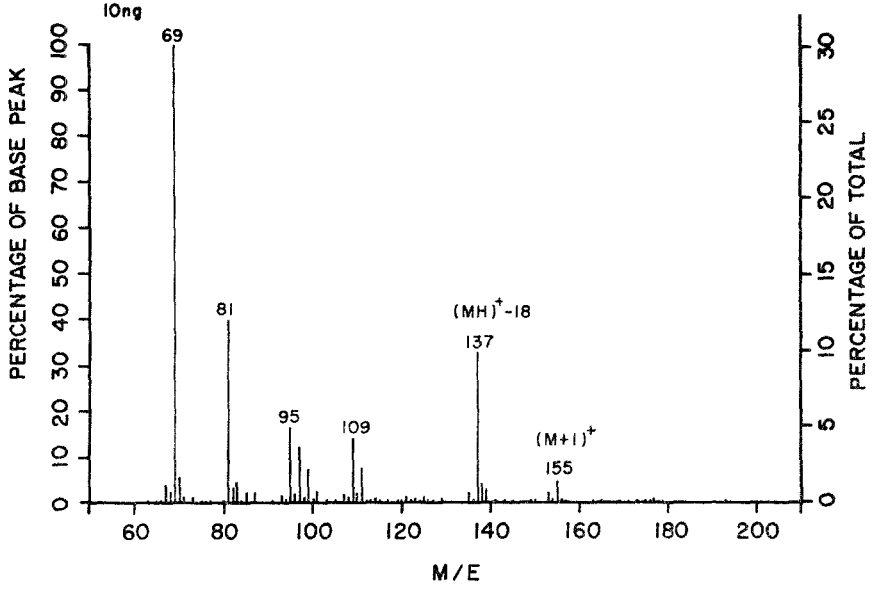

FIG. 4. Methane ionization mass spectrum of 10 ng of *cis*-2-isopropenyl-1-methyl-cyclobutaneethanol. Background peaks were subtracted by the computer.

The convenience and sensitivity of this technique is illustrated by Figure 4. A 10 ng sample of *cis*-2-isopropenyl-1-methyl-cyclobutaneethanol, a component of the boll weevil pheromone (Tumlinson et al., 1969), dissolved in about 0.2 μl of hexane was injected onto a 3% OV-1 on 100/120 mesh Varaport 30 column in a Varian Model 1400 gas chromatograph. The total effluent of the column was admitted to the source of a Finnigan Model 1015C chemical ionization mass spectrometer. Methane, the carrier gas, also served as the reagent gas at a source pressure of about 1 torr. Data acquisition and reduction were accomplished with a Systems Industries Disc System 150 computer interfaced to the mass spectrometer.

Infrared Spectroscopy

Infrared spectra are valuable in organic structure determinations, particularly in functional group analysis. Since others have published micro-infrared techniques recently, we will not discuss this at length. For example, Price et. al. (1967) reported good infrared solution spectra with 5 μg of benzyl acetate by using a specially designed cell. More recently, King (1973) published good solution spectra of 2,6-dimethoxyphenol with only 0.3 μg of sample in a microcavity cell by using a beam condenser and a Fourier transform mid-infrared spectrometer.

Micro-NMR Spectroscopy

High-resolution proton magnetic resonance (PMR) is probably the least sensitive of the three spectrometric techniques. Although Lundin et al. (1967) predicted that the minimum sample required for a compound of molecular weight 200 should be about 1 μg with Fourier transform high-resolution PMR, there have been no reports of usable spectra obtained with less than 20 μg thus far.

Our PMR spectra are obtained with a Bruker HX-90 spectrometer interfaced to a Nicolet 1080 Fourier transform data system and equipped with a high-sensitivity proton probe insert. Of several commercial PMR cells, we found a microtube prepared to our specifications by Wilmad Glass Co. (Figure 5) most compatible with our spectrometer. The optimum solvent volume in this tube was about 75 μl. Commercially available spectroquality carbon disulfide and carbon tetrachloride were purified by filtration through columns of silica gel Woelm (activity grade 1) and aluminum oxide Woelm

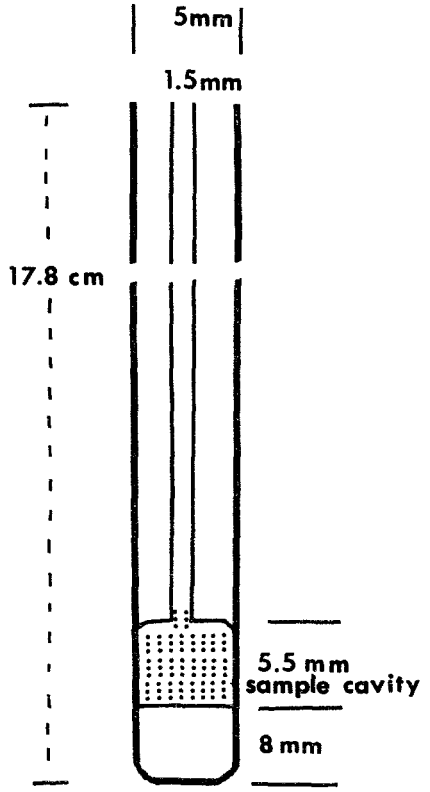

FIG. 5. Micro-NMR tube used to obtain spectra of 2-μg samples. Volume of the sample cavity is 75 μl.

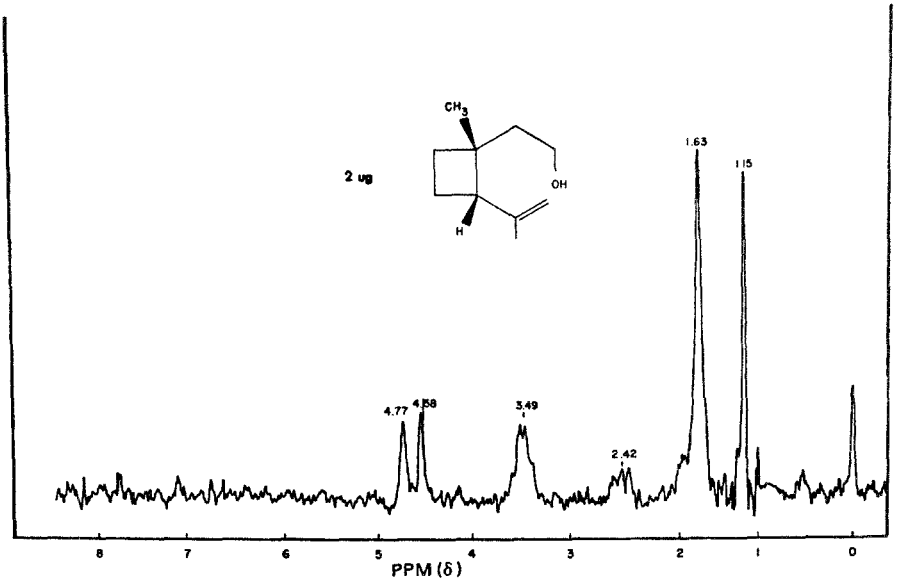

FIG. 6. 90-MHz proton NMR spectrum of 2 μg of *cis*-2-isopropenyl-1-methylcyclobutaneethanol. Concentration was 0.026% in 75 μl of CS_2. Tetramethylsilane was the internal standard. 60,000 transients of the sample were obtained and 60,000 background transients were subtracted by the computer. The free induction decay was stored in 4K memory. The sweep width was 1000 Hz. A high-sensitivity proton-receiver coil was used.

basic (activity grade 1[3]), followed by distillation in an all-glass system to remove particles of silica or alumina. Hexaflurobenzene (Thompson-Packard, NMR grade) at a concentration of about 5% (v/v) was used for the internal lock signal, and tetramethylsilane (Thompson-Packard, NMR grade) was used as an internal reference; in the small amounts used both produced no significant interference and needed no purification.

The PMR spectrum in Figure 6 illustrates the sensitivity of the technique. The design of the sample cell is critical because best results are achieved with the minimum amount of solvent that will fill the receiver coil area of the probe. If solvent impurities did not interfere, usable spectra could be obtained with less than 2 μg of sample. The major impurity in both carbon tetrachloride and carbon disulfide produces a singlet at 1.04 ppm downfield from TMS and interferes with the methyl signals of most organic compounds. This signal was decreased significantly by filtration of the solvent through silica gel, alumina, and distillation; the peak was smaller with carbon disulfide than

[3] "Purification of solvents by Woelm active aluminas," Waters Associates, Inc., Milford, Massachusetts.

with carbon tetrachloride. Although the addition of shift reagents [$Eu(fod)_3$, Penninsular ChemResearch] and trichloroacetyl isocyanate (TCAIC) shifted the impurity signal downfield, the impurity could not be removed by distillation of the solvent from TCAIC or 3-nitrophthalic anhydride. Also, addition of small amounts of water to the solvent produced a second peak, and the impurity peak remained at 1.04. We concluded that carbon disulfide is the best solvent for micro-PMR studies. Additionally, CS_2 facilitates sample recovery by gas chromatography since it produces a small signal and is very volatile.

Undoubtedly, techniques, procedures, and equipment will continue to improve and the amount of material necessary for identification of pheromones and other natural products will decrease even further. One of the promising new areas of investigation is ^{13}C nuclear magnetic resonance spectroscopy. This technique is presently limited by its sensitivity since mg samples are required. However, the vast amount of information that a ^{13}C spectrum yields, particularly with complex molecules, makes it a valuable method of structure elucidation (Nakanishi et al., 1973). In the near future, the problem of lack of sensitivity will be overcome and ^{13}C NMR will be a primary method of microstructure elucidation.

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ANAL SAC SECRETION OF THE RED FOX, *Vulpes vulpes*; VOLATILE FATTY ACIDS AND DIAMINES: IMPLICATIONS FOR A FERMENTATION HYPOTHESIS OF CHEMICAL RECOGNITION

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Abstract—Putrescine (1,4-diaminobutane) and cadaverine (1,5-diaminopentane) were identified in the anal sac secretions of the red fox, *Vulpes vulpes*, and of the lion, *Panthera leo*. Anal sac secretion samples obtained over a period of 10 weeks by sampling from within each sac of each of 6 captive foxes were analyzed and putrescine, cadaverine, and volatile fatty acid compositions and secretion pH values recorded. A significant ($P < 0.001$) negative correlation of pH (range 6.5–9.4) with total volatile fatty acid concentration was observed. Secretion compositions are discussed in the context of a fermentation hypothesis of chemical recognition. Secretion samples could not be unambiguously assigned to particular foxes on the basis of simple comparisons of volatile fatty acid profiles alone. Composition differences were noted between secretions obtained at a given time from corresponding right and left sacs.

Key Words—anal sac, volatile fatty acid, putrescine, cadaverine, profile, fermentation, recognition, *Vulpes vulpes*, *Panthera leo*.

INTRODUCTION

Saturated carboxylic acids (C_2 to C_5/C_6) occur together as major volatile constituents of red fox (*Vulpes vulpes*) and lion (*Panthera leo*) anal sac secretions (Albone and Fox, 1971; Albone et al., 1974), Indian mongoose

(*Herpestes auropunctatus*) anal pocket secretion (Gorman et al., 1974), guinea pig (*Cavia porcellus*) perineal gland secretion (Berüter et al., 1974), Rhesus monkey (*Macaca mulatta*) vaginal secretion (Michael et al., 1972), and in the vaginal secretions of other primates, including man (Michael et al., 1974). Microbiological studies have demonstrated the microbial origin of these acids in the red fox, lion, Indian mongoose, and Rhesus monkey secretions mentioned. Only in the case of the Rhesus monkey vaginal secretion has their behavioral significance been investigated in detail, although Gorman (1976) has suggested that the Indian mongoose recognizes conspecifics as individuals on the basis of odor differences between individually characteristic volatile fatty acid profiles of anal pocket secretions.

In this paper we report results concerning variations in red fox anal sac secretion volatile fatty acid and diamine composition, and we discuss the implications of these findings for fermentation hypotheses of chemical recognition.

METHODS AND MATERIALS

Secretion samples were obtained from 6 untamed red fox (Table 1) housed since August, 1973, in two adjoining pens, A and B (each having concreted outside run, 35 m^2; covered area, 7.5 m^2; height, 2 m), separated by a brick wall completely preventing physical and visual contact between the two pens. Samples, commonly in the range of 25–500 μl, were taken from within each sac via an irrigating cannula (Arnold's Veterinary Products Ltd., 0.762 mm OD; 0.254 mm ID), transferring the sample to a glass vial (chromic acid cleaned) using a disposable syringe. The animals were physically re-

TABLE 1. RED FOX SAMPLED

Fox code	Sex	Pen	Born (spring)
2[a]	F	A	1973
3[b]	M	B	1973
4	M	A	1972
5	M	B	1973
6[a]	F	B	1973
7[a, b]	F	B	1973

[a] Foxes 2, 6, 7 were litter-mates. Other foxes are unrelated.
[b] Fox 3 was castrated and fox 7 ovariohysterectomized, October 24, 1973.

strained and unsedated during sampling. Samples were stored at −20°C prior to analysis. Sampling dates were: 23 Dec. 1974 (code W), 8 Jan. 1975 (code X), 23 Jan. 1975 (code Y), 6 Feb. 1975 (code Z), 20 Feb. 1975 (code A), 5 Mar. 1975 (code B). Secretion samples are coded with a number identifying the fox (Table 1), a letter identifying the sampling date and R (or L) indicating right (or left) sac.

Volatile fatty acids were analyzed using a Pye-Unicam 104 gas chromatograph (FID) with a 2.3-m × 4-mm (ID) silanized glass column of 3.5% w/w Pegosperse S-9 (Glyco Chemicals Inc.) and 0.35% w/w *ortho*phosphoric acid on Chromosorb W-AW, 60/80 mesh (nitrogen carrier, 30 ml/min; column temperature 110°, isothermal; injector temperature 230°) by on-column injection of untreated aqueous secretion, or of secretion diluted 1:1, v/v, with aqueous *n*-hexanoic acid (0.018 M) as internal standard. Peak areas were estimated by cutting and weighing xerox copies of chromatograms. Gas chromatography of untreated secretion was preferred to that of the diethyl ether extract of acidified secretion, particularly where very small samples were available. A standard mixture of volatile fatty acids adjusted to various pH values up to 9.8 with ammonia yielded identical volatile fatty acid chromatograms. Gas chromatograms of diethyl ether extracts of selected acidified secretions were closely comparable with those obtained from untreated secretion in regard to the volatile fatty acid peaks, save that the more water-soluble acids (particularly acetic acid) were underestimated because of incomplete extraction.

Amines were detected and identified following derivatization with 1-dimethylaminonaphthalene-5-sulphonyl (dansyl) chloride (BDH Chemicals Ltd.). Authentic putrescine and cadaverine were purchased from Koch-Light Laboratories Ltd. and Ralph Emanuel Ltd., respectively. For analytical purposes, anal sac secretion (10 μl) was added to dansyl chloride (100 μl, 30 mg/ml acetone solution) followed by distilled water (30 μl) and sodium bicarbonate (50 mg). After standing (16 hr), proline (15 mg) was added and the sample left a further 2 hr. Distilled water (1 ml) was added and the sample extracted by agitation with ethyl acetate (2 ml) followed by centrifugation. The ethyl acetate extract was concentrated to 250 μl under nitrogen and examined (5 μl) by thin-layer chromatography (TLC).

TLC was performed with chloroform–triethylamine, 5:1, v/v, and with cyclohexane–diethyl ether, 1:9, v/v, on Merck silica gel 60 (0.25 mm) precoated plates for analytical purposes and on acetone preeluted Kieselgel G (0.5 mm) plates preparatively. Dansyl chloride derivatization of a standard mixture of all common amino acids revealed no artifacts moving in the region of putrescine and cadaverine didansyl derivatives. Analytical plates were sprayed with isopropanol–triethanolamine, 4:1, v/v, immediately after chromatography, dried over silica gel under vacuum at room temperature

for 16 hr and fluorescence assayed by scanning with a Vitatron densitometer, Model TLD 100 (507 nm filter, 0.25 mm diaphragm, scan speed 1 cm/min).

Mass spectrometry was performed using an AEI MS 902 mass spectrometer (direct insertion probe), source temperature 260–280°.

pH measurements were conducted, where sufficient sample was available, using a microelectrode (Activion Glass Ltd., Model 003-11-306) with an Electronic Instruments Ltd pH meter (Model 38B).

RESULTS

Components corresponding in TLC mobility to authentic *N,N'*-didansylputrescine and to *N,N'*-didansylcadaverine, obtained by subjecting dansylated fox anal sac secretion (2WL, 160 μl) to preparative TLC consecutively with chloroform–triethylamine, 5:1, and with cyclohexane–diethyl ether, 1:9, yielded the following mass spectral data ($m/e \geqslant 150$):

Component 1: R_f ($CHCl_3/Et_3N$, 5:1) 0.42; (C_6H_{12}/Et_2O, 1:9) 0.24, *m/e*; 555 (12.3), 554 (M^+, 40.0), 172 (13.9), 171 (100), 170 (82.2), 169 (45.7), 168 (45.7), 167 (8.7), 155 (17.5), 154 (21.0).

Component 2: R_f ($CHCl_3/Et_3N$, 5:1) 0.54; (C_6H_{12}/Et_2O, 1:9) 0.28, mass spectrum, very weak, with molecular ion, *m/e* 568 and intense *m/e* 170, 171.

These spectra indicate *N,N'*-didansylputrescine and *N,N'*-didansylcadaverine, respectively (Seiler et al., 1970; Creveling et al., 1968). Components of identical TLC properties were also observed in dansylated lion (*Panthera leo*) anal sac secretion (4-year-old male, sampled August, 1974). In this case, mass spectrometry yielded a strong *N,N'*-didansylcadaverine spectrum, *m/e*; 569 (9.7), 568 (M^+, 25.7), 250 (8.6), 172 (14.5), 171 (100), 170 (59.7), 169 (51.4), 168 (29.8), 155 (10.4), 154 (13.2), although insufficient component corresponding to the *N,N'*-didansylputrescine TLC zone was available to yield a satisfactory mass spectrum.

Two-dimensional TLC using the above solvent systems with selected dansylated fox and lion anal sac secretion samples confirmed that the fluorescent spots running with *N,N'*-didansylputrescine and *N,N'*-didansylcadaverine in chloroform–triethylamine each yielded predominantly one spot in the second dimension and that these corresponded with the same authentic compounds.

Anal sac secretion volatile fatty acid profiles are depicted in Figure 1, and total volatile fatty acid, putrescine, and cadaverine concentrations together with secretion pH values are given in Table 2. Blank entries indicate that insufficient secretion was obtained. In some cases, the secretion was seen

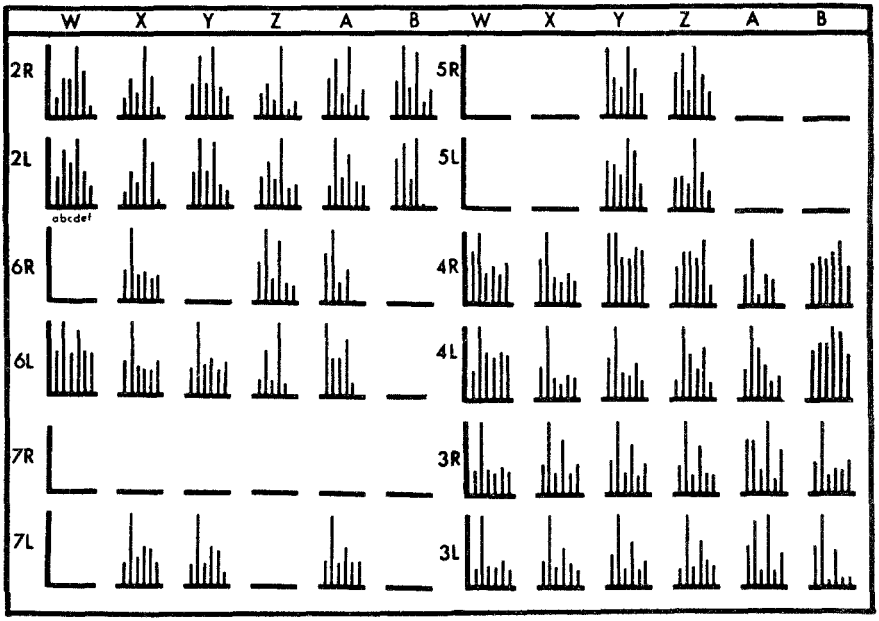

FIG. 1. Red fox anal sac secretion volatile fatty acid profiles. Each sample is coded with a number identifying the fox (Table 1), a letter indicating the sampling date, and R or L indicating right or left sac. Each profile records in sequence the relative molar concentrations of (a) acetic acid (concentration $\times 0.1$), (b) propionic acid, (c) isobutyric acid, (d) *n*-butyric acid, (e) isovaleric acid, (f) isocaproic acid. Blank entries indicate insufficient sample was obtained.

to be voided by the animal before sampling began. The volatile fatty acid profiles were calculated on the basis of the following identifications (in order): acetic, propionic, isobutyric, *n*-butyric, isovaleric, and isocaproic acids. The isovaleric acid peak may contain unresolved 2-methylbutyric acid (Albone and Fox, 1971). The *n*-butyric acid peak sometimes overlapped a poorly resolved unidentified component, and in such cases the two components were quantified together as *n*-butyric acid for purposes of profile comparison as it was not possible to estimate accurately the contribution of each component to the composite peak. When a composite peak was observed, its form was usually closely similar for corresponding right and left sac secretions.

n-Valeric acid was present in a number of samples, but always as a minor component and was not tabulated in the profiles.

DISCUSSION

In spite of the limited number of samples examined, a visual inspection

TABLE 2. RED FOX ANAL SAC SECRETION COMPOSITIONS: pH VALUES AND TOTAL VOLATILE FATTY ACID (VFA), PUTRESCINE [Put], AND CADAVERINE [Cad] CONCENTRATIONS

Sample[a]	pH	[VFA][b] (mM)	[Put] (mM)	[Cad]/[Put]	Sample[a]	pH	[VFA][b] (mM)	[Put] (mM)	[Cad]/[Put]	Sample[a]	pH	[VFA][b] mM	[Put] mM	[Cad]/[Put]
2RW	8.5	—	—	—	2LW	7.6	92	19	0.36	6RW				
2RX	8.1	44	9	0.31	2LX	8.7	39	6	0.21	6RX	8.5	14	4	0.75
2RY	6.6	132	29	0.08	2LY	7.4	137	30	0.25	6RY				
2RZ	6.6	102	18	0.34	2LZ	—	51	13	0.19	6RZ				
2RA	6.5	146	7	0.01	2LA	8.2	30	4	0.00	6RA	7.1	31	8	1.7
2RB	—	106	10	0.21	2LB	—	49	9	0.53	6RB				
3RW	8.3	72	10	0.15	3LW	8.7	25	4	0.33	6LW	8.7	9	3	0.20
3RX	8.2	60	13	0.26	3LX	7.3	114	25	0.05	6LX	9.0	19	2	1.4
3RY	8.1	60	8	0.25	3LY	8.3	—	8	0.32	6LY				
3RZ	7.0	126	26	0.03	3LZ	8.1	45	8	0.09	6LZ				
3RA	7.4	35	8	0.85	3LA	—	34	8	0.04	6LA	—	32	7	0.27
3RB	—	17	—	—	3LB	—	39	5	0.26	6LB				
4RW	8.4	25	2	2.7	4LW	8.4	21	2	3.3	7LW				
4RX	8.8	13	0.8	2.3	4LX	8.6	15	2	2.8	7LX	8.9	26	2	<0.1
4RY	9.4	6	0.5	3.2	4LY	8.6	7	0.6	3.9	7LY	9.0	34	—	—
4RZ	—	7	0.9	3.5	4LZ	—	12	1	2.9	7LZ				
4RA	8.7	8	0.6	0.74	4LA	8.6	17	4	3.2	7LA	—	20	2	0.20
4RB	—	14	1.5	2.3	4LB	—	29	3	2.9	7LB				

[a] Samples are coded with a number identifying the fox (Table 1), a letter indicating sampling date, and R or L indicating right or left sac.
[b] Calculated from gas chromatography profile data.

of the volatile fatty acid profiles (patterns of abundance), Figure 1, reveals the following features of importance.

1. The profiles of secretions obtained from the two sacs of a given fox at a given time were usually similar (e.g., 2XR/2XL, 3ZR/3ZL, 4XR/4XL), but not invariably so (e.g., 2BR/2BL, 3BR/3BL, 4AR/4AL). Odor differences between the secretions of the two sacs of a given animal at a given time have not previously been considered.

2. The profiles of secretions taken from a particular sac of a particular animal may remain relatively unchanged on consecutive sampling dates (e.g., 3XR/3YR/3ZR, 7XL/7YL), or they may change between sampling dates (e.g., 3ZR/3AR/3BR).

3. The short-term variability in the profile of a given animal may be greater than the profile difference between animals (e.g., 3ZR/6ZR), so that *it is not possible to assign unambiguously a particular sample to a particular fox on the basis of a simple visual profile comparison.* An exploratory computer analysis using a multivariate profile of the six peaks has revealed some evidence of a degree of clustering within the samples of some foxes (fox codes 2, 5, 7), whereas the others appeared to be randomly scattered. These studies are continuing.

4. No obvious features distinguish the profiles of the foxes from pen A from those from pen B (Table 1), nor the intact from the castrated and ovariohysterectomized animals, even though sampling was conducted during the breeding season [January, early February (Burrows, 1968; Creed, 1972)]. We observed no evidence of pregnancy in the study foxes, however. Although the possible existence of such fox anal sac secretion distinguishing features is the subject of continuing study, the recent finding by Doty and Dunbar (1974) that, for the beagle, anal sac secretions of estrous bitches were no more attractive to males than those of diestrous bitches, renders it less likely that sexually related effects will be found.

Throughout, the major amine component of anal sac secretion was ammonia, present in greater, and frequently in considerably greater, concentrations than those of the volatile fatty acids. Smaller concentrations of putrescine and cadaverine were also present and the relative proportions of these diamines also exhibited considerable variation, including right sac/left sac variation, e.g., 3AR/3AL (Table 2). Interestingly, all fox 4 samples (R and L) examined, except 4AR (ratio 0.74) exhibited a molar ratio of cadaverine to putrescine in the range 2.3–3.9, mean 3.0, $n = 11$, and were thus distinguished from all fox 2 (R and L) samples, range 0–0.55, mean 0.23, $n = 11$, and all fox 3 (R and L) samples, range 0.03–0.84, mean 0.24, $n = 11$.

When pH values and total volatile fatty acid concentrations of different secretions (Table 2) were compared, further differences were revealed between

certain secretions even of closely similar volatile fatty acid profile (e.g., 3ZR/3ZL).

The pH range of fox anal sac secretion extended from 6.5 to 9.4 and the total volatile fatty acid concentration from 6 mM to 146 mM. A linear regression of pH on total volatile fatty acid concentration was obtained, slope -0.015 pH unit/mM/liter and intercept (0 mM) 8.9 pH units (correlation coefficient -0.835, $n = 30$, $P<0.001$). The secretion pH values, being intermediate between the pK_a values of the volatile fatty acids (pK_a, 25°, acetic, propionic *n*-butyric isobutyric, isovaleric, and isocaproic acids being 4.76, 4.87, 4.82, 4.86, 4.78, and 4.85, respectively) and those of the amines [pK_a, 25°, ammonia, putrescine, and cadaverine being 9.25, 10.81 (pK_2 9.63), 10.93 (pK_2 10.05) respectively] indicated that the secretion acids and amines were present in solution predominantly in their odorless ionic forms. The free (un-ionized) acids and amines contribute to the odor of the secretion and, for a given acid/amine mixture, for every pH unit increase an approximately 10-fold increase in free amine and an approximately 10-fold decrease in free acid concentration results. The concentrations of any volatile nondissociable species (not investigated here) are pH independent. In this way the pH controls the odor of a given mixture by exerting differential effects on the partial vapor pressures of odorous acids, amines and nondissociable species.

The volatile fatty acids present in red fox anal sac secretion are the products of the resident microflora (Gosden et al., 1975; Albone et al., 1974) and it is most likely that the diamines present are, at least in part, similarly formed particularly by the sac *Clostridia* (Brooks and Moore, 1969; Hyatt and Hayes, 1975), although this has yet to be confirmed.

No evidence suggests that the two anal sacs of the fox are emptied to the same extent when secretion is expelled. Indeed, secretion volume differences were frequently noted between corresponding sacs so that, e.g., fox 7 never yielded sufficient secretion for analysis from its right sac, while the left sac yielded satisfactory samples on three out of six occasions. We therefore suggest that differences in secretion pH, including the substantial left sac/right sac differences frequently observed (Table 2), may reflect differences in secretion incubation (residence) time in the sac. This cannot be proved until techniques are available to follow the time dependence of sac processes without incurring the disturbance caused to the sac microenvironment by the sampling method presently employed. However it is commonly observed that incubations of proteinaceous substrate lead to increased pH with time whereas pH decreases are noted in carbohydrate fermentations.

Gorman (1976) has advanced a fermentation hypothesis for chemical recognition for the Indian mongoose by suggesting that this animal recognizes conspecifics as individuals on the basis of odor differences between individually characteristic profiles of microbiologically produced volatile fatty acids present in their anal pocket secretions.

As with the Indian mongoose, these same volatile fatty acids are also produced by fermentation in the anal sac secretions of the red fox and the lion and are present in the anal sac secretions of the bush dog, *Speothos venaticus*, of the tiger, *Panthera tigris*, of the maned wolf., *Chrysocyon brachyurus*, and of the domestic dog, *Canis familiaris* (Albone, unpublished observations) as well as of the domestic cat, *Felis catus* (Michael et al., 1972), although in these cases a microbial origin has yet to be investigated. Further emphasis of cross species similarities between such analogous fermenting systems is provided by the finding that the anal sac secretions of two such diverse carnivores as the red fox and the lion not only contain the same volatile fatty acids and the same diamines as major odorous constituents but also support closely similar microflora (Gosden et al., 1975).

Although the sensitivity of mammals to the odors of these diamines has yet to be studied, it is known that the domestic dog is sensitive to the volatile fatty acids (Moulton et al., 1960) and that the Indian mongoose can distinguish between different mixtures of these acids by olfaction (Gorman, 1976). Questions arise concerning the ways in which such odorous substances, the microbial formation of which appears to depend little on sex, sexual status or even, within limits, on species,[1] might acquire ecochemical significance.

A fermentation hypothesis of chemical recognition offers one possibility. By processes of cross-infection, a group of animals living together would be expected to come to share a common microflora characterizing that group. Even when the species of microorganisms are narrowly defined, the possibility of many biochemical strains provides sufficient group-distinguishing potential. The hypothesis argues that if these microorganisms produce substances detectable by the mammal in question, the odors of individuals from a particular group would possess certain recognizable common features characteristic of that group and its shared microflora (Albone et al., 1974). It is known that characteristically different volatile metabolite profiles are produced by different strains of microorganism incubated under standard conditions (Lewis et al., 1967) but the situation is complicated by observations that incubation (residence) time can affect profile (Moore et al., 1966), as can substrate (primary secretion) composition variations. Gorman (1976) has advanced a particular example of this fermentation hypothesis in relation to individual chemical recognition in the solitary Indian mongoose.

Results presented in this paper give an indication of the variability of odor profile to be expected in fermenting systems analogous to the red fox anal sac and point to the importance of such factors as pH variation and right sac/left sac differences which have not previously been considered. These observations make it more difficult to believe that odor profiles of fermenting

[1] Gross differences in anal sac secretion volatile composition with species do occur, *e.g.*, the dominance of C_4 and C_5 thiols in striped skunk (*Mephitis mephitis*) anal sac secretion (Andersen and Bernstein, 1974; Bernstein, 1974).

systems possess sufficient stability to form a basis for chemical recognition, although the proof must await further bacteriological, behavioral, and chemical studies, and they raise doubts which merit further examination in relation to Gorman's Indian mongoose studies.

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ANNOUNCEMENT

VERTEBRATE CHEMICAL SIGNALS SYMPOSIUM

A symposium on Chemical Signals in Vertebrates will be held during June 7–9, 1976, at the Gideon-Putnam Hotel, Saratoga Springs, New York. Additional information and registration forms forms are available from the Dean, School of Continuing Education, College of Environmental Science and Forestry, Syracuse, New York 13210.

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PHYSIOLOGICAL ACTIVITY OF WATER BEETLE DEFENSIVE AGENTS. I. TOXICITY AND ANESTHETIC ACTIVITY OF STEROIDS AND NORSESQUITERPENES ADMINISTERED IN SOLUTION TO THE MINNOW *Pimephales promelas* Raf.

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Abstract—By means of a bioassay employing the minnow *Pimephales promelas*, the anesthetic activities and toxicities of various defensive steroids and norsesquiterpenes produced by the Dytiscidae and Gyrinidae were compared with those of a wide selection of steroid standards. The most widely occurring major components of dytiscid secretions, 4-pregnen-3-ones and related derivatives, were among those compounds most active in minnow bioassays. The norsesquiterpenes gyrinidal and gyrinidione were among the most toxic compounds tested but they possessed little anesthetic action. The anesthetic activity of gyrinidone was comparable to that of testosterone. Steroid activity in the minnow bioassay was highly related to the degree of oxygenation; steroids oxygenated only at the termini of the molecule were most active. Less or additional oxygenation resulted in a loss of activity. When steroids were rapidly administered to minnows the activities of many of them were similar, suggesting they share a common mode of action.

Key Words—Dytiscidae, Gyrinidae, chemical defense, 4-pregnen-3-ones, gyrinidal, gyrinidione, gyrinidone, steroids, norsesquiterpenes.

INTRODUCTION

Aquatic beetles of the families Dytiscidae and Gyrinidae possess defensive glands which secrete agents repellent and toxic to vertebrate predators, i.e., fishes and amphibians. The prothoracic defensive secretions of numerous dytiscid species primarily contain steroids (Table 1, A), many of which are

TABLE 1. DEFENSIVE AGENTS OF AQUATIC COLEOPTERA
A. Dytiscid prothoracic defensive compounds

Compound	Species	Quantity (μg/beetle)[a]	References
4-pregnen-21-o1-3,20-dione (deoxy-corticosterone, DOC)	*Acilius semisulcatus*	(M)	Miller and Mumma (unpublished)
	Acilius sulcatus	19 (m)	Schildknecht et al. (1967b) Schildknecht (1970)
	Agabus bipustulatus	(M)	Schildknecht and Hotz (1970)
	Agabus seriatus	40 (M)	Miller and Mumma (1973)
	Cybister confusus	(m)	Chadha et al. (1970)
	Cybister limbatus	133 (M)	Sipahimalani et al. (1970)
	Cybister tripunctatus	143 (M)	Chadha et al. (1970)
	Dytiscus marginalis	400 (M)	Schildknecht et al. (1966)
	Graphoderus liberus	20 (M)	Miller and Mumma (1973)
4-pregnen-20α-o1-3-one	*Acilius sulcatus*	1 (m)	Schildknecht et al. (1967b) Schildknecht (1970)
	Cybister limbatus	8 (m)	Sipahimalani et al. (1970)
	Dytiscus marginalis	(m)	Schildknecht and Hotz (1967)
4-pregnen-20β-o1-3-one	*Cybister tripunctatus*	100 (M)	Chadha et al. (1970)
	Ilybius fenestratus	1 (t)	Schildknecht and Birringer (1969)
4,6-pregnadien-21-o1-3,20-dione	*Acilius sulcatus*	56 (M)	Schildknecht et al. (1967b) Schildknecht (1970)
	Cybister literalimarginalis	(m)	Schildknecht (1968)
	Cybister limbatus	13 (m)	Sipahimalani et al. (1970)
	Cybister sp.	(t)	Schildknecht and Körnig (1968)
4,6-pregnadien-20α-o1-3-one (cybisterone)	*Acilius sulcatus*	7 (m)	Schildknecht et al. (1967b) Schildknecht (1970)
	Cybister lateralimarginalis	167 (M)	Schildknecht et al. (1967c)

	Cybister limbatus	17 (m)	Sipahimalani et al. (1970)
	Dytiscus marginalis	(t)	Schildknecht and Hotz (1967)
4,6-pregnadien-3,20-dione	*Acilius sulcatus*	6 (m)	Schildknecht et al. (1967b)
			Schildknecht (1970)
	Cybister lateralimarginalis		Schildknecht (1968)
4,6-pregnadien-12β,20-diol-3-one	*Cybister lateralimarginalis*	(m)	Schildknecht (1968)
4-pregnen-12β-o1-3,20-dione	*Cybister limbatus*	80 (m)	Chadha et al. (1970)
4,6-pregnadien-12β-o1-3,20-dione	*Cybister limbatus*	37 (M)	Chadha et al. (1970)
(cybisterol)	*Cybister* sp.	1000 (M)	Schildknecht and Körnig (1968)
4-pregnen-15α,20β-diol-3-one	*Platambus maculatus*	10 (M)	Schildknecht et al. (1969b)
4,6-pregnadien-3,20-dione-15α isobutyrate	*Agabus sturmi*		Schildknecht and Hotz (1971)
4,6-pregnadien-15α-o1-3-one-20β-isobutyrate	*Agabus sturmi*		Schildknecht and Hotz (1971)
4-pregnen-15α-o1-3,20-dione-7α isobutyrate	*Agabus sturmi*		Schildknecht and Hotz (1971)
4-pregnen-15α-o1-3,20-dione-7α hydroxyisobutyrate	*Agabus sturmi*		Schildknecht and Hotz (1971)
4-androsten-17β-o1-3-one	*Ilybius fenestratus*	28 (m)	Schildknecht and Birringer (1969)
(testosterone)	*Ilybius fuliginosus*		Schildknecht et al. (1967a)
1,4-androstadien-17β-o1-3-one (1-dehydrotestosterone)	*Ilybius fenestratus*	16 (m)	Schildknecht and Birringer (1969)
1,3,5-androstatrien-3β,17β-diol (estradiol)	*Ilybius fenestratus*	19 (m)	Schildknecht and Birringer (1969)
1,3,5-androstatrien-3β-o1-17-one (estrone)	*Ilybius fenestratus*	2 (t)	Schildknecht and Birringer (1969)

B. Gyrinid pygidial defensive compounds

Compound	Species	Quantity (μg/beetle)[a]	References
gyrinidial	*Dineutus assimilis*	118 (M)	Miller et al. (1975)
	Dineutus hornii		Meinwald et al. (1972)
	Dineutus nigrior	66 (M)	Miller et al. (1975)
	Dineutus serrulatus	ca. 100 (M)	Meinwald et al. (1972)
	Gyrinus marinus		Schildknecht et al. (1972)
	Gyrinus minutus		Schildknecht et al. (1972)
	Gyrinus natator	ca. 84 (M)	Schildknecht et al. (1972)
	Gyrinus substriatus		Schildknecht et al. (1972)
	Gyrinus ventralis	80 (M)	Meinwald et al. (1972)
gyrinidione	*Dineutus assimilis*	95 (M)	Miller et al. (1975)
	Dineutus nigrior	54 (M)	Miller et al. (1975)
gyrinidone	*Dineutus assimilis*	22 (m)	Miller et al. (1975)
	Dineutus discolor	(M)	Wheeler et al. (1972)
	Dineutus nigrior	12 (m)	Miller et al. (1975)

[a] μg per beetle are presented when reported. The relative concentration per beetle is as follows: M = major, m = minor, and t = trace.

stored in surprisingly high quantities. Although dytiscid secretions may contain a wide variety of steroid structures, including such well-known mammalian hormones as deoxycorticosterone (DOC), testosterone, and estradiol, the compounds in greatest quantity are generally 4-pregnen-3-ones, 4,6-pregnadien-3-ones, or the many modifications of these basic structures. Several nonsteroidal compounds have been isolated as major components of prothoracic defensive secretions of several dytiscid species of the subfamily Colymbetinae (Schildknecht et al., 1969a; Schildknecht and Tacheci, 1971). The pygidial secretions of the Gyrinidae are composed largely of oxygenated norsesquiterpenes (Table 1, B), which have been shown by Benfield (1972) to be repellent to fishes and amphibians and by Miller et al. (1975) to be toxic as well.

The discovery that aquatic beetles utilize steroids and norsesquiterpenes as defensive agents raises the question of how these compounds function in a defensive context. In addition to their classical hormonal actions, various pharmacological effects of steroids on animals are recognized, such as steroid anesthesia, allergy, fever, and immunological reactivity as well as steroid hematopoietic, hemolytic, and cytotoxic properties (Kappas and Palmer, 1963).

Although it is not yet certain which toxicological action(s) of steroids is being exploited by the beetles in their defense against predation, observations (Schildknecht et al., 1966; Schildknecht et al., 1967a; 1967b; Miller et al., 1975) indicate that anesthesia may be a primary effect of the agents elaborated by water beetle defensive glands. Fish and frogs, force-fed beetle defensive secretions, show signs of excitation followed by varying degrees of narcosis, which is usually reversible. Likewise, when fish are placed in solutions of defensive steroids at concentrations of 5–10 μg/ml they enter a state of deep narcosis in ca. 30 min. The effects may be completely reversed if fish are then placed in fresh water. Fish that remain in such steroid solutions are usually killed (Miller et al., 1975).

As a means of screening steroids for their comparative anesthetic potency, Selye and Heard (1943) exposed the redfin shiner, *Notropis cornutus*, to solutions of various steroids and recorded the minimal dosage required to cause minnows to lose their ability to orient in water currents. These experiments proved that just as various neutral steroids are anesthetic to mammals (Selye, 1941a; Selye, 1941b; Selye, 1942) they are also capable of anesthetizing fish.

The present study was undertaken in order to expand the available information on the physiological action of steroids on fish. A minnow bioassay similar to that of Selye and Heard (1943) was used to measure both the relative anesthetic activity and relative toxicity of a wide selection of steroids to the minnow, *Pimephales promelas*. Various types of steroids similar to those produced by dytiscids were tested as were the gyrinid norsesquiterpenes. The

activities of these compounds were measured at concentrations far in excess of the AC_{50} (anesthetic concentration) and LC_{50} (lethal concentration) values by recording lag times for anesthesia and death. It was hoped that a measure of commonness in the mode of action of steroids could be obtained by quantifying their activities when administered at rates where varying detoxification mechanisms and rates would be inconsequential. The data obtained from the study taken together with those of Selye and Heard (1943) will hopefully serve to further characterize the physiological activity of steroids and norsesquiterpenes in their role as defensive agents.

METHODS AND MATERIALS

Experimental Animals

The fathead minnow, *Pimephales promelas* Raf., was used in all experiments because of its desirable small size and commercial availability. Minnows of a mean body length of 4.5 cm and a mean weight of 1.2 g (range 0.7–1.7 g) were purchased from live-bait dealers in the vicinity of State College, Pennsylvania. In order to standardize them, all batches of minnows were stored at 4°C in 40-gal aquaria for at least 48 h before they were used in experiments. During the period of storage, minnows were constantly aerated but were not fed. Experiments conducted on such minnows upon their acclimatization to near room temperatures yielded uniform results. Once minnows were used in an experiment they were discarded, since it had been shown (Kappas and Palmer, 1963) that adaptation to the anesthetic effect of steroids could occur in animals repeatedly treated with these substances.

Test Compounds

Steroids of a purity >99% were purchased from various commercial sources. Their purity was checked by thin-layer chromatography employing multiple development.

Norsesquiterpenes were isolated from the gyrinids, *Dineutus assimilis* and *Dineutus nigrior* (Miller et al., 1975). Purities of norsesquiterpenes used in this study ranged from 97% to 99% as ascertained by gas–liquid chromatography.

Experimental Procedure

In order to determine the physiological activities of the test compounds both at threshold levels and above, minnows were exposed to a range of toxicant concentrations from ca. 1 to 100 μg/ml. The lag time from administra-

tion of the compounds until minnows lost their equilibrium and could no longer right themselves was taken to be a measure of anesthetic activity. The lag time until respiratory arrest (RA) or cessation of gill-pumping was taken to be a measure of toxicity. Although in some cases heartbeat continued for at least 15 min after RA, cessation of gill-pumping was selected as the end-point of the toxicity bioassay because it was far more readily observed. Curves of lag times vs. toxicant concentration were obtained along with a concentration range bounding the AC_{50} and LC_{50} values.

In the experimental procedure ca. 80 minnows at a time were allowed to equilibrate from 4°C to 25±0.5°C over the period of 1 h and were held at 25°C for another 2 h. Three minnows were then transferred to each of a series of 250-ml glass beakers containing 50 ml of aerated tap water held at 25± 0.5°C by means of a water bath. From stock solutions of the steroids and norsesquiterpenes prepared in ethanol or DMF (67 μg/μl solvent), the appropriate quantities of toxicant needed to produce the desired range of concentrations were injected into the beakers containing fish and the solutions were stirred thoroughly. Control experiments demonstrated no measurable difference in the toxicity of solutions of these compounds when they were prepared using the carrier ethanol, the carrier DMF, or when the solution was prepared without carrier by exhaustively shaking crystalline compounds in water.

Using a completely randomized experimental design, 40 compounds were tested on *P. promelas* in the above manner. Each concentration was replicated at least three times. Additionally, the constancy of response of each batch of minnows acclimated to 25°C was monitored by measuring their survival time in a standard solution of the steroid DOC at 10 μg/ml. The mean survival time of all batches of minnows exposed to 10 μg/ml DOC was 27±3.1 (SD) min.

RESULTS AND DISCUSSION

Data typical of those obtained in this study are presented graphically in Fig. 1 as a log–log plot of lag times for anesthesia and RA vs. toxicant concentration. Such a double logarithmic plot yields a straight line over the higher range of toxicant concentrations, and a straight line in this case is characteristic of an absorption process, following the Freundlich absorption isotherm (Rothblat et al., 1966). As the AC_{50} or LC_{50} values were approached, lag times progressively diverged from the Freundlich isotherm, presumably because at these concentrations detoxification mechanisms significantly influenced the effects of the substances tested. The effects of all compounds administered to *P. promelas* were short term. Death rarely occurred after the

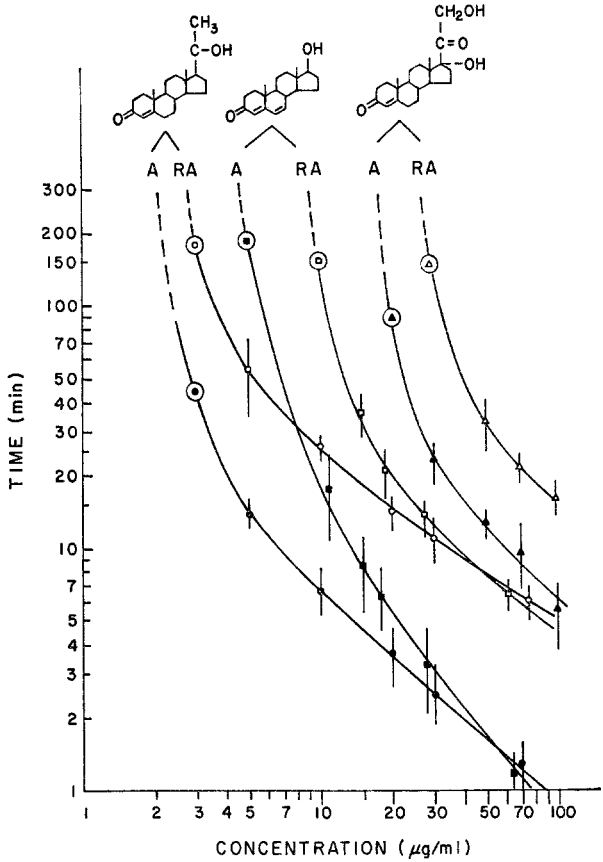

FIG. 1. Dose–response curves typical of highly active, active and slightly active steroids. Highly active = 4-pregnen-20β-ol-3-one (2), active = 4,6-androstadien-17β-ol-3-one (13), and slightly active = 4-pregnen-17α, 21-diol-3,20-dione (17). A = anesthesia and RA = respiratory arrest. Circled means were calculated upon exclusion of some (<50%) unanesthetized or surviving minnows.

5-h period over which data were recorded for these experiments. In fact, minnows anesthetized but not killed usually had made or were making a recovery by 5 h.

Although the activities of the test compounds were measured at seven or eight different concentrations, the findings are adequately summarized in the abbreviated Table 2. Compounds are arranged according to increasing toxicity and are arbitrarily grouped into these categories: highly active, active,

slightly active, and inactive. The data for the gyrinid norsesquiterpenes are presented separately at the end of Table 2.

AC_{50} values were found to parallel LC_{50} values, and in both cases the following generalizations can be made concerning structure–activity relationships: The activity of compounds tested by the *P. promelas* bioassay was highly dependent on their water solubility. Compounds roughly fell into three categories: those very water insoluble (< 5 μg/ml) and inactive, those slightly soluble in water (ca. 10–100 μg/ml) and highly active or active, and those fairly soluble in water (> 200 μg/ml) and inactive or slightly active.

Several types of compounds fell into the very insoluble group; included are those compounds oxygenated at only one terminus of the molecule (C-3 and C-17 or -20) whether androstanes (31,32) or pregnanes (39), the androstan- and androsten-3,17-diols (30,33), most of the estratrienes (36, 37, 38), and the singly oxygenated cholestenes (34, 35). Since the uptake of steroids into the fish is concentration dependent (see Fig. 1), the rate of uptake for these compounds, even from a saturated solution, may have been too slow to effect any accumulation of these materials within the fish. Therefore, no judgement can be passed as to the potential activity of highly water-insoluble compounds. Judging from the comparative studies of the anesthetic activity of steroids injected intraperitoneally into rats (Selye, 1942), some of these steroids are weakly anesthetic.

The compounds generally anesthetic and toxic to *P. promelas* were oxygenated at the termini (C-3, C-17, C-20, or C-21) of the molecule. The active androstanes were oxygenated at C-3 and C-17, while the active pregnanes were oxygenated at C-3 and C-20 or C-21. The highly active steroids of those tested in this study were terminally oxygenated pregnenes and 5α(*cis*)-androstanes. Although not included here, terminally oxygenated 5α and β pregnanes are even more active in mammals than corresponding pregnenes (Selye, 1942; Selye et al., 1943; Figdor et al., 1957). Terminally oxygenated androstenes were consistently less active than the pregnenes and androstanes (see compounds 10–15).

The degree of ring unsaturation beyond the $\Delta 4$ unsaturation, as just discussed, influenced activity only slightly. The activities of testosterone (12), 1-dehydrotestosterone (15), and 6-dehydrotestosterone (13) were very similar. Unsaturation of progesterone (1) in the D ring at C-16 did result in a loss of activity (7).

The steric positions of hydroxyl groups were also of only slight importance as testosterone (12) and epitestosterone (11) possessed similar activity. The activities of (2) and its α isomer (tested only at a few selected concentrations, and these data are not included in Table 2) were also very similar.

Oxygenation of steroids in positions in addition to the termini can greatly reduce activity; however, some positions on the rings are much more critical

TABLE 2. ANESTHETIC ACTIVITY AND TOXICITY OF SELECTED STEROIDS AND NORSESQUITERPENES AS MEASURED BY A MINNOW BIOASSAY

Code No.	Compound	Lag time (min)[a] 70 μg/ml A	70 μg/ml RA	20 μg/ml A	20 μg/ml RA	10 μg/ml A	10 μg/ml RA	AC_{50} (μg/ml)	LC_{50} (μg/ml)
	Highly active steroids								
(1)	4-pregnen-3,20-dione (progesterone)	1.0	6.0	2.6	15.1	5.9	22.6	1–2	1–2
(2)	4-pregnen-20β-ol-3-one	1.3	8.0	3.7	13.4	6.8	26.8	1–3	1–3
(3)	5α-androstan-3α-ol-17-one (androsterone)	1.0	5.7	1.0	9.7	1.5	13.2	1–2	2–3
(4)	4-pregnen-21-ol-3,20-dione (deoxycorticosterone)	1.1	6.7	2.6	14.2	5.8	28.0	1–3	2–3
(5)	3,4-di-*p*-hydroxyphenylhex-3-ene (diethylstilbestrol)	1.3[b]	5.7[b]	2.0[b]	8.4[b]	2.9	21.1	1–3	3–5
(6)	5α-androstan-3,17-dione (androstanedione)	1.1	7.1	2.4	12.7	6.5	23.9	1–3	3–5
(7)	4,16-pregnadien-3,20-dione (16-dehydroprogesterone)	1.1	6.9	2.8	14.1	5.5	32.7	3–5	3–5
	Active steroids								
(8)	5β-pregnan-12α-ol-3,20-dione	2.2	9.4	6.9	25.8	17.0	39.1	3–5	5–10
(9)	1,3,5(10)-estratrien-3,17α-diol (estradiol)	2.4[b]	17.9[b]	4.8[b]	27.4	16.2	58.5	5–10	5–10
(10)	4-androsten-3,17-dione (androstenedione)	1.1	6.7	3.5	21.6	14.5	99.6	3–5	5–10
(11)	4-androsten-17α-ol-3-one (epitestosterone)	1.2[b]	8.3[b]	4.2	18.7	17.0	126.2	3–5	5–10
(12)	4-androsten-17β-ol-3-one (testosterone)	1.3	6.5	4.4	16.2	16.4	s	3–5	5–10
(13)	4,6-androstadien-17β-ol-3-one (6-dehydrotestosterone)	1.2	6.3	6.0	17.9	22.0	s	3–5	5–10
(14)	5-androsten-3β-ol-17-one (dehydroisoandrosterone)	2.0	7.0	6.3	18.6	23.1	s	5–10	5–10
(15)	1,4-androstadien-17β-ol-3-one (1-dehydrotestosterone)	1.1	6.9	6.3	18.8	23.3	s	3–5	5–10
(16)	4-pregnen-3,11,20-trione (11-ketoprogesterone)	1.4	9.8	11.3	35.6	65.3	s	5–10	5–10

Slightly active steroids								
(17) 4-pregnen-17α,21-diol-3,20-dione (deoxycortisol)	9.4	21.0	u	S	U	S	10–20	20–30
(18) 4-pregnen-21-ol-3,11,20-trione (11-dehydrocorticosterone)	60.4	129.0	U	S	U	S	30–50	30–50
(19) 4-pregnen-11β,21-diol-3,20-dione (corticosterone)	49.3	83.5	U	S	U	S	30–50	30–50
(20) 4-androsten-3,11,17-trione (androstenetrione)	11.5	116.4	U	S	U	S	30–50	50–70
(21) 4-androsten-17β-ol-3,11-dione (11-ketotestosterone)	43.0	s	u	S	U	S	30–50	50–70
Inactive steroids[c]								
Norsesquiterpenes								
(27) gyrinidione	6.1	10.1	16.2	24.0	25.2	34.9	1–3	1–3
(28) gyrinidal	8.3	11.5	20.4	26.4	31.2	38.2	1–3	1–3
(29) gyrinidone	2.5	12.5	4.0	45.3	8.9	s	5–10	10–15

[a] A = anesthesia; RA = respiratory arrest; s = < 50% survived; S = > 50% survived; u = < 50% not anesthetized; U = > 50% not anesthetized.

[b] Concentration exceeds the solubility limit.

[c] Water soluble and inactive: 5β-cholanic acid-3α,7α,12α-triol (cholic acid) (22); 4-pregnen-18-ol-11β-21-diol-3,20-dione (aldosterone) (23); 4-pregnen-3,20-dione-21-sulfate (24); 4-pregnen-17α,21-diol-3,11,20-trione (cortisone) (25); 4-pregnen-11β,17α,21-triol-3,20-dione (cortisol) (26). Water insoluble and inactive: 5α-androstan-3β,17β-diol (30); 5α-androstan-3β-ol (31); 5α-androstan-17-one (32); 5α-androsten-3β,17β-diol (33); 5-cholesten-3β-ol (34); 4-cholesten-3-one (35); 1,3,5(10)-estratrien-17α-ol-3-benzoate (36); 1,3,5(10)-estratrien-3,16α,17β-triol (37); 1,3,5(10)-estratrien-3β-ol-17-one (38); 5α-pregnan-3β-ol(39); 5-pregnen-3-ol-20-one (40).

than others. The effect of oxygenations at the C-11 β position in reducing activity can be appreciated by comparing these compound pairs: (1) and (16), (4) and (18), (4) and (19), (10) and (20), and (12) and (21). Addition of oxygen to the C-17 resulted in a lesser reduction in activity (compare 4 and 17). Compound (8) contains a C-12 α-OH but yet is quite active. The parent 5β-pregnan-3,20-dione could be expected to be slightly more active than progesterone (1) (Figdor et al., 1957). Likewise, addition of OH at C-6 of progesterone and DOC acetate caused only a moderate reduction in activity in rat tests (Selye, 1942).

Soluble but inactive compounds were multiple oxygenated. It has already been pointed out that the addition of 17 α-OH, 11-keto, or 11-OH to DOC (4) greatly reduced activity. As seen by compounds 25 and 26, addition of the combination of 11-keto or OH plus a 17 α-OH resulted in a total loss of activity. Aldosterone (23) and cholic acid (22) are multiple oxygenated and inactive. The addition of the very polar sulfate group to the C-21 hydroxyl of DOC (4) completely inhibited activity (24).

Despite the fact that norsesquiterpenes (Table 1, B) are structurally dissimilar to steroids, their toxicities as measured by *P. promelas* were comparable. The straight chain gyrinidal and the monocyclic gyrinidione, each possessing an aldehyde and two keto moieties, fall into the highly active group of compounds. The bicyclic gyrinidone, possessing a keto and hydroxyl group in a hemiacetal linkage and one additional keto group, falls into the active group.

Like the steroids, gyrinidone was capable of inducing a prolonged and very deep anesthesia that was completely reversible. Judging from the ratio of AC_{50} to LC_{50}, the therapeutic index for gyrinidone compares favorably with that of many of the steroids (compare 29 with 12–16). When the ratios of lag times for respiratory arrest to lag times for anesthesia are compared at a given concentration such as 70 μg/ml it can be seen that values for steroids are about 5–6 and that of gyrinidone is also about 5.

Although gyrinidal and gyrinidione were highly toxic to *P. promelas*, the anesthetic qualities of these compounds were less evident than those for gyrinidone or the steroids. When gyrinidal and gyrinidione caused minnows to lose their equilibrium, minnows never became totally quiescent and RA occurred very soon after equilibrium loss. Ratios of lag times for RA to lag times for anesthesia at 70 μg/ml are much lower for gyrinidal and gyrinidione than gyrinidone (1.4 and 1.6 vs. 5). Once equilibrium loss had occurred in minnows exposed to gyrinidal and gyrinidione, recovery was poor even after these minnows were then transferred to fresh water. On various occasions gyrinidal and gyrinidione treated minnows bled from the gills at about the time death occurred. From our observations these two compounds are more toxic in nature than anesthetic.

The lag times for steroid anesthesia and RA presented in Table 2 provide a means for comparing the activities of compounds when they are administered at increasing concentrations. As toxicant concentration increased, a convergence of lag times of the highly active and active steroids occurred [see Fig. 1 where compound (2) is representative of the highly active group and compound (13) of the active group]. At 70 μg/ml the lag times for a great number of terminally oxygenated steroids were nearly identical. Lag times of multiple oxygenated compounds such as (17) did not converge with those of the active compounds.

It can be suggested, then, that provided deactivation mechanisms are not a factor, either the absorption rates and activities of active compounds are quite similar or else differences in absorption rates happened to be offset by compensating differences in activity. We favor the former explanation and its implication that the activity of steroids is a very nonspecific phenomenon where similar quantities of many steroids elicit the same generalized effect.

Even though their AC_{50} and LC_{50} values were similar, lag times for a few steroids in the highly active group were distinctly different from the others. Most notable is androsterone (3) for which at 10 μg/ml lag times were significantly shorter than for compounds (1), (2), (4), (6), and (7) (P0.01). Figdor et al. (1957) reported similar findings in the mouse, where the onset of anesthesia for some steroids varied quite widely from others. It was proposed that varying times for the onset of anesthesia were due to differences in rates of steroid uptake brought about by differences in molecular configuration and solubilities; however, no clear-cut relationship was outlined. Likewise, compounds 3 and 5 in Table 2 may have been absorbed more rapidly than the others by *P. promelas*.

Reexamination of Table 1 in light of the data presented in Table 2 leads one to conclude that the defensive arsenal of dytiscids has been so selected to include only those steroids highly anesthetic and toxic to fish. The compounds most widely occurring as major components of dytiscid secretions, 4-pregnen-3-ones and 4,6-pregnandien-3-ones, were among those compounds most active in minnow bioassays. Although steroids oxygenated in positions in addition to the termini are produced as major components of the defensive secretions of some dytiscid species, such oxygenation never occurs at C-11 or C-17 in the pregnenes or pregnadienes. Interestingly, the C-7, -12, and -15 positions are additionally hydroxylated, and in some cases such hydroxyl groups occur as isobutyrate derivatives.

Preliminary bioassays conducted by Schildknecht and co-workers indicate that the biological activities of dytiscid 4-pregnenes, 4,6-pregnadienes, and their ring hydroxylated derivatives are very similar (Schildknecht et al., 1967b; Schildknecht and Hotz, 1971). When administered in solution to goldfish these compounds have little or no effect at 1 μg/ml. At 2 μg/ml

goldfish are slightly narcotized, and at 10 μg/ml they are deeply anesthetized.

It is rare that 4-androstenes are found in dytiscid secretions, and they are never the major components. As previously suggested in this paper, their apparent higher rate of detoxification may make them less efficient toxins. Estradiol and estrone were the only highly water-insoluble steroids found in dytiscid secretions. In the one beetle in which they were found, *Ilybius fenestratus*, they occurred only in minor or trace amounts.

CONCLUSIONS

Under natural conditions water beetles presumably administer their defensive agents to a predator via the digestive track (Blunck, 1917). In the present study, test compounds were administered in external solution to fish, and the results may therefore not be directly applicable to the natural situation. However, on the basis of the data that have now been accumulated, it can be suggested that the Dytiscidae are exploiting the anesthetic action of steroids in their defense. Dytiscids produce a range of 4-pregnen-3-ones and 4,6-pregnadien-3-ones as major defensive steroids but these compounds all possess certain physico-chemical properties that place them among the more highly anesthetic steroids known. The toxicity of these steroids presumably results from the depression of the respiratory center of the brain.

The gyrinid norsesquiterpenes apparently act in several ways. Gyrinidal and gyrinidione are highly toxic to fish but possess little definitive anesthetic quality. Judged from the hemorrhaging that takes place in minnows treated with these compounds, they may be membrane active, i.e., possibly hemolytic agents. Gyrinidone possesses anesthetic qualities similar to those of the steroids. To our knowledge, the anesthetic quality of oxygenated norsesquiterpenes has not previously been recognized. Further investigation of gyrinidione and its analogs could lead to the discovery of compounds that would be useful medicinally.

When minnows are placed in concentrated aqueous solutions of steroids where the rate of steroid absorption is far more rapid than deactivation mechanisms, many steroids apparently have similar activities suggesting that they may exert their effects via a very nonspecific process, possibly membrane stabilization (Seeman, 1969). However, certain limits on the physiochemical properties of steroids do govern activity, as not all steroids are active against fish. Water solubility appears to be of great importance in determining the activity of steroids administered in solution to fish. Other properties such as the partition coefficient of steroids across the aqueous–lipid interface of membranes may be of even greater importance. Until the actual rates of uptake of steroids into fish are measured it is impossible to state whether the

steroids found to be inactive in minnow bioassays appear so because they fail to reach the target tissues or whether they are innately less toxic even though they gain access to the cells of the body.

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PHYSIOLOGICAL ACTIVITY OF WATER BEETLE DEFENSIVE AGENTS. II. ABSORPTION OF SELECTED ANESTHETIC STEROIDS AND NORSESQUITERPENES ACROSS GILL MEMBRANES OF THE MINNOW *Pimephales promelas* Raf.

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Abstract—The uptake of selected steroids and norsesquiterpenes by live minnows, *Pimephales promelas*, was studied when the compounds were administered externally in aqueous solution. The gills of minnows absorbed 80% of the steroid removed from solution. Steroid absorption across minnow gills is apparently a nonmediated process as rate saturation could not be demonstrated. Initial absorption rates of test compounds were inversely correlated with (1) the degree of oxygenation, (2) water solubility, and (3) polarity on silica gel thin-layer chromatography. These findings support the Stein model of nonmediated transport. The majority of compounds anesthetic and toxic to minnows exerted their effects at a similar internal concentration. Anesthesia occurred at ca. 10^{-6}–10^{-5} M and death at 10^{-4}–10^{-3} M. Since various agents causing membrane stabilization and lysis in in vitro systems act in an identical concentration range, it is proposed that the defensive steroids and norsesquiterpenes of water beetles act via membrane stabilization and lysis.

Key words—minnow, *Pimephales promelas*, chemical defense, steroids, norsesquiterpenes, gyrinidae, anesthesia, toxicity.

INTRODUCTION

Bioassays in which the anesthetic activity and toxicity of aqueous solutions of steroids were measured using minnows (Miller and Mumma, 1976; Selye and

Heard, 1943) demonstrated that the physiological activity of steroids was greatly dependent on the degree of nuclear substitution in the steroid molecule. Steroids oxygenated only at the termini of the molecule (C-3, -17, -20, or -21) were generally active, while those oxygenated at positions such as C-11 in addition to the termini were considerably less active or inactive. Since they were administered in the external solution bathing the minnows, the steroids tested in the minnow bioassays first had to cross the membranes of the gills or skin of the fish before gaining access to the target tissues. It was not known whether compounds possessing high nuclear substitution were absorbed by the fish but were innately less active than steroids oxygenated at the termini, or whether such compounds appeared inactive only because their rates of uptake were very low.

The transport of various steroids and norsesquiterpenes across the gill membranes of live minnows is the subject of this report. These experiments were designed to provide basic information on steroid transport through animal membranes as well as to elucidate further the physiological activity of beetle defensive agents.

METHODS AND MATERIALS

Experimental Animals and Test Compounds

Fathead minnows, *Pimephales promelas* Raf., weighing 0.9 ± 0.2 g were used in all experiments. Minnows were standardized and allowed to equilibrate to 25°C (Miller and Mumma, 1975). Chromatographically pure steroids were purchased commercially while the norsesquiterpenes were isolated from the pygidial secretions of the gyrinid beetles *Dineutus assimilis* and *Dineutus nigrior* (Miller et al., 1975). The purity of norsesquiterpenes used was 97–99%. Stock solutions of terminally oxygenated steroids and of norsesquiterpenes were prepared at 67 μg/μl ethanol while stock solutions of steroids oxygenated at C-11 or C-17α were prepared at 34 μg/μl 50% aqueous ethanol. Aqueous solutions of steroids used for uptake studies were prepared by injecting the appropriate quantity of stock solution into 50 ml tap water.

Experiment 1—*Site of Steroid Uptake by Minnows*

Before studies were undertaken of the rates of steroid absorption by minnows held in aqueous solution of these compounds, the site(s) of steroid uptake was determined. The apparatus shown in Figure 1 was designed to separate the anterior gill-bearing portion of minnows from the remainder of the body and thereby provide a means of monitoring the comparative rates at which the respective body portions depleted a solution of deoxycorticosterone

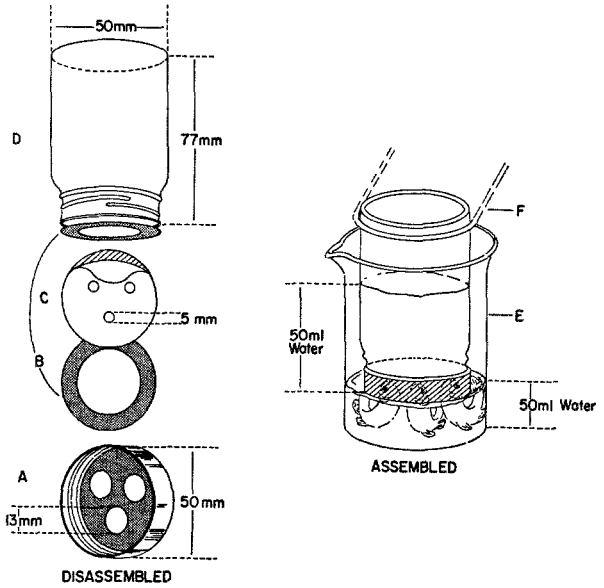

FIG. 1. Apparatus used to determine the site of DOC absorption by *Pimephales promelas*. A = plastic bottle cap; B = rubber washer; C = two thicknesses of rubber sheeting; D = glass bottle having bottom removed; E = 250-ml beaker; and F = supporting clamp.

(DOC). The apparatus itself was first checked and found not to absorb any measurable quantity of DOC from 50 ml of a 45 μM solution over 2 h. Also, the rubber sheeting (Hygenic Dental Manufacturing Company, Akron, Ohio) separating the compartments was found to be completely impermeable to DOC at the highest concentration gradient tested (90 μM vs. tap water).

Minnows were inserted through the 5-mm holes in the rubber sheeting of the assembled apparatus (Figure 1) so that the portion of the body from the pectoral fins forward was on the inside of the bottle. When the bottle was inverted, the pectoral fins were instrumental in holding the minnows in a position where a water-tight seal was maintained. Fifty milliliters of aerated aqueous DOC (90 μM) was poured into the inverted bottle and the apparatus was inserted into a 250-ml beaker containing an identical 50-ml DOC solution (See Figure 1). One milliliter aliquots were removed from each compartment at timed intervals and their DOC concentration was determined by reading them directly in the uv at 247 nm on a Bausch and Lomb Spectronic 600 spectrophotometer. The experiment was continued for 60 min even

though the minnows were killed by the DOC solution in ca. 15 min. Upon termination of the experiment the volume of water remaining in each compartment was measured to ascertain that no leakage had occurred. The amount of DOC uptake per gram of fish from time 0 to any time t was computed as follows: let Q_t = total quantity (nmoles) DOC absorbed per gram fish during the interval 0 to t, A = starting quantity DOC in the containers = [starting concentration × starting volume], c_t = concentration of the aliquot taken at t, V_t = volume in the assay container before aliquot t is withdrawn, v = volume of aliquots withdrawn (always 1 ml in this study), and W = total weight of the minnows. Then the quantity of DOC absorbed at time 1 = $Q_1 = (A - c_1 V_1)/W$, and for $t > 1$, Q_t is given by the formula

$$Q_t = \frac{A - [(c_t V_t) + \Sigma_{i=1}^{t-1} c_i v]}{W}$$

where the term $\Sigma_{i=1}^{t-1} c_i v$ takes into account the quantity of DOC removed for the purpose of UV determinations of concentration.

As a control, samples were withdrawn and read when minnows were exposed only to tap water in both compartments. Such experiments provided a measure of the changes in optical density (OD) at 247 nm that minnows effected independent of the presence of DOC. Experiment 1 with its controls was replicated three times.

Experiment 2—Rate of DOC Uptake vs. External Concentration

The results of Experiment 1 indicated that the gills of minnows were the major site of steroid uptake but that the skin of the body was responsible for some of the absorption. Subsequent absorption studies were limited to the gills of minnows so that the results obtained could be attributed to one specific tissue and would not be confounded by absorption phenomenona of other tissues. Although no evidence was observed that DOC was adsorbed in the mucous layer coating minnows, we chose to eliminate this possibility as much as possible.

Further absorption studies were conducted as follows (Miller, 1975). By means of a short length of cotton thread tied to the base of the caudal fin, three minnows were suspended vertically from wire hangers. At a time 0 the three minnows were lowered into a 250-ml beaker containing 50 ml of an aerated steroid solution of known concentration. Minnows were left hanging vertically so that only their heads and gills were submerged in the steroid solution. One-milliliter samples were removed at carefully timed intervals and calculations of steroid uptake were performed as in Experiment 1.

In order to characterize the kinetics of steroid absorption across minnow gill membranes, uptake profiles of DOC were measured at concentrations of

3, 15, 30, 45, 60, 90, 150, and 210 μM. Absorption profiles at higher levels were not obtained owing to the error limitations inherent in the method. The design of Experiment 2 was randomized complete block with three replications of blocks. Two controls where minnows were exposed only to tap water were conducted in each block in order to correct for OD changes independent of DOC uptake.

Experiment 3—*Comparative Uptake of Selected Steroids and Norsesquiterpenes*

Gill uptake of the following series of compounds was measured by the methods of Experiment 2 (the UV λ max in nm of each compound in water is presented in parentheses with the trivial names); 4-androsten-3,17-dione (androstenedione, 247), 4-androsten-17β-ol-3-one (testosterone, 248), 4-pregnen-3,20-dione (progesterone, 247), 4-pregnen-3,11,20-trione (11-keto-progesterone, 244), 4-pregnen-21β-ol-3,20-dione (DOC, 247), 4-pregnen-17α, 21-diol-3,20-dione (11-deoxycortisol, 247), 4-pregnen-11β,21-diol-3,20-dione (corticosterone, 247), 4-pregnen-21-ol-3,11,20-trione (11-dehydrocorticosterone, 247), 4-pregnen-17α,21-diol-3,11,20-trione (cortisone, 244), 4-pregnen-11β,17α,21-triol-3,20-dione (cortisol, 247), (*E,E,E*)-3,7-dimethyl-8,11-dioxo-2,6,9-dodecatrienal (gyrinidal, 241), and (*E,Z*)-2-hydroxy-5,9-dimethyl-4(but-1′-ene-3′-one)-3-oxobicyclo[4.3.0]-non-4-ene (gyrinidone, 318). Uptake of the compounds that were active in minnow bioassays was measured at both 45 and 90 μM, while uptake of those that were only slightly active or inactive was measured at only 90 μM. In each case, absorption was monitored for 2 h. Experiment 3 was conducted using a randomized complete block design where blocks were replicated three times. Within each block three controls were conducted where minnows received no steroid so that corrections could be made for OD changes that occurred independent of steroid depletion. The aliquots taken in these controls were read at the λ max of each of the compounds tested. Data were submitted to an analysis of variance following transformation to $\sqrt{x+0.5}$.

RESULTS

Experiment 1—*Site of Steroid Absorption*

Comparative uptake of DOC by the head and body portions of minnows is presented in Figure 2. At 90 μM the initial rate of DOC uptake by the head was 40 nmole g^{-1} min^{-1} while that for the body was 6 nmole g^{-1} min^{-1}. As expected, absorption rates began to decrease after several minutes as DOC began to anesthetize the minnows. Head and body absorption rates progressively decreased, reached zero at about the time of respiratory arrest (RA), and

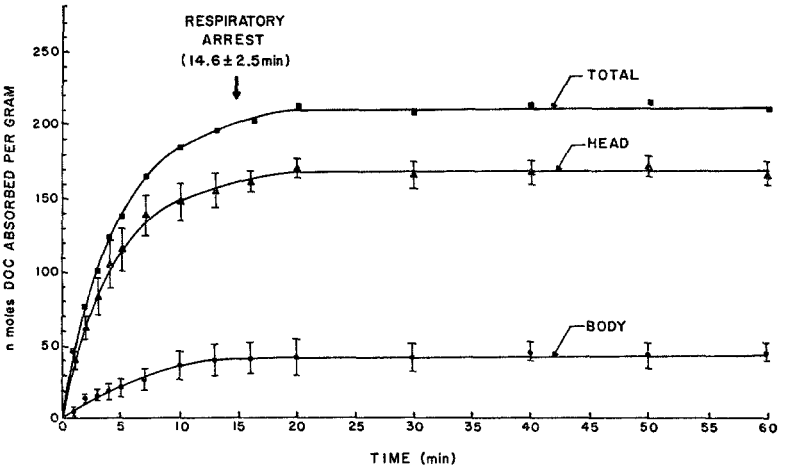

FIG. 2. Comparative uptake of DOC by the head and body portions of minnows.

remained at zero for the remainder of the experiment. By the time of death a total of 210 nmoles DOC/g was absorbed; 168 nmole/g by the head and 42 nmole/g by the body portions of minnows. Accordingly, 80% of the absorption occurred via the gills, and 20% through the skin of the body. Experiment 1 demonstrated that the gills are the primary site of steroid uptake by minnows held in aqueous steroid solutions.

In the control experiments where minnows were exposed only to tap water, the OD at 347 nm of aliquots taken from both compartments increased slightly over 1 h. The OD increase in the head compartment was equivalent to the addition into the assay container of 26 nmole DOC/g fish, while that in the tail compartments was equivalent to the addition of 41 nmole DOC/g. In order to correct for the increase in OD in the steroid absorption experiments, the change in OD/g fish in the controls was plotted against time and the appropriate correction was applied to the steroid absorption experiments up to the point of RA. Such corrections were made in all absorption data that will be reported.

Experiment 2—Rate of DOC Uptake vs. Concentration

Absorption profiles for DOC at various concentrations are presented in Figure 3. At 3 μM, minnows absorbed 55 nmole DOC/g (virtually 100% of the steroid administered to them) by ca. 1 h and that dose was not lethal. At 15 μM minnows absorbed a lethal dose of DOC (102 nmole/g) with RA occurring in ca. 45 min. Therefore, the LD_{50} for DOC administered across the

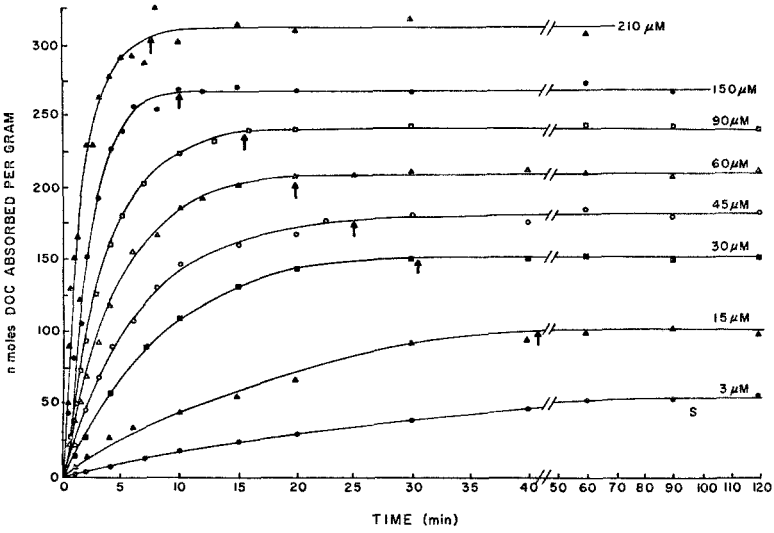

FIG. 3. Absorption profiles for DOC at various concentrations. Arrows indicate mean time of death. S indicates minnows survived.

gills lies between 55 and 102 nmole/g. Assuming that 1 g of fish is roughly equivalent to 1 ml water, the internal concentration of DOC that was lethal lay between 5.5×10^{-5} and 1.1×10^{-4} M. It was also observed that in a 3 μM DOC solution less than 50% of minnows tested were anesthetized but that at 15 μM all minnows were anesthetized in ca. 20 min. Judging from the 15-μM DOC absorption profile (Figure 3) approximately 70 nmole DOC are

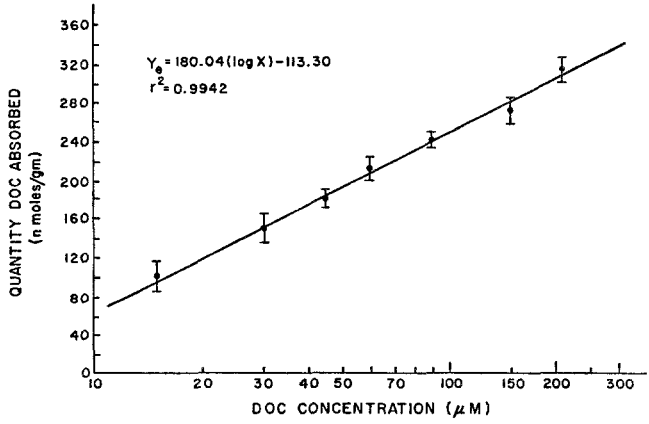

FIG. 4. Quantity of DOC absorbed vs. log of concentration.

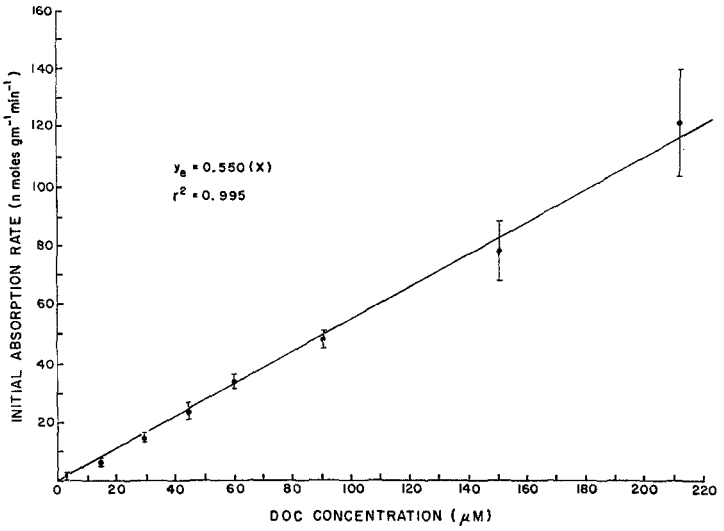

FIG. 5. Rate of DOC uptake vs. concentration.

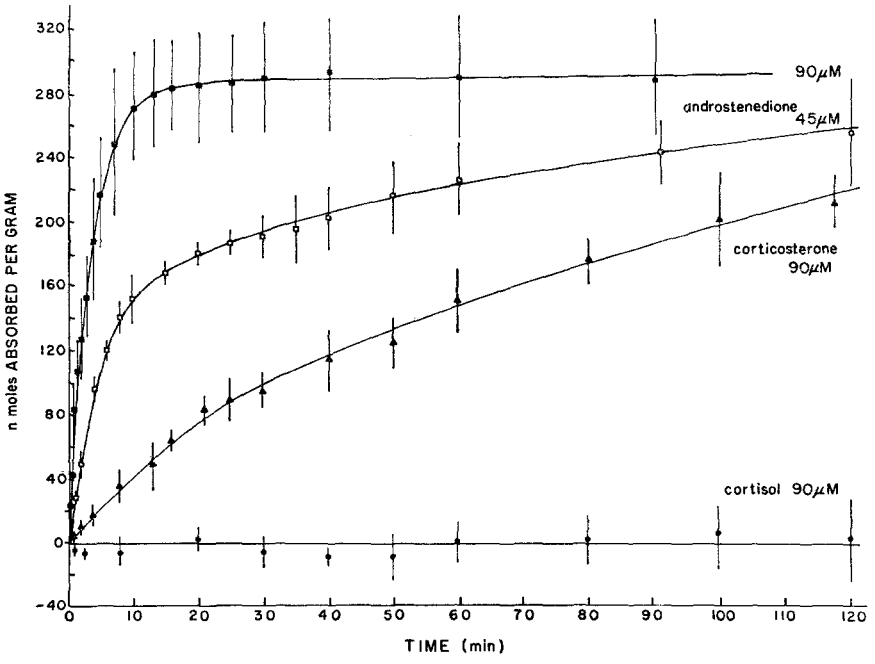

FIG. 6. Absorption profiles for androstenedione, corticosterone, and cortisol.

absorbed per gram by 20 min. Therefore, the dosage required to anesthetize minnows was 55–70 nmole/g ($5.5–7.0 \times 10^{-5}$ M internal concentration).

At concentrations of 15–210 μM the quantity of DOC absorbed by the time of RA increased linearly with the log of concentration (Figure 4). At the high concentrations, considerably more steroids were absorbed before RA than proved lethal at the low concentrations. These data indicate that a lag exists between the time that DOC was taken into the fish and the time that it acted on the target tissues. This would suggest that the target tissue of these compounds must be distant from the gills, possibly the central nervous system.

Initial absorption rates increased linearly with increasing concentration (Figure 5). Even at the highest concentration examined (210 μM), there was no indication of rate saturation.

Experiment 3—*Comparative Uptake of Selected Steroids and Norsesquiterpenes*

Absorption profiles typical of the compounds tested are presented in

TABLE 1. INITIAL RATES OF STEROID AND NORSESQUITERPENE ABSORPTION BY THE GILLS OF *Pimephales promelas*

Compound	External concentration (μM)	Initial absorption rate[a] (nmole g^{-1} min^{-1})
Progesterone	90	80.5±7.4 a
	45	42.3±1.8
Androstenedione	90	63.9±8.1 a
	45	26.9±2.0
Testosterone	90	52.0±3.5 b
	45	28.8±7.2
DOC	90	49.3±4.0 b
	45	25.1±1.8
Gyrinidone	90	47.9±2.5 b
	45	19.1±2.1
11-Ketoprogesterone	90	36.5±6.1 c
Gyrinidal	90	32.9±2.5 c
	45	13.7±2.9
11-Deoxycortisol	90	11.5±8.2 d
Corticosterone	90	3.5±2.1 e
11-Dehydrocorticosterone	90	2.9±0.6 e
Cortisone	90	0 e
Cortisol	90	0 e

[a] Means followed by the same letter are not significantly different from each other as determined by Duncan's (Bayesian) least significant difference test.

Figure 6. Some compounds were very rapidly absorbed (androstenedione), some were absorbed at moderate rates (corticosterone), and in some instances no absorption was detected (cortisol). Initial absorption rates are presented in Table 1; compounds are listed according to decreasing initial absorption rates at 90 μM.

Of the compounds whose absorption rates were measured at two concentrations, absorption rates at 90 μM were nearly double the rates at 45 μM. Apparently, absorption rates of these compounds increase linearly with concentration, as was shown to be true for DOC (Figure 5).

From Table 1 it can be seen that steroids oxygenated only at the termini of the molecule were more readily absorbed by the minnows than were steroids possessing additional oxidation. Of these compounds examined, progesterone was absorbed most readily. However, the addition of a keto moiety to the C-11 position (11-ketoprogesterone) resulted in a reduction of 50% in uptake rate. Similar effects of nuclear substitution in the steroid rings

TABLE 2. QUANTITIES OF STEROIDS AND NORSESQUITERPENES ABSORBED BY THE GILLS OF *Pimephales promelas*

Compound	External concentration (μM)	Quantity absorbed by 120 min[a] (nmole/g)	
Gyrinidone	90	509.8 ± 58.1[b]	a
Testosterone	45	326.9 ± 49.9	b
Gyrinidal	90	324.8 ± 57.7	b
11-Deoxycortisol	90	297.3 ± 30.6[b]	bc
Gyrinidone	45	296.7 ± 51.3[b]	bcd
Androstenedione	90	295.7 ± 36.7	bcd
Progesterone	90	279.9 ± 38.1	bcde
Androstenedione	45	253.9 ± 34.9[b]	cdef
DOC	90	239.0 ± 18.2	defg
11-Ketoprogesterone	90	239.4 ± 14.0	defg
Progesterone	45	228.0 ± 36.7	efg
Testosterone	90	216.0 ± 8.7	fg
Corticosterone	90	211.6 ± 17.6[b]	fg
11-Dehydrocorticosterone	90	199.5 ± 37.7[b]	fg
DOC	45	186.7 ± 9.3	g
Gyrinidal	45	179.5 ± 15.9	g
Cortisone	90	0[c]	h
Cortisol	90	0[c]	h

[a] Means followed by the same letter are not significantly different from each other as determined by Duncan's modified (Bayesian) least significant difference test.
[b] Some minnows alive at 120 min.
[c] All minnows alive at 120 min.

can be seen by examining the absorption rates of DOC and its nuclear substituted derivatives. The addition of C-17α-hydroxyl group to DOC (11-deoxycortisol) resulted in a reduction in absorption rate of 77%. Addition of an hydroxyl or keto group to the C-11 position of DOC (corticosterone, 11-dehydrocorticosterone) resulted in a reduction in absorption rate of ca. 94%. Addition of the combination of C-17α-hydroxy and C-11-hydroxy or keto groups to DOC resulted in a 100% reduction in uptake (cortisone, cortisol).

The norsesquiterpenes were absorbed at rates slightly lower than DOC. The bicyclic gyrinidone, possessing one hydroxyl and keto group in a hemiacetal linkage plus one additional keto group, was absorbed slightly faster than was the straight chain gyrinidal, possessing an aldehyde and two keto groups.

Information on compound toxicities can be deduced from measurements of the quantity of compound absorbed by the time of RA or by the 2-h limit of the experiment (Table 2). At 90 μM a lethal dosage of below 300 nmole/g was recorded for progesterone, androstenedione, testosterone, DOC, and 11-ketoprogesterone. When these five steroids were administered to fish at rates of at least 35 nmole g^{-1} min^{-1} their toxicities were similar. However, at 45 μM some minnows treated with androstenedione and testosterone survived while those exposed to progesterone and DOC were killed. Even though androstenedione, testosterone, and DOC were all entering the fish at ca. 25 nmole g^{-1} min^{-1}, the former two compounds were less toxic than the latter. As was suggested by Miller and Mumma (1976) androstenedione and testosterone may be more readily metabolized or otherwise detoxified by the fish than is DOC.

The data in Table 2 gave no clear indication that the toxicities of 11-deoxycortisol, corticosterone, and 11-dehydrocorticosterone are greatly different from the other steroids once these compounds are absorbed. 11-Deoxycortisol administered at 300 nmole/g over 2 h was lethal to ca. 50% of the minnows tested. Corticosterone and 11-dehydrocorticosterone administered at 200 nmole/g over 2 h also killed some minnows. Had these compounds been administered more rapidly the actual dosage of absorbed compound required to kill could well have been comparable to the other steroids.

Gyrinidal was shown to be considerably more toxic than gyrinidone. The dosage of gyrinidal required to kill all minnows falls below 180 nmole/g while that of gyrinidone is greater than 500 nmole/g. Although absorbed gyrinidone was less toxic than most of the steroids, the toxicity of absorbed gyrinidal was comparable to that of DOC and progesterone.

DISCUSSION

The gills of fish are the primary site of exchange of materials between the

blood and external medium; however, the skin of fish is an important auxillary site of exchange (Lagler et al., 1962). It is not surprising, therefore, that the gills of *P. promelas* were responsible for 80% of the uptake of steroids from solution and the skin 20%.

A number of indications were found that uptake of steroids across fish gill membranes is a nonmediated process occurring by passive diffusion. First, no evidence for rate saturation was observed for DOC when absorption rates were measured at concentrations up to 210 μM. Further, a log–log plot of minnow survival time vs. DOC concentration was found to be linear (followed a Freundlich absorption isotherm) from a concentration of 30 μM to 500 μM. Therefore, no evidence of rate saturation for DOC uptake was observed over the concentration range bounded by the water solubility of DOC.

Second, the gills show very little selectivity in steroid uptake. Based on minnow bioassays (Miller and Mumma, 1976; Selye and Heard, 1943) a great number of steroids of diverse structure are readily absorbed into fish. Third, no evidence for inhibition of steroid uptake could be demonstrated. The presence of those steroids such as cortisol which are not absorbed into fish had no effect on the uptake of active compounds when steroids were administered in combination. Therefore, it can be concluded that as was found for sterols (Rothblat et al., 1966) the uptake of steroids administered at pharmacological dosages is a nonmediated process.

From in vitro studies the hemolytic and cytotoxic properties of steroids and other anesthetic agents are well known (Kappas and Palmer, 1963; Weissman 1955; Weissman and Keiser, 1965). Seeman has characterized the initial action of steroids as being a part of the broad phenomenon of membrane expansion and stabilization (Seeman, 1966; Seeman, 1969). Like the various other membrane stabilizers, steroids demonstrate the typical biphasic effect on membranes. At extracellular concentrations of ca. 10^{-6}–10^{-5} M, steroids stabilize membranes, causing them to be resistant to hypotonic lysis. Such stabilization is accompanied by a degree of anesthesia as cell membranes become less permeable to ions and become depolarized. However, at concentrations of 10^{-4}–10^{-3} M steroids cause lysis and membrane deterioration of cells and organelles of in vitro systems.

Such studies on membrane stabilization provide the basis for proposing the mode of action of water beetle defensive agents. The anesthetic action and toxicity of steroids on live minnows can be attributed to their stabilizing and lytic action since our calculations show that, as in in vitro studies, minnows are anesthetized at internal concentrations of 10^{-6}–10^{-5} M and are killed at 10^{-4}–10^{-3} M. The norsesquiterpenes are also apparently acting via membrane stabilization and lysis as they are active at the same internal concentration range.

One of the most widely accepted models of nonmediated transport across

membranes is advanced by Stein (1967). In this model, transport through the bimolecular leaflet occurs when the permeant species dissolves in the lipid layer of the cell membrane by a partitioning process, penetrates through a lattice formed by the spaces between lipid chains by a diffusion process, and partitions into the aqueous medium on the far side of the membrane. Therefore, any physicochemical property of the permeant species that influences the partitioning or diffusing processes will affect the rate of transport, i.e. degree of hydrogen bonding, molecular polarity, van der Waals interactions, molecule size and dimensions, etc. However, it is generally agreed that since most membranes are very thin, the partitioning step is usually rate limiting (Lieb and Stein, 1971; Kotyk and Janacek, 1970).

The data reported in this study are consistent with the Stein model of nonmediated transport. Those compounds most highly oxygenated were least readily absorbed. Multiple oxygenation retards the partitioning of these compounds into the cellular lipid in at least two ways. First, with each additional site of oxygenation additional hydrogen bonds have to be broken for entry into the membrane. The activation energy is therefore raised and net transport is decreased. Second, each oxygenation of the ring causes masking of CH_2 groups that contribute greatly toward stabilizing the transition state of the permeant species as it enters the membrane lipid (Stein, 1967).

Judging from the Stein model, uptake of a series of compounds should

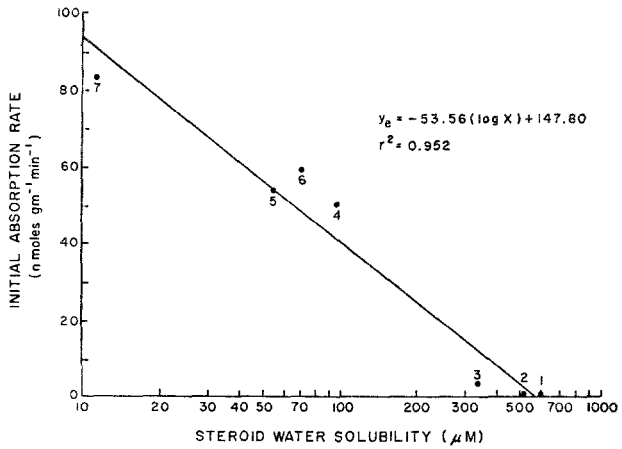

FIG. 7. Correlation of steroid water solubility with uptake rates into *Pimiphales promelas*. 1 = cortisol; 2 = cortisone; 3 = corticosterone; 4 = DOC; 5 = testosterone; 6 = androstenedione; 7 = progesterone.

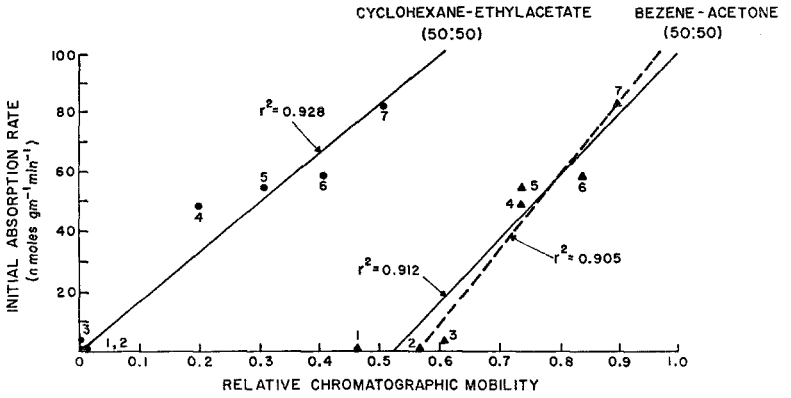

FIG. 8. Correlation of steroid chromatographic mobility with uptake rates into *Pimephales promelas*. Values for steroid chromatographic mobility were taken from Stahl (1969). All points are included in solid regression lines; compounds not absorbed are deleted in the broken regression line. 1 = cortisol; 2 = cortisone; 3 = corticosterone; 4 = DOC; 5 = testosterone; 6 = androsterone; 7 = progesterone.

be highly dependent on their partition coefficients between water and lipid phases. Although data on the partition coefficients of the steroids studied here were not available, some values of steroid water solubilities were found in the literature (Slaunwhite et al., 1963), and plotted against steroid uptake rates into fish (Figure 7). Although solubility data were available for only seven of the steroids for which we had determined uptake rates, it can be seen that a plot of uptake rates vs. the log of steroid water solubility resulted in a highly significant inverse correlation. The more highly water soluble compounds were absorbed most slowly.

If indeed the absorption of steroids across membranes is largely influenced by a partitioning process, it can be reasoned that a correlation should exist between the rates of steroid uptake and mobility in partition chromatography. When the rates of steroid uptake were plotted against steroid mobility on Silica Gel-G, a highly significant correlation was in fact found (Figure 8). The correlation holds in various solvent systems, two of which are shown in Figure 8. Our data strongly support the Stein model of nonmediated transport.

Various pharmacological effects of steroids are somewhat structure specific (Kappas and Palmer, 1963; Weissman, 1955; Weissman and Keiser, 1965; Figdor et al., 1957) in that the most highly anesthetic, hemolytic, and cytotoxic compounds are those oxygenated only at the termini of the molecule. In all cases additional oxygenation at positions C-11 and C-17α causes a reduction in activity. Based upon the data presented here, such differences in

steroid activity can be explained by differences in uptake rates. In many cases, compounds with *cis* AB ring fusion are physiologically most active. Such compounds might simply be most rapidly absorbed.

The data presented here are of some use in understanding the biology of water beetle defense against predation. In the natural setting, beetle defensive agents are presumably administered to predators via the digestive tract. Predators force-fed beetles are narcotized (Blunck, 1917). No data are presently available on the comparative uptake rates of defensive agents across the gut as opposed to the gills. Such information would be more difficult to obtain but would undoubtedly be of greater value in evaluating the efficacy of these defensive secretions under natural conditions. Assuming the uptake rates into the tissues are similar to those found here once the compounds reach the bloodstream, the most highly active compounds would be the terminally oxygenated pregnanes and pregnenes. Dytiscid beetles do preferentially secrete terminally oxygenated pregnenes (Miller and Mumma, 1976).

In the natural setting of a pond or lake, fish are probably not poisoned by gill-absorbed beetle toxins. Based on the rates of gill absorption of steroids and norsesquiterpenes reported here, it is doubtful whether sufficiently high concentration gradients could be established for significant gill uptake of toxins. At 25°C, DOC and gyrinidal concentrations of at least 3 μM were required to poison minnows.

On the other hand, beetle defensive agents are known to be highly repellent to fish (Benfield, 1972). It is not yet certain whether the repellent action of beetle defensive agents is independent of their anesthetic or toxic action. Fish may develop an aversion to these beetles after experiencing illness due to consuming them. Even though the ultimate effect of a meal may be delayed for hours, it is known that only a few trials are required for animals to develop a long-term aversion to noxious prey (Garcia et al., 1974). That such repellency in fish might be related to gill absorption of small quantities of toxins has not been ruled out.

The structural similarity of the norsesquiterpenes to various monoterpene defensive agents of arthropods (Weatherston and Percy, 1970) raises the question of whether such compounds might also act as toxins via membrane stabilization and lysis. The findings that beetles are exploiting membrane stabilization also raises the possibility that an examination of arthropod defensive agents could lead to the discovery of agents whose membrane activity could be exploited for medicinal purposes.

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TRAIL-FOLLOWING PHEROMONES OF THE RHINOTERMITIDAE: APPROACHES TO THEIR AUTHENTICATION AND SPECIFICITY

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Abstract—A method for determining the authenticity of subterranean termite trail pheromones is suggested and utilized to verify the presence of trail pheromones in *Reticulitermes virginicus*, *R. flavipes*, and *R. tibialis*. In addition, a possible trail pheromone has been demonstrated for *Coptotermes formosanus*. A choice bioassay method shows that the above trail pheromones are species specific.

Key Words—subterranean termites, chemical ecology, insect behavior, bioassay, trail pheromone, *Reticulitermes virginicus*, *Reticulitermes flavipes*, *Reticulitermes tibilias*, *Coptotermes formosanus*.

INTRODUCTION

The presence and utilization of trail-following pheromones (hereafter referred to as trail pheromones) has been known for years (Stuart, 1963; 1969). Recently several such chemicals have been isolated and identified from *Zootermopsis nevadensis* (Hagen) (Hummel, 1968), *Reticulitermes virginicus* (Banks) (Tai et al., 1969), and *Nasutitermes exitiosus* (Hill) (Birch et al., 1972). Partial chemical characterization has been achieved for trail pheromones of *Nasutitermes* spp. (Moore, 1966; 1974), *Trinervitermes trinervoides* (Sjostedt) (Tschinkel and Close, 1973) and *Anacanthotermes ahngerianus* Jacobson (Shatov, 1974a,b). Tschinkel and Close's work is significant in that extracts of trails laid on a paper substrate were utilized rather than whole-organism extracts. In addition, recent nonchemical studies on termite trail pheromones have been reported for the species *Z. nevadensis*, *N. corniger*

(Motschulsky), *Z. angusticollis* (Hagen) (Stuart, 1963, 1964), *Hodotermes mossambicus* (Hagen), and *T. trinervoides* (Hewitt et al., 1969).

Elucidation of the nature and function of these chemicals, however, has been hampered by methodological problems, not the least of which has been lack of agreement on a critical method for distinguishing a true termite trail pheromone from a potent, nonpheromonal attractant (such as those reported by Verron and Barbier, 1962; Watanabe and Casida, 1963; Becker and Petrowicz 1967; Karlson et al., 1968; Birch et al., 1970; Tai et al., 1971; and Matsumura et al., 1972). A further complication has been a lack of agreement as to what constituted an adequate bioassay (Stuart, 1969; Shatov, 1974a,b).

An interesting but as yet unanswered question concerns the specificity of termite trail pheromones at various taxonomic levels. Moore (1974) has suggested that, at least for the *Nasuititermes*, a single common trail pheromone is utilized and enriched with secondary chemicals to provide specificity at the species level. Details of the relevant experiments have not been published, however, so that an evaluation of this conclusion is not possible. Matsumura et al. (1972) noted that several Rhinotermitids showed selective trail-following responses to (*Z,Z,E*)-3,6,8-dodecatrien-1-ol (the trail pheromone of *R. virginicus*) and several analogs (albeit at different concentration levels) but concluded that their data did not allow an unequivocal assessment of species specificity.

In our opinion, the lack of an adequate method for ascertaining termite trail pheromone authenticity represents a major hurdle to progress in chemical ecological studies of subterranean termites. Our efforts at developing the necessary methods have utilized the subterranean termites *R. virginicus*, *R. flavipes* (Kollar), *R. tibialis* Banks, and *Coptotermes formosanus* Shiraki. In addition, we have addressed ourselves to the question of species specificity of the trail pheromones from each of the above four species.

MATERIALS AND METHODS

All termites were from stock cultures maintained in our laboratories, either in 20-gallon galvanized trash cans held at room temperature (*R. flavipes* and *C. formosanus*) or in plastic shoeboxes in an incubator held at 25°C (*R. virginicus* and *R. tibialis*). Sound southern pine, *Pinus* spp., was provided as food. The stock cultures of *R. flavipes* were obtained on June 5, 1973 from Janesville, Wisconsin and May, 1975 from Gulfport, Mississippi; those of *R. virginicus* were obtained during fall, 1968 and spring, 1975 from different sites in Gulfport, Mississippi; that of *R. tibialis* was obtained during May, 1970 from Galena, Illinois; and that of *C. formosanus* was obtained during the fall, 1973 from Lake Charles, Louisiana. *Gloeophyllum trabeum* Pers. ex Fr.

(=*Lenzites trabea*) was obtained from Dr. G. Esenther, U.S. Forest Products Research Labs, Madison, Wisconsin (strain number 5096–15 cultured on southern pine, *Pinus* spp.) and was used as a source of (*Z,Z,E*)-3,6,8-dodecatrien-1-ol since the pure synthetic trienol was unavailable. Termites used for extraction were held for 48–72 h on moistened filter paper prior to extraction. Termites used for bioassays were held under constant overhead fluorescent illumination in petri dishes with moistened filter paper for 24–48 h prior to their use. Bioassays were conducted in a room maintained at 26–30°C and 40–45% relative humidity and provided with direct overhead fluorescent lighting. No termite was used for more than one bioassay in 24 h. Separate groups of termites were used to establish threshold levels and to perform choice bioassays.

Thin-layer chromatography (TLC) plates were prepared using Brinkman silica gel HF-254 and were activated for 1 h at 110°C. Solvents were reagent grade. Anhydrous ethyl ether was used directly, whereas other solvents were once-distilled and subsequently filtered through activated Florisil (1 h at 110°C).

A modification of the Matsumura et al. (1969) bioassay was used to establish threshold response levels for trail-following. This consisted of the use of 2 μl disposable pipettes and in having a petri dish lid on the ground-glass plate to minimize air currents. A given extract was considered to have elicited a positive trail-following response if at least three of five termites immediately followed the trail upon contact and remained on it to its terminus. It also was required that the termites, upon reaching the terminus, hesitate,

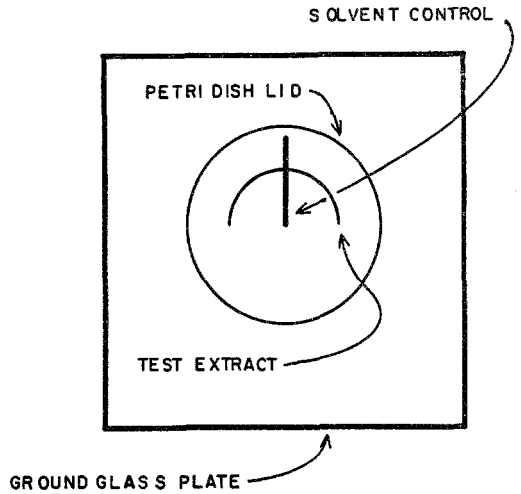

FIG. 1. Open-Field bioassay apparatus used for assessing termite trail-following per se and for determining threshold concentration levels of test extracts to be used in the Choice bioassay.

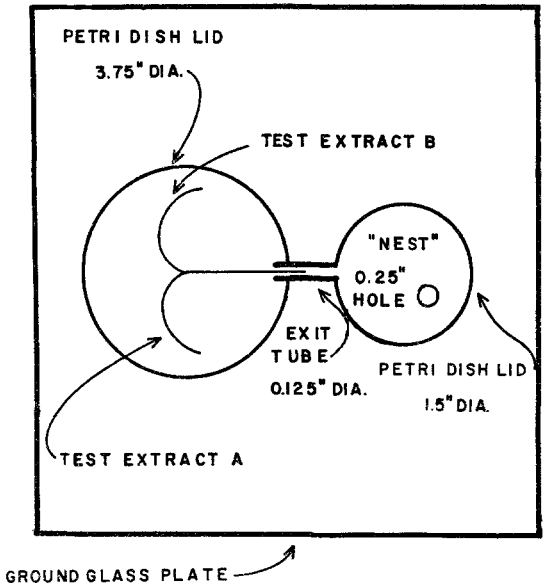

FIG. 2. Choice bioassay apparatus used for testing the ability of a termite to discriminate between two test chemicals.

explore the area immediately in front of them with their antennae, and then turn around and follow the trail in the opposite direction. A solvent control trail perpendicular to the sample trail was always present. This bioassay procedure is referred to as an Open-Field bioassay (Figure 1). The other bioassay (Figure 2), referred to as a Choice bioassay, also utilized trails of threshold activity level extracts streaked with disposable 2-μl pipettes on a ground-glass surface. As indicated in Figure 2, either test extract A or B was streaked from the junction to the midpoint of the exit tube. Preliminary experiments showed that the observed distributions did not depend on which test solution the termites first encountered in the exit tube. For each Choice bioassay, 15 test termites were placed into the "nest" via the 1/4-in. hole. The number of termites reaching the terminus of each trail was counted. Generally, all termites left the "nest" and reached the termini before the number of returning termites became too confusing for accurate counting. If the sum of respondents was less than 15, then either not all of the termites were drawn out in the 10-min time interval, or some of the termites failed to follow the trail far enough to register a choice. In all cases the threshold trail-following activity for each of the test extracts was determined with the species to be tested via the Open-Field bioassay immediately prior to the Choice bioassay.

In addition, test solutions containing 1/10 the activity of the threshold solution at no time elicited trail-following responses. This eliminated any question of variable response by the termites on a day-to-day basis. Each Open-Field bioassay was replicated at least twice. All termites used were larvae. The basic assumption[1] of the Choice bioassay is that if two chemicals are the same and are at equal activity levels, the termites should show equal response to them, generating a 50/50 distribution. The corollary is that if the chemicals are different, then the termites will choose their own chemicals, and a skewed distribution will result. Also implicit in the above assumption is a lack of significant reinforcement of the existing trail by the termites. Such a lack of reinforcement has been demonstrated (see Experimental Procedures and Results). All distribution data were analyzed by a chi-square statistical analysis using the Yates Correction for Continuity (Yates, 1934). Comparison of activity levels were made using the *t* test. Dissections were performed on freshly frozen termites which had been held on moistened filter paper for 48 h.

TABLE 1. CONSPECIFIC CHOICE BIOASSAY TEST FOR ASSESSING IMPORTANCE OF ACTIVE TRAIL REINFORCEMENT[a]

Species	Trial	Distribution (left/right)	χ^2	Direction chosen by first termite
R. Virginicus	1	8/7	0	Left
	2	8/7	0	Right
	3	6/9	0.27	Right
R. flavipes	1	8/7	0	Left
	2	8/7	0	Right
	3	9/6	0.27	Right
R. tibialis	1	8/7	0	Right
	2	9/6	0.27	Left
	3	9/6	0.27	Right
C. formosanus	1	6/9	0.27	Left
	2	5/8	1.23	Right
	3	6/8	0.07	Right

[a] Fifteen termites per trial. Distributions compared by chi-squared test to expected 1:1 situation assuming no trail reinforcement. All observed distributions fit the expected one at the 0.05 level of significance.

[1] One of the reviewers has questioned the basic assumption, asserting that unrelated chemicals might be more potent stimulants than the natural pheromone. While we concede that such, in principle, is possible, we have not encountered any such examples in our work and consider it to be a rather remote possibility.

EXPERIMENTAL PROCEDURES AND RESULTS

Demonstration of A Lack of Significant Reinforcement of Test Extract Trails by the Termites During Choice Bioassays

Choice bioassays were performed for each of the four species utilizing identical conspecific whole-organism extracts for test solutions A and B. In all cases, the observed distributions corresponded to the expected 50/50 distribution at the 0.05 level of significance (Table 1).

Demonstration of Trail-Laying. A

Ten termites which had been previously held on moistened filter paper for 24 h were released into the entrance of a maze (Figure 3) and allowed to move freely through selected galleries for 1 h. The termites were removed, the plugs at each junction removed, and then 10 fresh termites of the same species introduced one at a time and each allowed 1 min to complete the maze. *Reticulitermes virginicus*, *R. flavipes*, and *R. tibialis* all readily negotiated the maze with correct choices being made at each of the three junctions. *Coptotermes formosanus*, however, showed no evidence of trail-following,

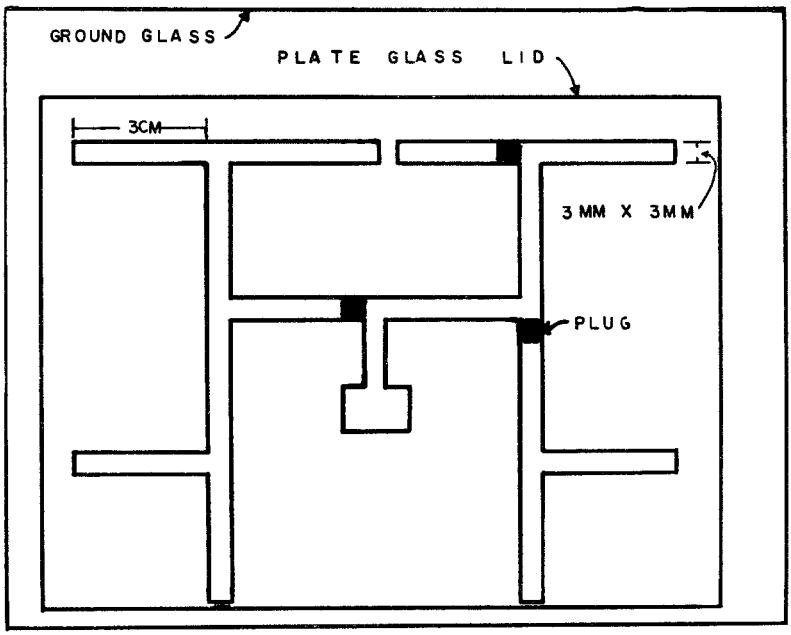

FIG. 3. Maze bioassay apparatus used to demonstrate active trail pheromone deposition.

with essentially random choices being made at each junction. It is thus clear that under these experimental conditions the three *Reticulitermes* spp. actively deposited a trail pheromone, whereas *C. formosanus* did not. It should be noted, however, that upon presenting *C. formosanus* with any of the *Reticulitermes* spp. maze trails, active and correct trail following resulted.

Demonstration of Trail-Laying. B

Twenty termites of each of the four species (previously held on moistened filter paper for 24 h) were placed in $6 \times \frac{1}{4}$ in. ID glass tubing lined on the bottom with filter paper and plugged on each end with glass wool. These tubes were kept in a chamber held at 85% RH for 24 h, along with a fifth control tube lacking termites, and were occasionally shaken to induce movement by the termites. The termites were then removed, the papers extracted twice with 1-ml portions of ether, and each combined extract concentrated to dryness, made up to 0.1 ml with hexane, and assayed for biological activity using the Open-Field bioassay. The extracts of *R. virginicus*, *R. flavipes*, and *R. tibialis* elicited positive trail-following behaviors, when tested conspecifically. A 1:10 dilution of these extracts did not elicit trail following. The extract from the *C. formosanus* paper did not elicit trail following in the bioassay, nor did the extract from the control paper. It is again clear that under these experimental conditions the *Reticulitermes* spp. actively deposited a trail pheromone, whereas *C. formosanus* did not.

Each of the above *Reticulitermes* paper extracts were spotted onto a silica gel HF-254 TLC plate and developed with ether:hexane (1:1) for 15 cm. The zones corresponding to the R_f of known *R. virginicus* pheromone [R_f 0.2–0.25 with respect to the reference standards cinnamyl alcohol ($R_f = 0.0$) and cinnamyl acetate ($R_f = 1.0$)] were removed and extracted twice with 1-ml portions of ether. Each combined 2-ml extract was concentrated to dryness, made up to 0.1 ml with hexane, and bioassayed conspecifically using the Open-Field bioassay. Positive trail-following was obtained in all uses. A 1:10 dilution of these extracts elicited no trail following. The remainder of the TLC plate was extracted with ether, concentrated to dryness, and made up to 0.1-ml solutions with hexane. Conspecific Open-Field bioassays of these materials elicited no trail-following responses. Clearly, for each of the *Reticulitermes* spp., all of the trail activity is located in a single band on the TLC plate.

Demonstration of Identity of Trail Extract and Whole-Organism Extracts

To ascertain if the biologically active trail chemicals deposited by the termites correspond to the chemicals isolated from whole-body extracts, a

TABLE 2. CHOICE BIOASSAY OF PARTIALLY PURIFIED TERMITE TRAIL EXTRACTS VS. PARTIALLY PURIFIED WHOLE-BODY EXTRACTS[a]

Test organism	Experiment No.	Number of termites choosing		χ^2
		Trail extract	Whole body extract	
R. virginicus	1	7	8	0
	2	9	6	0.27
R. flavipes	1	9	6	0.27
	2	7	8	0
R. tibialis	1	6	4	0.10
	2	5	5	0

[a] Chi-square analysis made on the hypothesis that if the termite trail extract trail is the same as the whole body extract trail, a 1:1 distribution will result, and χ^2 will be less than 3.84 at the 0.05 level of significance.

conspecific Choice bioassay was carried out for each species, using its once-TLC-purified "trail" extract against a once-TLC-purified whole-body extract. For both preparations it was established that all of the biological activity applied to the TLC plate was recovered from a single, identical, active zone on the plate. The results (Table 2) clearly indicate that these termites cannot differentiate between their trail extract and whole-body extract, suggesting the identicalness of the active principles in these preparations.

Morphological Site of Trail Pheromone Storage in Termites

Dissections were made of each of the four species to test the generally accepted hypothesis that the fifth abdominal sternite of the Rhinotermitidae was the source of the trail pheromone. Four termites of each species were used for each anatomical test region. Regions selected for study were abdominal sternites 4–6, abdominal tergites 4–6, the head, and the legs. The abdominal preparations undoubtedly contained portions of the insects' fat body and muscle. Each group of tissues was placed in 100 μl of hexane and soaked for 1 min. Each extract was then conspecifically tested for trail-following activity using the Open-Field bioassay. The results clearly indicated that only the abdominal sternite preparation elicited trail-following. In addition, for each species a quantitative comparison was made of the biological activity present in whole-organism extracts versus sternal segment extracts. For each species five larvae and five larval abdominal sternal segments were suspended in 1 ml of hexane, and 1:10 dilutions were prepared.

TABLE 3. QUANTITATIVE COMPARISON OF TRAIL PHEROMONE ACTIVITY CONTAINED IN WHOLE ORGANISM EXTRACTS VS. ABDOMINAL STERNITE EXTRACTS[a]

Species	Extract[b]	Number of termites that follow trail Trial 1	2	3	4	5	Total No.	t
R. virginicus	Whole body	3/5	2/5	3/5	1/5	3/5	12/25	
	Sternites	4/5	2/5	3/5	0/5	2/5	11/25	0.253
R. flavipes	Whole body	5/5	4/5	4/5	3/5	3/5	19/25	
	Sternites	4/5	2/5	3/5	3/5	4/5	16/25	1.133
R. tibialis	Whole body	5/5	2/5	3/5	3/5	2/5	15/25	
	Sternites	1/5	3/5	4/5	4/5	3/5	15/25	0
C. formosanus	Whole body	2/5	3/5	4/5	1/5	3/5	13/25	
	Sternites	1/5	4/5	2/5	2/5	3/5	12/25	0.277

[a] Comparisons of averages by t test. All values significant at the 0.05 level.
[b] Bioassays used 2-μl streaks of stock solution of extracts in 1 ml hexane. Preliminary experiments showed that at the time of the experiments 1:10 dilutions failed to elicit trail-following (less than two termites out of 25 responded).

In all cases the 1:10 dilutions failed to elicit trail-following, whereas the stock extracts did. For all four species the trail-following activity present in the whole-body extracts was identical to that contained in the abdominal sternite extracts (Table 3).

Assessment of Ability of Termites to Recognize Their Own Extracts from Fungal Extracts.

Ten larvae each of *R. virginicus*, *R. flavipes*, *C. formosanus*, and five larvae of *R. tibialis* were suspended in 1-ml volumes of hexane. Approximately 2 g of *G. trabeum* infected wood blocks were extracted five times with 1-ml portions of hexane. The combined hexane solutions were filtered to remove sediment and concentrated to 1 ml. Four serial dilutions of each of the above five stock extracts were prepared and utilized for Choice bioassays. The results (Table 4) show that *R. virginicus* was the only species which could not distinguish the *G. trabeum* extract trail from its own.

The apparent ability of *R. flavipes*, *R. tibialis*, and *C. formosanus* to discriminate between their extracts and the fungal extracts possibly reflects the presence of additional chemicals in the extracts. If this is so, the ability to discriminate between extracts should disappear or lessen upon partial purification. Each of the five stock solutions was chromatographed on a silica gel HF-254 TLC plate (ether–hexane, 1:1). The plate was divided into 10 sec-

TABLE 4. CHOICE BIOASSAYS OF CRUDE TERMITE EXTRACTS VS. CRUDE *G. trabeum* EXTRACTS

Test Organism	Experiment No.	Number of termites choosing: Termite extract	Number of termites choosing: *G. trabeum* extract	χ^{2a}
R. virginicus	1	6	9	0.27
	2	7	8	0.0
	3	6	4	0.10
	4	5	3	0.13
	5	7	8	0
R. flavipes	1	15	0	13.06
	2	15	0	13.06
R. tibialis	1	8	0	6.13
	2	10	0	8.10
C. formosanus	1	15	0	13.06
	2	15	0	13.06

[a] Chi-square analysis made on hypothesis that if the termite extract trail is the same as the G. trabeum extract trail, a 1:1 distribution will result and χ^2 will be less than 3.84 at the 0.05 level of significance.

TABLE 5. CHOICE BIOASSAYS OF ONCE-TLC-PURIFIED TERMITE EXTRACTS VS. ONCE-TLC-PURIFIED *G. trabeum* EXTRACTS

Test organism	Experiment No.	Number of termites choosing: Termite extract	Number of termites choosing: *G. trabeum* extract	χ^{2a}
R. virginicus	1	8	7	0
	2	9	6	0.27
R. flavipes	1	15	0	13.06
	2	15	0	13.06
R. tibialis	1	15	0	13.06
	2	15	0	13.06
C. formosanus	1	11	2	4.92
	2	12	0	10.08

[a] Chi-square analysis made on hypothesis that if termite extract trail is the same as the *G. trabeum* extract trail, a 1:1 distribution will result and χ^2 will be less than 3.84 at the 0.05 level of significance.

TABLE 6. INTERSPECIES CHOICE BIOASSAYS USING CRUDE TERMITE EXTRACTS

Test organism	Experiment No.	*R. virginicus*			*R. flavipes*			*R. tibialis*			*C. formosanus*		
		A[a]	B[b]	χ^{2c}	A[a]	B[b]	χ^{2c}	A[a]	B[b]	χ^{2c}	A[a]	B[b]	χ^{2c}
R. virginicus	1	—	—	—	15	0	13.06	15	0	13.06	9	4	1.23
	2	—	—	—	15	0	13.06	10	1	5.82	13	2	6.66
R. flavipes	1	15	0	13.06	—	—	—	15	0	13.06	15	0	13.06
	2	15	0	13.06	—	—	—	15	0	13.06	15	0	13.06
R. tibialis	1	13	2	6.66	15	0	13.06	—	—	—	12	3	4.27
	2	12	3	4.27	15	0	13.06	—	—	—	15	0	13.06
C. formosanus	1	14	1	9.60	10	0	8.10	15	0	13.06	—	—	—
	2	13	2	6.66	10	0	8.10	15	0	13.06	—	—	—

[a] Number of test termites choosing their species trail.
[b] Number of test termites choosing the other species trail.
[c] Chi-square analysis made on hypothesis that if the tested species extract trail is the same as the other species extract trail, a 1:1 distribution will result and χ^2 will be less than 3.84 at the 0.05 level of significance.

tions of 0.1 R_f unit each, and each band was extracted with five 1-ml portions of ether. The combined extracts for each band for each of the five species were concentrated to 1 ml and checked for biological activity using the Open-Field bioassay. Only those bands corresponding in R_f to the known *R. virginicus* pheromone possessed any biological activity. Serial dilutions of each of these biologically active fractions were prepared and used to obtain the proper concentrations of solutions for the Choice bioassays. The results (Table 5) are in agreement with the previous data (Table 4), i.e., that *R. virginicus* is the only species whose members did not differentiate their extract trail from that of the fungus extract trail.

Interspecific Trail-Following Responses Using Crude Extracts of Termites

Stock whole-organism extracts of each termite species were prepared and corresponding serial dilutions made. Each species was used as the test organism against the extracts of the other three species in the Choice bioassay. The results (Table 6) show that each species preferred its own extract trail.

Interspecific Trail-Following Responses on Partially Purified Termite Extracts

Fifty larvae each of *R. virginicus*, *R. flavipes*, *C. formosanus*, and 33 larvae of *R. tibialis* were suspended in 1 ml of hexane. After 10 min, each extract solution was concentrated and spotted on a silica gel HF-254 TLC plate and developed with ether–hexane (1:1) for 10 cm. The bands corresponding to the known R_f of *R. virginicus* pheromone were scraped off and each extracted with 1 ml of ether. The extracts were concentrated and spotted on a fresh silica gel HF-254 TLC plate and developed for 15 cm with hexane–ether–chloroform (4:3:3). The bands at R_f 0.2–0.25 with respect to the standards were again removed and extracted with ether. The ether solutions were concentrated to dryness and made up to 1 ml with hexane to produce the stock solutions of twice-TLC-purified termite extracts. One hundred microliters of each of the four solutions were used to prepare 1:10 and 1:100 dilutions for Choice bioassays. The results of the bioassays (Table 7) also show a strong preference by each species for its own extract trail, with the possible exception of *R. tibialis* tested against the extract of *R. virginicus*.

DISCUSSION

Central to any study of termite trail pheromones is one's confidence in the authenticity of the material at hand, with several workers having used various criteria for assessing this. Such criteria have included high biological

TABLE 7. INTERSPECIES CHOICE BIOASSAYS USING TWICE-TLC-PURIFIED TERMITE EXTRACTS

Test organism	Experiment No.	*R. virginicus*			*R. flavipes*			*R. tibialis*			*C. formosanus*		
		A[a]	B[b]	χ^{2c}	A[a]	B[b]	χ^{2c}	A[a]	B[b]	χ^{2c}	A[a]	B[b]	χ^{2c}
R. virginicus	1	—	—	—	15	0	13.06	12	1	7.70	15	0	13.06
	2	—	—	—	15	0	13.06	14	1	9.60	10	1	5.82
R. flavipes	1	14	1	9.60	—	—	—	8	0	6.13	14	1	9.60
	2	13	2	6.66	—	—	—	10	1	5.82	13	2	6.66
R. tibialis	1	10	5	1.07	12	3	4.27	—	—	—	12	3	4.27
	2	10	5	1.07	15	0	13.06	—	—	—	15	0	13.06
C. formosanus	1	12	1	7.70	10	0	8.10	11	4	2.40	—	—	—
	2	13	2	6.66	14	1	9.60	9	1	4.9	—	—	—

[a] Number of test termites choosing their species trail.
[b] Number of test termites choosing the other species trail.
[c] Chi-square analysis made on hypothesis that if the tested species extract trail is the same as the other species extract trail, a 1:1 distribution will result and χ^2 will be less than 3.84 at the 0.05 level of significance.

activity (all workers), inducement of a mass exodus from a "nest" (Karlson et al., 1968; Stuart, 1963; 1969; Moore, 1969), morphological localization in the termite (Stuart, 1963; 1969; Smythe and Coppel, 1966; Mertins et al., 1971; Shatov, 1974a; Hewitt et al., 1969) and direct demonstration of pheromone deposition on a substrate (Tschinkel and Close, 1973; Hewitt et al., 1969). No single criterion, however, by itself is sufficient to guide one in both the biological and chemical aspects of trail pheromone work.

A critical factor in the development of authenticity criteria is one's choice of bioassays. Several alternative approaches to our earlier Open-Field bioassay have been suggested in the literature (Stuart, 1969; Shatovh, 1974a; Tschinkel and Close, 1973), and arguments have been made for their superiority. It must be emphasized, however, that none of the bioassays described in the literature (including ours) is complete in terms of reproducing a "natural" situation for subterranean termites. In particular, they require the termites to be exposed to some combination of light, low humidity, abnormal caste composition, and a lack of gallery surfaces, which undoubtedly provide them with important thigmotrophic feedback. Such deficiencies, however, do not mean that all the bioassays are useless. Once one has worked with subterranean termites it is easy to recognize a "normal" trail-following response, which must then become the crucial component of any bioassay. A prime consideration, however, is to ensure that the bioassay measures a single quantity—trail-following—and not a mixture of factors such as attractancy from a distance and trail-following. We have compared Stuart's (1969) and Shatov's (1974a,b) bioassay methods to our Open-Field bioassay and found equivalent results (using a *G. trabeum* extract and *R. flavipes* as a test animal). The major difference was in the length of time required to complete the bioassay, with the Open-Field method being much faster. We also have noted occasional mass exoduses from the "nest" such as described by Stuart, but these seemed to reflect more upon the number of termites close to the exit at the moment of emergence by the first few termites rather than upon any special property of the bioassay. We also evaluated a maze-type bioassay of our own design (Figure 3) which provided this thigmotropic feedback to the termites; it too elicited positive trail-following responses in every instance that the Open-Field model did. Interestingly, the threshold response for the termites was approximately two orders of magnitude lower with the maze bioassay than with the Open-Field bioassay. The time required for a positive response was much slower with a maze bioassay and its physical manipulation requirements were more cumbersome than those of the Open-Field bioassay.

We found no bioassays that elicited a positive trail-following response by *R. flavipes* that did not also elicit a positive response with our Open-Field bioassay, and vice versa. The simplicity of the Open-Field bioassay compared

to the others therefore led us to use it exclusively for demonstrating trail-following per se, and for determining threshold response levels for use in the Choice bioassay.

The genesis of our Choice bioassay methodology arose from Blum and Brand's (1972) discussion of trail pheromones. The proper choice of activity levels for the test chemicals, i.e., that level which just elicits a normal trail-following response by 50% of the test animals, is crucial. Preliminary experiments showed that if both test extracts were at activities 10–100-fold greater than threshold levels, *R. flavipes* could not discriminate between its extract and the *R. virginicus* extract. Conversely, if one chemical was at threshold level, and the other was 10–100-fold higher, then the more active extract trail was always chosen. A further consideration in the Choice bioassay design was an arrangement that would allow the termites a few moments to settle down before being presented with a test chemical. The small "nest," with its exit gallery, fulfills this requirement. Another initial concern was that the termites, as they traversed the trail, might reinforce it, thus biasing the results. The consistent 50/50 distribution in the experiments where both test extracts were identical (Table 1), removed any doubt. We have found some evidence for trail reinforcement by *R. flavipes* if large (>50) numbers of termites are used. The possibility of reinforcement by small numbers of termites must be considered (and tested for) in every species examined. In the event that a particular species does show significant trail reinforcement, then an alternative choice bioassay design utilizing only one termite per assay would be required.

The following set of tests, which we believe will prove to be workable in assessing the authenticity of subterranean termite trail pheromones, has been developed: (i) high biological activity; (ii) localization in a distinct morphological region of the termite; (iii) active deposition on a substrate; (iv) chemical demonstration that the material deposited by the termite is the same as that contained in the unique tissue area; (v) biological demonstration that the chemical deposited on the substrate is indistinguishable to the termite from that contained in the distinct morphological region. This set of tests may of course require additions or deletions if future studies indicate other parameters to be critical.

Criteria (i) and (ii) were clearly met by *R. virginicus*, *R. flavipes*, *R. tibialis*, and *C. formosanus*. Matsumura et al. (1972) showed that these species would follow the pure *R. virginicus* trienol at approximately picogram per centimeter concentrations, and our partially purified extracts were active at 10^{-3}–10^{-4}-fold dilutions of the stock solutions. Assuming a weight of 1 mg per termite and a concentration of 0.1% of pheromone per termite (a rather high estimate in our opinion), then our extract solutions also contained pheromone at approximately picogram per microliter concentrations.

Exact quantitative threshold levels are strongly dependent, however, on the prior history of the tested termites. In assessing biological activity it is therefore important to utilize termites that have been uniformly conditioned. In our experience, holding the termites on moistened filter paper for 24 h yielded reproducible results with no obvious resulting stress on the termites.

The dissection experimental results were unambiguous and agree with the common assertion that only an abdominal sternite contains trail pheromone. None of the other extracts evoked the slightest suggestion of trail-following, or, for that matter, of any reaction whatsoever. Stuart (1969) suggested that, if a termite is presented with a choice of no chemical trail versus any chemical trail, it will choose the latter. Such has not been our experience. In addition our data show that the sternal segments contained trail activity equal to that found in whole-organism extracts, thus ruling out the possibility that trail activity might have been present in body regions not examined (Table 3).

Criterion (iii) was easily demonstrable for *R. virginicus*, *R. flavipes*, and *R. tibialis*, but, as noted earlier, it was not demonstrable for *C. formosanus*. We do not know why the *Reticulitermes* spp. deposited a trail under our experimental conditions, whereas *Coptotermes* did not, but note that little is known about the environmental factors that promote deposition of trail pheromone. Such information is sorely needed for an understanding of the role of trail pheromones in subterranean termite biology, as well as for an aid in demonstrating criterion (iii).

Thin-layer chromatography was used for assessing criterion (iv). Whole-organism extracts were used in place of sternal gland extracts since the dissection experiments had clearly demonstrated their equivalence. For all *Reticulitermes* spp. tested, the biologically active fraction from the trail extracts coincided exactly in R_f position with the biologically active fraction from the whole-organism extracts. Earlier experiments had shown that no other regions of the TLC plate contained biologically active material from either extract source. Additional chemical data on the active principles from the TLC plate would clearly be desirable, but the minute quantities of chemical available via excised sternal segments present formidable obstacles.

Last, criterion (v) provides the ultimate test of the authenticity of a termite trail pheromone. The results in Table 1, in conjunction with the first four criteria and previously published data (Tai et al., 1969; Matsumura et al., 1968), clearly establish that (*Z*,*Z*,*E*)-3,6,8-dodecatrien-1-ol is the authentic trail pheromone of *R. virginicus*, and that it is not an artifactual contaminant as suggested as a possibility by Stuart (1969), Blum and Brand (1972), and Tschinkel and Close (1973). Our data further confirm the presence of true trail pheromones in *R. flavipes* and *R. tibialis* and suggest the possibility of such a pheromone in *C. formosanus*. Confirmation of the latter awaits elucida-

tion of the necessary stimuli for elicitation of trail pheromone deposition in this species.

Consideration of the data in Tables 4–7 leads us to the following four conclusions: (i) *R. flavipes*, *R. tibialis*, and *C. formosanus* are able to distinguish their trail pheromone, and biologically active component, respectively, from the biologically active component found in *G. trabeum*. *R. virginicus* is *not* able to distinguish its trail pheromone from the *G. trabeum* component. (ii) Each species of *Reticulitermes* examined, except *R. tibialis*, is able to distinguish its trail pheromone from that of the other two *Reticulitermes* spp. as well as from the biologically active component of *C. formosanus*. *R. tibialis* clearly distinguished its trail pheromone from the others when crude extracts were used, but when tested with twice-TLC-purified extracts they were not able to distinguish between their pheromone and the *R. virginicus* pheromone at the 0.05 level of significance. Discrimination against the other species was still evident. (iii) *C. formosanus* is able to distinguish its biologically active component from the trail pheromones of the three *Reticulitermes* spp. (iv) Each species trail pheromone possesses physical and chemical properties similar to that of the others.

The chemical ecological relationships between brown-rot fungi and subterranean termites are intriguing, but poorly understood. One such relationship, that between *Gloeophyllum trabeum* (= *Lenzites trabea*) and *Reticulitermes* spp. has special interest since it was demonstrated (Matsumura et al., 1969) that *R. virginicus* and *G. trabeum* contained the same termite trail pheromone (not *R. flavipes* and *G. trabeum* as reviewed by Blum and Brand, 1972). Matsumura et al. (1968) established that other Rhinotermitids would also respond to the *G. trabeum* extracts as well as to the *R. virinicus* trail pheromone. As noted earlier, the above findings led Stuart (1969), Blum and Brand (1972), and Tschinkel and Close (1973) to conclude that possibly the trienol isolated from *R. virginicus* was present as gut tract contamination by *G. trabeum*. It is important to realize, however, that field studies have not demonstrated a causal relationship between ingestion of brown rot fungi and termite trail pheromone production. Indeed, such a demonstration would be extremely difficult. Likewise, attempts to culture termites in fungal-free environments would likely be impossible with current technology.

Most of the studies of *G. trabeum-Reticulitermes* spp. interactions have been done in a laboratory under artificial conditions. The field work that has been done (Esenther and Gray, 1968; Esenther and Beal, 1974; Beard, 1974) involved artificially placing *G. trabeum* infected wood blocks in areas known to contain termites and observing a feeding response, not a trail-following response. These considerations, taken in conjunction with our clear demonstration of the authenticity of the trail pheromone of *R. virginicus*, strongly suggest that the possession of the *R. virginicus* trienol by *G. trabeum* is a case

of accidental cooccurrence, analogous to the cooccurrence of ecdysones in plants and arthropods (Siddall, 1970). Since we have shown that *R. flavipes* and *R. tibialis* also have authentic trail pheromones and that they prefer their pheromones to the *G. trabeum* chemical (Tables 2 and 3), it follows that the trail pheromones of these two species are *not* the same as the *G. trabeum* chemical, and hence, not the same as the *R. virginicus* trail pheromone. This conclusion is further substantiated by the data from Tables 5–7, which demonstrate a consistent preference of each species for its own trail pheromone, even after repeated purifications. A similar preference for its biologically active component was shown by *C. formosanus*.

The question of whether the species specificity observed results from each species possessing a single unique trail pheromone or a common pheromone reinforced with secondary constituents remains unresolved. Although the two thin-layer chromatographic purifications should have greatly cleaned up each preparation, one cannot rule out the possibility that secondary components with physical and chemical properties similar to the known trienol were present.

No matter what the mechanism of species specificity, however, our findings pose some interesting ecological problems. Although the geographical ranges of some *Reticulitermes* spp. overlap to some degree, it is not known whether they are in direct contact with each other through their trail pheromones. The stimuli which elicit trail pheromone deposition are basically unknown and may vary for each species. The biogenetic origins of the trail pheromones are totally unknown and may or may not be involved with fungal food sources. And little is known of the presence or absence of the trail pheromone within the various castes, even of a single species. Considerable laboratory and field investigations will be required to settle these interesting questions.

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THE SUPRACAUDAL SCENT GLAND OF THE RED FOX *Vulpes vulpes*

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Abstract—The supracaudal gland of the red fox consists of both tubular "apocrine" sweat glands and massively developed sebaceous glands. The gland is characterized by a high level of histochemically demonstrable hydroxysteroid dehydrogenase activity (particularly β–3β HSD) and by the presence of naturally fluorescent photolabile sebum constituents. Evidence suggests that these components may be carotenoid. Results are presented in the context of histological observations and are discussed in relation to scent production.

Key Words—supracaudal gland, fluorescence, hydroxysteroid dehydrogenase, carotenoid, scent, sebaceous gland, *Vulpes vulpes*, red fox.

INTRODUCTION

The supracaudal or tail gland of the red fox, *Vulpes vulpes*, occupies a circumscribed ovoid area, about 2.5 × 0.75 cm, arranged longitudinally on the dorsum of the tail some 5 to 6 cm from the base, its position being marked by localized dark hair pigmentation. Waxy yellowish-brown particles of stale secretion which possess a characteristic odor adhere to the surface of the skin and the base of the hairs in the glandular region. Volatile terpenes have been observed in a distillate of this secretion, possibly accounting for early descriptions of this gland as "the violet gland" (Albone, 1975). Schaffer (1940), in his histological observations on the gland, suggests that secretion may be expelled from the gland by contraction of the arrector muscles of the hair. The

supracaudal gland is present in most Canidae, although it varies greatly in size from species to species (Hildebrand, 1952). The supracaudal gland of the domestic dog has been described by Lovell and Getty (1957).

In spite of the growing interest in the function of skin glands in chemical communication in mammals (Mykytowycz, 1970), almost nothing is known of the behavioral significance of the supracaudal gland. Sniffing the glandular area is reported to be common during social investigation in the red fox, the arctic fox, and the grey fox, rare in the wolf, and absent in the domestic dog (Fox, 1971a). In the arctic fox, secretory action has been observed to increase after group play. The odor of the gland is said to be more apparent in the least social Canidae (arctic fox, red fox,) less apparent in the more social species (wolf, jackal, coyote), and absent in the domestic dog (Fox, 1971b).

We report here results of preliminary studies on the supracaudal gland of the red fox that have been undertaken as part of a larger multidisciplinary investigation of chemical communication in this species (Albone et al., 1974).

METHODS AND MATERIALS

All tissues were taken from wild fox. Details are given in Table 1. The histology of the gland was investigated using 7-μm hemotoxylin and

TABLE 1[a]. SOURCES OF FOX SUPRACAUDAL GLAND TISSUE FOR HISTOLOGICAL AND HISTOCHEMICAL EXAMINATION[b]

Fox code[c]	Sex	Date killed	Age (yr/mo)	Sexual status
33	M	20.3.72	0/11[d]	Fecund
134	M	17.3.72	3/11[d]	Fecund
233	F	18.3.72	5/11[d]	Anestrous
1	M	2.2.74	0/10	Fecund
2	M	2.2.74	> 1/10	Fecund
3	F	2.2.74	0/10	Pregnant (8 cell tubal ova)
4	M	6.2.74	0/10	Fecund
5	F	6.2.74	> 0/10	In estrus

[a] Tissues and data by courtesy of H.G. Lloyd and staff, Ministry of Agriculture, Fisheries and Food, Pest Infestation Control Laboratory.

[b] Tissues dissected for gland weight data and ether extraction were obtained from foxes taken in Wales, December 1971–March 1972 and February–March, 1974.

[c] Foxes 33, 134, 233 taken in Pembrokeshire, 1, 2, 3 in Cardiganshire. 4, 5 in Breconshire. Tissues for foxes 1, 2, 3, 4, 5 were quenched in liquid nitrogen in the field and stored over liquid nitrogen until required for examination.

[d] Ages determined by dental cementum annulation method.

eosin stained sections of tissues from foxes 33, 134, and 233 fixed in Helly's fluid. Cryostat sections of fixed tissue from fox 233 were also stained with oil red O to demonstrate lipid droplets.

Hydroxysteroid dehydrogenase activity was demonstrated histochemically in 8-μm cryostat sections of supracaudal gland, back skin, lip, scrotal skin, circumanal skin, anal sac, and fore- and hind-pad tissue from foxes 1, 2, 3, 4, 5 using the method described by Baillie et al. (1966). The medium contained NAD^+ (0.25 mg/ml), $NADP^+$ (0.25 mg/ml), nitro blue tetrazolium (0.1 mg/ml), and the substrate (0.15 mg/ml) in a 0.05M phosphate buffer at pH 7. The following substrates were used: androsterone (α-3α), Δ5-androsten-3β-ol-17-one and Δ5-pregnen-3β-ol-20-one (Δ5-3β), 5β-androstan-3β-ol-17-one (β-3β), 17α-oestradiol (17α), 17β-oestradiol and testosterone (17β) and cortisol (11β), all supplied by Sigma Chemical Company Ltd. and not subjected to further purification. The substrate solvent was dimethylformamide and incubations lasted one hour at 37°C. Control sections were incubated in medium lacking substrate. The strengths of reactions were scored on an arbitrary scale from 1 to 5.

Tissues were examined for fluorescence using a UVS-11 short wavelength Mineralite uv lamp. Tissues from foxes, 1, 2, 3, 4, 5 were also examined by fluorescence microscopy as 10-μm cryostat sections mounted in glycerol (Leitz Ortholux II, 380–430 nm irradiation).

For chemical examination, freshly dissected glandular tissue samples were homogenized with distilled water ($\sim$10% homogenate) using a Gallenkamp tissue grinder for individual glands and a Polytron homogenizer (Kinematica GmbH) for 1–2-g samples bulked according to sex, each sample being cooled in ice water. Homogenates were extracted at room temperature with ANALAR diethyl ether (twofold volumes, twice) by agitation on a Rotamixer (Hook & Tucker Ltd.) followed by centrifugation. Each ether extract was evaporated down to a convenient volume ($<$5 ml) at $<$30°C and stored at $-$20°C.

Thin layer chromatography (TLC) was performed using Kieselgel G plates, applying the sample under nitrogen and eluting with *n*-hexane/diethyl ether, 9/1. Preparative plates were pre-eluted twice with ethyl acetate. Plates were visualized by brief exposure to uv light, and, when required, with iodine vapor.

Ultraviolet spectra were obtained using a Pye-Unicam SP800 spectrophotometer and fluorescence spectra using a Farrand Optical Co. Ltd. Mark I spectrofluorometer (150-W. xenon lamp; slit width 5 nm, uncorrected for lamp output and photomultipler response variation with wavelength).

Throughout, care was taken to keep samples from direct sunlight and to minimize oxygen exposure. The fluorescent components were most susceptible to degradation after purification.

RESULTS

Morphology, Histology, and Histochemistry

The dissected supracaudal gland was found to be significantly heavier in the dog fox (average wet weight, 214.7 mg; standard error 18.0 mg, 45 samples) than in the vixen (average wet weight, 146.5 mg; standard error, 11.5 mg, 39 samples) ($P<0.01$).

The present histological findings support those of Schaffer (1940). The supracaudal gland area was found to consist almost exclusively of large sebaceous glands, though these were interspersed with a few coiled tubular "apocrine" sweat glands. The sebaceous glands contained cells of two main types. In the first, the nucleus was large, central, and vesicular and the cytoplasm was uniformly and brightly eosinophilic. In the second, the nucleus was pyknotic and was often displaced from the center of the cell by large lipid droplets of up to about 5 μm diameter. These doubtless correspond to the osmiophilic droplets reported by Schaffer. The former cells presumably give rise to the latter. Both supracaudal gland sebaceous cell types differed from those of the sebaceous glands of the remainder of the hairy skin, which were smaller and possessed a weakly eosinophilic, foamy cytoplasm.

As in the dog (Lovell and Getty, 1957) the follicles in the supracaudal gland area were large and simple, and, in our material, many did not contain hairs. These follicles and the ducts of the sebaceous glands were packed with a lipid-rich holocrine secretion.

TABLE 2. HISTOCHEMICAL EVIDENCE OF HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN THE SEBACEOUS GLANDS OF THE RED FOX

Substrate[a]	Supracaudal gland	Back skin	Other areas (see text)
α–3α	++	++	++
β–3β	+++++	+++	++
Δ^5–3β[b]	++	++	++
11β	t[c]	t	t
17α	+	t	t
17β[b]	+	+++	+++
Control	–	–	–

[a] Substrates used were androsterone (α–3α), 5β-androstan-3β-ol-17-one (β–3β), Δ5-androsten-3β-ol-17-one and Δ5-pregnen-3β-ol-20-one (Δ5-3β), cortisol (11β), 17α-oestradiol (17α) and 17β-oestradiol and testosterone (17β).

[b] Identical reactions were given by both substrates.

[c] t = trace.

FIG. 1. Photomicrograph of red fox supracaudal gland tissue. Histochemical evidence for hydroxysteroid dehydrogenase activity; substrate 5β-androstan-3β-ol-17-one. The staining was restricted to the cytoplasm of the sebaceous gland cells. The skin surface is uppermost. ($\times$60) (Reduced 45% for reproduction.)

(*Facing p.* 170)

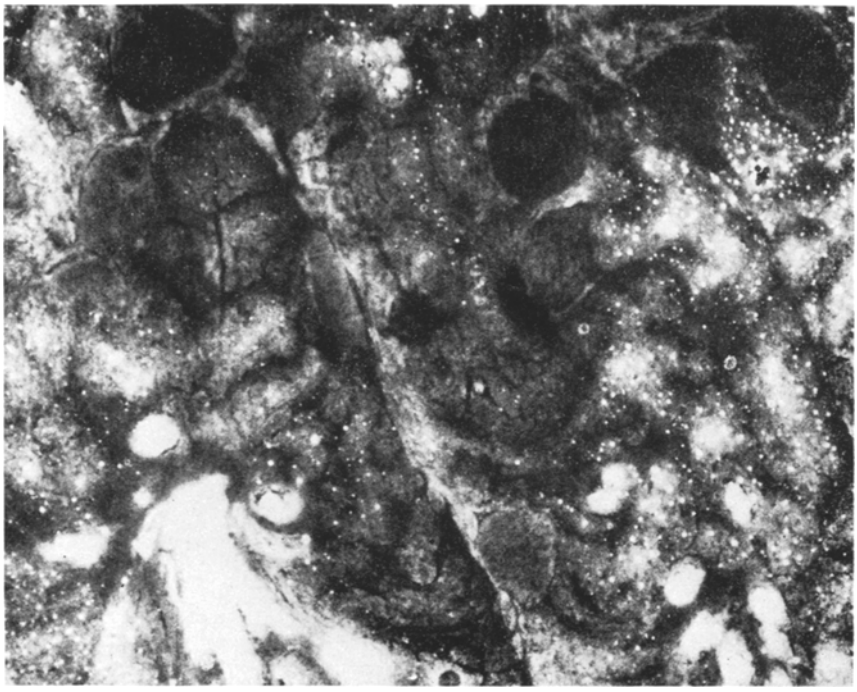

FIG. 2. Photomicrograph of red fox supracaudal gland tissue. Autofluorescence; longitudinal section through sebaceous tissue. Part of a duct system is visible at the lower left and numerous intracellular lipid droplets can be seen on the right. The diffuse fluorescence in the center of the field comes from sebaceous cells lacking large cytoplasmic droplets. Two gland lobules are cut tangentially. (×90) (Reduced 50% for reproduction.)

(*Facing p.* 171)

Hydroxysteroid dehydrogenase (HSD) activities in supracaudal gland tissues from foxes 1, 2, 3, 4, 5 (Table 1) were compared with those in the anal sac and circum-anal skin (foxes 1, 2, 4, 5), back skin (foxes 1, 2), lip (fox 2), scrotal skin (foxes 1, 2), and fore- and hind-pads (foxes 1, 2, 4). The results are summarized in Table 2.

Regardless of the substrate used, HSD activity was confined to the sebaceous glands of the skin areas examined. It was present throughout the cytoplasm but could not be ascribed to any particular organelle. It was absent from the nuclei and lipid droplets. In the supracaudal gland tissue, HSD activity was greatest in cells possessing large nuclei, and declined in relation to intracellular lipid droplet accumulation. No activity was noted in the most degenerate cells or in the secretion. The only staining observed in control slides was associated with nonspecific reduction of staining reagent in certain hair shafts, particularly in the interdigital region.

Table 2 shows that utilization of 17β-hydroxy substrates predominated in all tissues other than those of the supracaudal gland. Here, dehydrogenation at the 3 position in the β–3β configuration was strongest (Figure 1). Steroid dehydrogenation by subaceous glands from the skin of the back was intermediate in its pattern of substrate use. In the small number of samples examined there was no histochemical evidence of sex differentiation.

Fluorescence

A strong yellow-green fluorescence, clearly visible on illuminating supracaudal gland tissue with ultraviolet light, was most apparent immediately after an incision was made in the gland. In general, dog fox tissues fluoresced more strongly than those from vixens. Some fluorescence was also noted at the skin surface and on the hairs of the glandular region.

Microscopically, the tissue fluorescence was strongest in the intracellular lipid droplets and the accumulated secretion within the sebaceous ducts and hair follicles (Figure 2) but fainter diffuse fluorescence, possibly attributable to submicroscopic lipid particles, was also present in the cytoplasm of the supracaudal gland sebaceous cells with large central nuclei. Similar though less intense fluorescence was present in the sebaceous glands of all the other skin areas examined in foxes 1, 2, 3, 4, 5. No fluorescence was detected in the sebaceous component of the supracaudal gland of a mature male Labrador retriever, although a punctate fluorescence was seen in the cytoplasm of the coiled parts of the sweat glands of the area.

Thin layer chromatography of supracaudal gland diethyl ether extracts using 9/1 *n*-hexane/diethyl ether on Kieselgel G revealed at least three major fluorescent components; component I (R_f, 0), component II (R_f, 0.04) and component III (R_f, 0.53). Further TLC examination with 70/30/1 petroleum

spirit (60–80°C)/diethyl ether/acetic acid showed component II to move with a cholesterol standard. All three components were noted in extracts from both dog fox and vixen bulked samples, although one such (vixen) extract showed intense fluorescence only in component I, none in component II, and but weak fluorescence in component III. Usually, however, component III produced the most intensely fluorescent TLC zone, particularly in dog fox gland extracts. Extracts of hair and the waxy particles from the skin surface of the glandular region generally showed but weak component III fluorescence and an intense, bluer fluorescence at R_f, 0.

The fluorescent components were photolabile. Thus, under the fluorescence microscope, lipid droplet fluorescence was seen to decay substantially within 10 sec illumination. Component III fluorescence decayed within 5 min yielding a polar (presumably photoxidation) product when a developed TLC plate was irradiated with a UV visualizing lamp. In contrast, fluorescence was maintained for months when intact tissue was stored deep-frozen in the dark.

A further fluorescent component (component IV) was revealed as a faint, redder fluorescence at R_f, 0.74 (9/1 *n*-hexane/diethyl ether; β-carotene, R_f, 0.76) when bulked dog fox tissue extracts were repeatedly chromatographed by TLC. This substance exhibited an ultraviolet absorption spectrum, λ_{max} (*n*-hexane) (253.5), 263, 272.5, (337) 351.5, 370, 390.5 nm, suggesting, with the polarity data, an isoprenoid polyene hydrocarbon of six conjugated double bonds (Zechmeister, 1958). The spectral fine structure term (Ke et al., 1970) varied in the range 52%–69% from sample to sample, probably as the result of varying degrees of *cis–trans* isomerization.

Component III exhibited an ultraviolet absorption spectrum, λ_{max} (dry Et_2O), (316), 328.5 nm; ($CHCl_3$), (322) 335 nm, lacking fine structure and resembling that expected for a conjugated carbonyl function. This possibility is supported by polarity data and by the removal of this uv absorption on treatment with sodium dihydro-bis(2-methoxyethoxy)aluminate.

Following partial TLC purification, component II was observed to possess a similar structureless UV absorption, λ_{max} (EtOAc), 326 nm.

The UV absorption, $\lambda > 275$ nm, of crude diethyl ether extracts of dog fox supracaudal gland, $\lambda_{max}(Et_2O)$, (316.5) 327.5 nm is very largely attributable to component III and other substances of closely similar absorption spectra. Crude diethyl ether extracts of vixen supracaudal gland showed weaker and more complex absorption in this region. Crude extracts of wax and hair from the gland surface showed the 327.5 nm absorption only as a weak shoulder. The main absorption was at short wavelength with a subsidiary maximum at λ_{max} 281 nm.

Fluorescence spectra of components III (fluorescence maximum, 485 nm) and IV (fluorescence maximum, 532 nm) are given in Figure 3.

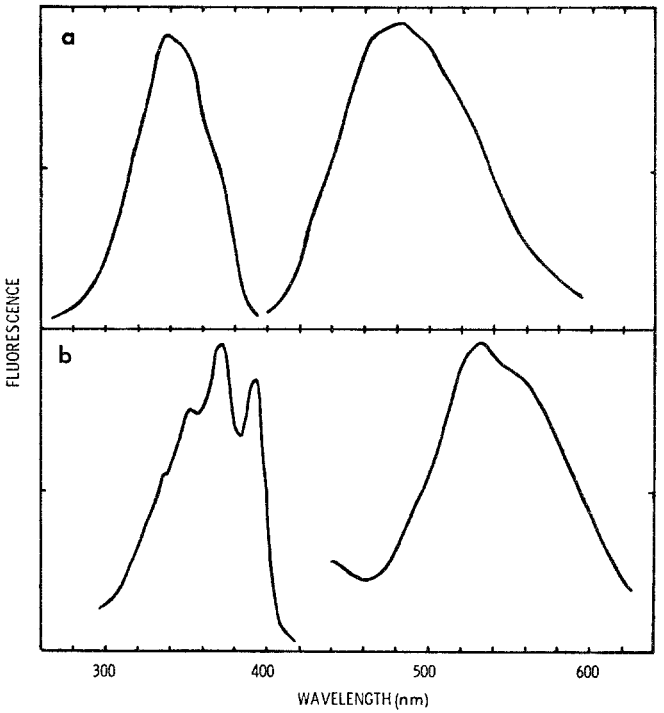

FIG. 3. Excitation and fluorescence spectra of (a) component III ($CHCl_3$ solution, OD 0.15 at 335 nm) with excitation at 337 nm and emission at 475 nm, and of (b) component IV (*n*-hexane solution, OD 0.22 at 370 nm) with excitation at 373 nm and emission at 530 nm.

DISCUSSION

The supracaudal sebaceous glands exhibit an unusually high histochemically demonstrable hydroxysteroid dehydrogenase (HSD) activity characterized by an intense β–3β HSD reaction.

Histochemical HSD activity has been reported in the sebaceous glands of human skin (Baillie et al., 1966), in the chin gland of the male cuis (*Galea musteloides*) (Holt and Tam, 1973), in the antebrachial scent organs of certain lemurs (Sisson and Fahrenbach, 1967) and in the mandibular gland of the boar (Flood, 1973). Both the sweat glands and the mandibular glands of the mature boar contain high concentrations of steroids (Stinson and Patterson, 1972) of recognized pheromonal activity (Melrose et al., 1971). The present finding that the pattern of steroid utilization by the supracaudal gland of the red fox is very different from, and much stronger than, that seen in other

sebaceous glands in this species suggests that the supracaudal gland has unique steroid metabolizing properties; further, the presence of activity at the 3 position could be indicative of steroidogenesis. The production of pheromonal steroids by the supracaudal gland would accord with the suggestion of Kloek (1961) that mammalian pheromones would frequently prove to be steroidal. However, steroid metabolism by this gland has yet to be investigated biochemically.

The fluorescent components described also appear to be characteristic of the sebum of the red fox, and particularly of the supracaudal gland. Similar fluorescence was not observed in supracaudal gland tissue of a domestic dog. Natural fluorescence in animal tissues is usually less intense than that reported here and is often associated with polar tissue components (Pearse, 1972). Many reports of skin gland fluorescence lack sufficient detail for comparison with the photolabile lipid-soluble fluorescence of the spectral properties described here. A yellow fluorescence occurs in the sebum of certain anal sac sebaceous glands in the dog (Montagna and Parks, 1948) and in certain other sebaceous glands (Montagna and Parakkal, 1974), and a yellow-orange fluorescence has been noted in the lipids of certain apocrine and eccrine glands (Montagna et al., 1951). Clearly distinct from the supracaudal gland fluorescence are the red fluorescence, probably due to microbially produced porphyrin, observed in certain skin infections (Noble and Somerville, 1974) and the water-soluble fluorescent pterins detected in the skin of amphibians (Hama and Obika, 1958).

The significance of supracaudal gland fluorescence is uncertain. It is unlikely that the fluorescence itself can be visible to the fox, even though light of wavelengths necessary to excite it is present in daylight, because of the unfavorable ultraviolet/visible flux ratio, particularly when the sun is low in the sky (Leighton, 1961). Field desorption mass spectrometry of component III suggests the presence of compounds in the carotenoid mass range (Albone, 1975). The presence of carotenoids would also accord with ultraviolet spectral and polarity data and the observed lability of these compounds to light and air and on electron-impact mass spectrometry.

If the fluorescent compounds are carotenoid, their presence may be associated with that of the volatile terpenes in the secretion of the supracaudal gland. Thus, under certain circumstances, dihydroactinidiolide, one of the terpenes identified in the secretion, is formed from β-carotene (Isoe et al., 1969).

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VOLATILE CONSTITUENTS OF DOG (*Canis familiaris*) AND COYOTE (*Canis latrans*) ANAL SACS

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Abstract—The volatile organic compounds from the anal sac secretions of male and female dogs and coyotes were examined using gas chromatography and gas chromatography–mass spectrometry. Short chain (C_2–C_6) acids and trimethylamine were major constituents. Changes in the type and abundance of the volatiles were examined across state of estrus, species, and gender. No consistent difference in the pattern of volatiles was detected that was indicative of estrus state or gender. Dogs displayed larger amounts of all constituents. The anal sac secretions of a third carnivore, the cat, were examined to see if they contained trimethylamine: none was found.

Key Words—anal sacs, canids, GC-MS, chemical communication, metabolic profiling.

INTRODUCTION

Although various biological functions have been ascribed to the odorous

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secretions from the anal sacs of canids, none has been definitely established. Qualitative observations of dog (*Canis familiaris*) behavior have suggested to some authors that these anal sac secretions may function in sexual attraction (Donovan, 1967), individual recognition, territorial demarcation (Baker, 1962), and alarm or defense (Donovan, 1969). However, scientific reports suggest that canids mark mainly with urine (Kleiman, 1966), and that anal sac secretions from estrous females elicit no more investigatory behavior from male dogs than those from diestrous females (Doty and Dunbar, 1974a).

No rigorous studies have been conducted with coyotes (*Canis latrans*); however, anal sac secretions often constitute a major portion of coyote lures (Young and Jackson, 1961), suggesting that the secretion may have attractive properties. Although little is known about population dynamics and/or the role of odor in the reproductive biology of the coyote, such information is important since it may assist in developing coyote management techniques (Cain, 1972).

The anal sac secretion of dogs is reported to consist of 88% water with proteins, lipids, and inorganic substances secreted into the sac by surrounding glands (Montagna and Parks, 1948). The color, the odor, and the rate at which the secretion accumulates vary from dog to dog, and appear not to depend upon the extent of exogenously produced early androgenization (Doty and Dunbar, 1974b).

The chemistry and microbiology of anal sac secretions from the red fox (*Vulpes vulpes fulva L*), have been studied (Albone and Fox, 1971; Albone and Eglinton, 1974). They established the presence of C_2–C_5 aliphatic acids, and 4-methylvaleric acid as well as phenylacetic, 3-phenylpropionic, *p*-hydroxyphenylacetic, and *p*-hydroxyphenylpropionic acids in the secretions from this canid. Trimethylamine was also identified as a constituent of these secretions. The nature of these constituents suggested that they may be formed by flora in the sac utilizing available substrates (Albone and Eglinton, 1974).

The purpose of this study was to determine if changes in the nature and abundance of volatiles in the secretions from the anal sacs of beagles and coyotes occur across species, gender, or state of estrus. The work of Donovan (1967) suggested that dogs in estrus possess odors, attractive to conspecifics, in their anal sac secretions. Preliminary data suggested to us that trimethylamine was found only in the anal sacs of female beagles and coyotes. Consequently, we determined the amount of this compound in each sample. Albone and Fox (1971) reported this amine as a constituent of female red fox anal sacs. In order to determine if it is found in glandular secretions of a noncanid, secretions from the anal sacs of the cat (*Felis domesticus*) (Greer and Calhoun, 1966) were examined for trimethylamine. These secretions have been shown to contain large amounts of the C_2–C_5 acids discussed above (Berüter, unpublished, 1972).

METHODS AND MATERIALS

Sixteen pure-bred beagles and 14 coyotes used in this study were 2–5 yr old and were housed at the Denver Wildlife Research Center, Denver, Colorado. Two of the female coyotes were born in captivity while the remainder were trapped in Colorado or Texas. Each animal was housed individually in an outdoor, concrete-lined run containing weather-proofed wooden shelters. The animals were all maintained under routine dog colony procedures with dry dog food (Wayne "Krumettes")[5] and water provided ad libitum. The nutritional status and health of the animals used were excellent for the duration of the study.

The secretions were collected by two procedures. The first (Method I) was performed on coyotes only after anesthetization using a 4% thioseconal solution (to effect). The beagles were not anesthetized. The sac contents were then directly expressed into beakers held over the sac orifices. After collection, the contents of the anal sacs were washed into a glass vial using 1.0 ml of 0.15 M saline. The vials were then frozen at −18°C and sealed. Using Method I, samples were collected from each animal at 2–4 wk intervals over a period of 4 mo. Almost every attempt to express secretions from coyote anal sacs yielded little or no material (see Table 1). Consequently, secretions were aspirated from the sacs using 0.3–0.5 ml of 0.15 M saline. The aspirated material was placed in vials, frozen, and sealed. The difficulty in obtaining anal sac secretions from coyotes via direct expression led to development of a second collection procedure (Method II), designed to collect equal volumes of aspirated sac contents from both species.

In Method II, all animals were first anesthetized with a 4% thioseconal solution (to effect) and then 0.15 ml of 0.15 M saline were injected into the opening of each sac (left and right) using a blunt, stainless steel, 20-gauge needle attached to a glass syringe. After 10 min, the saline plus secretion were withdrawn by the same syringe, transferred to individual glass vials which were then frozen in liquid nitrogen and sealed. Two collections were made within 2 mo of each other using Method II.

In both procedures, a number of saline blanks of 1.0 ml volume were prepared after being exposed to the collection room atmosphere. All samples and blanks were frozen at −18°C until shipment to Philadelphia.[6]

The gender, state of estrus, and number of samples obtained from each animal using Methods I and II are shown in Table 1. Vaginal cytology was used to determine the state of estrus.

Before gas chromatography (GC) analysis was performed, each tube was

[5] Use of trade names does not imply a United States Government endorsement.

[6] Samples were shipped packed in dry ice via air freight and never defrosted until analysis. Samples collected by Method I were shipped at three separate intervals in the 4-mo period. Those from Method II were shipped within 2 wk of collection.

TABLE 1. GENDER, STATE OF ESTRUS, AND THE NUMBER OF SAMPLES OBTAINED FROM EACH ANIMAL USING METHODS I AND II[a]

Animal		Method I					Method II	
		Sample No.						
		1	2	3	4	5	1	2
Beagle ♀	1	E	Ps	—	—	—	—	—
	2	E	P	—	—	—	An	M
	3	Pr	M	—	—	—	An	M
	4	E	E	—	—	—	—	—
	5	Pr	E	P	—	—	—	—
	6	Pr	E	E	P	—	—	—
	7	Pr	—	—	—	—	—	—
	8						An	M
	9						—	M
	10						—	M
Coyote ♀	1	Pr	Pr[b]	Pr[b]	Pr[b]	E	M	An
	2	Pr	—	—	—	—	—	—
	3						M	An
	4						M	An
	5						—	An
	6						—	An
		Number of samples						
Beagle ♂	1	2					1	1
	2	2					1	1
	3	2					—	—
	4	—					1	1
	5	—					—	1
	6	—					—	1
Coyote ♂	1	5[b]					—	—
	2	3[b]					—	—
	3	4[b]					1	1
	4	4[c]					—	—
	5	—					1	1
	6	—					1	1
	7	—					—	1
	8	—					—	1

[a] E represents estrus; Ps, pseudopregnant; P, pregnant; Pr, proestrus; M, metestrus; An, anestrus.
[b] Anal sac contents aspirated using saline.
[c] Two of four samples were aspirated.

cracked open and a 5 μl aliquot removed for injection. The remaining secretion was transferred to a screw-top vial with a Teflon-lined cap and refrozen at -10°C. Analysis of several samples after 3–4 mo under such storage conditions showed no decrease in the levels of volatiles.

The four cats used in the study were housed at the Monell Center. Each animal was individually quartered in a $2\frac{1}{2} \times 2\frac{1}{2} \times 2\frac{1}{2}$ ft stainless steel cage having a 12-in-wide shelf and a "kitty litter" box. A 12-hr light–12-hr dark cycle was used in the cat quarters. Eight ounces of dry Purina Cat Chow was provided each day, with water provided ad libitum. Two intact cats and two castrates (one from each gender) were used as donors. The ovariectomized female was given 50 μg/day of estradiol benzoate.

Anal sac secretions from the cats were obtained via Method I (i.e., direct expression). Due to the viscosity of the secretion it had to be washed into the bottom of glass vials using 0.5 ml of 0.15 M saline. The vials were then sealed and frozen (-10°C) until analysis (3 days later). One sample from each cat was used for analysis.

Analyses were performed with a Perkin-Elmer 990 gas chromatograph equipped with a flame ionization detector (FID). The gas chromatograph was fitted with a 6 ft $\times$ 0.25 in ID or a 12 ft $\times$ 2 mm ID stainless-steel column each packed with Porapak Q-S. The 6-ft column was operated isothermally at 215°C with a 40-ml/min helium flow. All samples were chromatographed on this column. The 12-ft column was programmed from 100 to 215°C at 4°/min using a 50-ml/min helium flow. Mass spectra were routinely obtained using a Perkin-Elmer 990 GC interfaced to a Hitachi/Perkin-Elmer RMU-6L mass spectrometer. High resolution spectra were run on a CEC-21-110B high resolution mass spectrometer interfaced with a Varian-Aerograph model 600 GC located at the Mass Spectrometer Laboratory at MIT. Identifications were confirmed by comparison of mass spectra and GC retention times with those from commercially available samples. Quantitation of volatile constituents was performed using samples collected with Method II. Except for trimethylamine (see below) individual constituent amounts were determined by first calculating their response factors with standard solutions and then calculating peak areas from the chromatograms.

RESULTS

The chromatogram shown in Figure 1 was obtained from a female beagle but is representative of the chromatographic pattern from both genders of the two species. However, there was a great deal of variation in the amounts of volatiles found in individual samples. Samples from males or females of both species could be volatile rich or poor from one collection

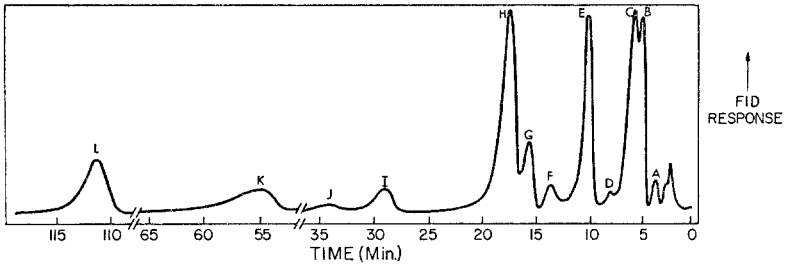

FIG. 1. Chromatogram produced by the volatile materials from a female beagle anal sac. Peaks B through H were attenuated (× 5) to keep them on scale. Flame ionization detector response is plotted on the ordinate. Peak A = ethanol, B = trimethylamine, C = acetic acid and acetone (see Table 2), D = 2-methylpropanal, E = propionic acid, F = 2- and 3-methylbutanal (see Table 2), G = isobutyric acid, H = *n*-butyric acid, I = 2-methylbutyric and isovaleric acids, J = *n*-valeric acid, K = 4- and 2-methylvaleric acids (see Table 2), and L = 2-piperidone. Because acetic acid has a low response in a FID, it does not appear larger than peaks E and H.

TABLE 2. COMPOUNDS IDENTIFIED IN THE DOG AND COYOTE ANAL SAC SECRETIONS

Chromatographic peak	Compound	Molecular weight	Characteristic ions seen in the mass spectrum[a]
A	Ethanol	46	*m/e* 46, 45, and 31
B	Trimethylamine	59	*m/e* 59, 58, 42, and 30
C	Acetic acid and	60	*m/e* 60, 45, and 43
	Acetone[b]	58	*m/e* 58 and 43
D	Isobutanal	72	*m/e* 72, 43, 41, 29, 27
E	Propionic acid	74	*m/e* 74, 73, 57, and 45
F	2- and 3-Methylbutanal[c]	86	*m/e* 86, 71, 58, 57, 44, 41, 29
G	Isobutyric acid	88	*m/e* 88, 73, 45, 43, and 41
H	*n*-Butyric acid	88	*m/e* 88, 73, 55, and 60
I	2-Methylbutyric acid and		*m/e* 87, 74, 57, and 41
	iso-valeric acid	102	*m/e* 87, 60, 43
J	*n*-Valeric acid	102	*m/e* 73 and 60
K	4-Methylvaleric acid and		*m/e* 87, 60, and 43
	2-Methylvaleric acid[b]	116	*m/e* 87, 74, and 43
L	2-Piperidone	99	*m/e* 99, 98, 70, 69, and 30

[a] Most intense ion underlined.
[b] Tentatively identified by mass spectrometric fragmentation patterns.
[c] Both compounds have identical retention times on Porapak Q-S, and ions for both are present in the mass spectra.

to the next. Although male and female beagles displayed far larger amounts of volatiles than their coyote counterparts, no consistent differences were found in the nature and abundance of one or more of the volatiles across estrous state, gender, or species.

All of the compounds identified are listed in Table 2. Owing to the large amounts of acids present in the samples and their tailing on Porapak Q-S, combination GC–high resolution mass spectrometry of several samples was needed to identify peaks D and F as aldehydes.

Acetic acid was generally the most abundant acid present in the secretion. In the sample which produced the chromatogram in Figure 1 its concentration was $\simeq 6$ μg/μl of sample. Other acids in this sample were present in these amounts: propionic (0.84 μg/μl); isobutyric (0.1 μg/μl); *n*-butyric (0.70 μg/μl); isovaleric/2-methylbutyric (0.17 μg/μl); *n*-valeric (0.016 μg/μl); and 4-methylvaleric/2-methylvaleric (0.25 μg/μl).

Mass spectra obtained of peaks C, I, and K consistently showed that these peaks contained two compounds, as listed in Table 2. Peak C consisted almost entirely of acetic acid; however, no effort was made to distinguish the ratios of the two acids comprising I and K. Peak F undoubtedly consisted of the two methylbutanals, since ions for both were present and the GC retention times were identical on our column. Peak L (2-piperidone), as seen in Figure 1, had an extremely long retention time, and unless it was present in large concentrations it was difficult to detect during routine GC assays owing to peak spreading.

Measurement of trimethylamine in our samples was complicated by a co-elution phenomenon in which this amine co-eluted with acetic acid below a 25:1 weight ratio of acid to amine. In samples where no amine was seen in the GC trace, it was searched for by continuously obtaining mass spectra every 5 sec as acetic acid eluted into the ion source of the mass spectrometer. By comparison of the abundance of m/e 58 in these samples to known injected amounts of amine and acetic acid mixtures we found that as little as 30–50 ng of amine could be detected in the presence of up to 10 μg of acetic acid.

The male–female difference in the amounts of trimethylamine was not statistically significant. This was shown by a 2(sex) $\times$ 2(species) $\times$ 2(left sac–right sac) analysis of variance with repeated measures on the third factor for the data from collection Method II, second collection. The analysis did indicate a statistically significant species difference with respect to the amine ($F = 5.65$, $df = 1/16$; $P < 0.05$). Typically, the amounts of amine found in the beagle anal sacs was from 0.14 μg to 0.70 μg/μl of secretion (estimated, as discussed above). On the average these amounts were 10 times more than the amount of amine found in the coyotes and may be a reflection of the lower amounts of secretion found in coyote's anal sacs.

No trimethylamine was detected in the samples obtained from the cats.

In addition, we have found that the two aldehydes listed in Table 2 as well as 2-piperidone were present in the cat anal sac secretions. No evidence for any of the compounds found above was seen in the blanks.

DISCUSSION

As noted above, no consistent changes indicative of the estrous state could be found in the patterns of volatiles from the beagle or coyote anal sacs. In addition, no one compound or ratio of volatiles appeared to be characteristic of gender. Species differences do, however, exist in that the beagles had greater amounts of volatiles than the coyotes. The amount of volatiles from the same animal appeared to be variable, using both collection methods. Because of this variability, subtle changes in the patterns of volatiles may have gone undetected. Doty and Dunbar (1974a and 1974b) found that the color, odor, and secretory rate of beagle anal sac secretion appears to be independent of hormonal status of the animal. In addition, urine and vaginal secretions from estrous females, but not anal sac secretions, elicited attraction by sexually experienced male beagles. Consequently, our results are in agreement with this behavioral study.

The anal sacs of dogs have been shown to possess numerous apocrine tubules and sebaceous glands in the tissue lining them (Montagna and Parks, 1948). Histochemical studies have shown that these secrete proteins, carbohydrates, and lipids into the sacs (Montagna and Parks, 1948). Short chain aliphatic acids, trimethylamine, ethanol, and acetone may all arise via microbial action on proteins, carbohydrates, and lipids (Wood, 1961; Sokatch, 1969; Hayward and Stadtman, 1960; Holdeman and Moore, 1972; Thimann, 1963). The indigenous microflora of the fox anal sac, when incubated in vitro, produce the C_2–C_5 acids (Albone and Eglinton, 1974). In addition, Gorman et al. (1974) have shown that the series of short chain aliphatic acids present in the anal sacs of the Indian mongoose (*Herpestes auropunctatus*) are products of bacterial metabolism. Hence, the presence of the available substrates, in addition to the volatiles formed, strongly suggests a microbiological origin for these compounds.

The short chain aliphatic acids are widely distributed in biological fluids. In addition to the three canids discussed above, they have also been reported to be constituents of cats' anal sacs (Berüter, unpublished, 1972), guinea pig (*Cavia porcellus*) perineal glands (Berüter et al., 1974), Beagle and coyote vaginal secretions (Preti, Muetterties, Furman, Kennelly, and Johns, unpublished results, 1973), rhesus monkey vaginal secretions (Michael et al., 1971), and human vaginal secretions (Michael et al., 1974; Preti and Huggins, 1975).

Trimethylamine has been confirmed as present in the anal sac secretions of three canid species: dog, coyote, and red fox. It was not detected in the four secretion samples from cats' anal sacs and was not reported as present in these secretions from the lion (Albone and Eglinton, 1974) or the Indian mongoose (Gorman et al., 1974), which are noncanid carnivores. In addition, the perineal gland of the guinea pig, a site of volatile acid production and storage, does not contain the amine. These findings suggest that the amine will not be found in all glands where volatile acids are produced and/or stored.

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A CHEMICAL TRAIL FACTOR FROM THE SILK OF THE EASTERN TENT CATERPILLAR *Malacosoma americanum* (LEPIDOPTERA: LASIOCAMPIDAE)

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Abstract—The leaf-feeding larvae of the eastern tent caterpillar *Malacosoma americanum* (Fabricius) follow silk trails laid down on branches leading from their communal tent to distant foraging sites. The response of colonies reared in the laboratory under seminatural conditions to silk trails washed in methylene chloride and to chemical trails prepared from a solvent extract of their tent or trail silk, showed that one or more soluble components of their trail is essential to the elicitation of the following response. The demonstrated ability of the caterpillars to distinguish between old and newly reinforced silk trails most likely occurs in response to a temporal change in the detectable chemical properties of their trail.

Key Words—*Malacosoma americanum*, tent caterpillar, chemical trail substance, pheromone, trail-following behavior.

INTRODUCTION

The phytophagous larvae of the eastern tent caterpillar *Malacosoma americanum* (Fabricius) construct a communal silk tent in the branches of cherry or apple trees. The caterpillars feed gregariously on the new foliage of the host and return to the tent to rest after each forage. Larvae lay down single strands of silk as they move over the branches of the tree and frequently used routes soon become thickly silked trails that enable the larvae to find their way back to the tent. Although the various species of tent caterpillars are widely distributed and the subject of numerous scientific and semitechnical articles, little is known of either the silk trail system employed by these insects or the

sensory basis of trail following. The present study describes a technique for studying the behavior of the eastern tent caterpillar under seminatural conditions in the laboratory and investigates the role of a chemical component of the silk in eliciting the trail-following response.

METHODS AND MATERIALS

Rearing Techniques

Egg masses of the eastern tent caterpillar were collected in the field or obtained from second generation laboratory reared moths. Egg diapause was terminated by storing the eggs at 2°C for at least 15 wk (Bucher, 1959). The eggs were washed in a 5.25% solution of sodium hypochlorite just prior to hatching to protect the larvae from virus infection (Grisdale, 1967). Newly hatched larvae were maintained in a petri dish until they achieved the second instar, when they were transferred to an inverted tripod (Figure 1). Laboratory colonies ordinarily consisted of from 50 to 200 caterpillars. The larvae established a permanent tent on the tripod and moved en masse via a connecting bridge to feeding sites on a small cherry tree, *Prunus serotina*, during discrete activity periods occurring up to four times per day. Since *M. americanum* grows well only when fed young leaves, saplings of the host tree were maintained year-round in a greenhouse and periodically cut back to induce new growth.

Colonies were maintained at 21 ± 1°C under a 15:9 light:dark daylight fluorescent light regime. Observations of caterpillars foraging during the dark period were made under subdued red light. Forty-four colonies were observed in the laboratory during the course of this study. Food requirements of whole colonies often exceeded our supply of leaves so that observations were largely limited to the first four or five of the six larval instars.

Trail Factor Bioassay

A removable glass rod, 8 cm long, was attached to the bridge connecting the tent and tree (Figure 1). When the caterpillars had laid down a silk trail over the rod, it was removed and washed in methylene chloride for 5–10 min, then replaced. The treatment removed soluble components of the silk but had no readily discernible effect on the appearance of the silk itself. The response of the larvae to the washed silk was observed directly during their next activity period.

A solvent extract of tent or trail silk, prepared by soaking approximately 5 mg of silk in 0.1 ml of methylene chloride for 5 min, was placed on silk

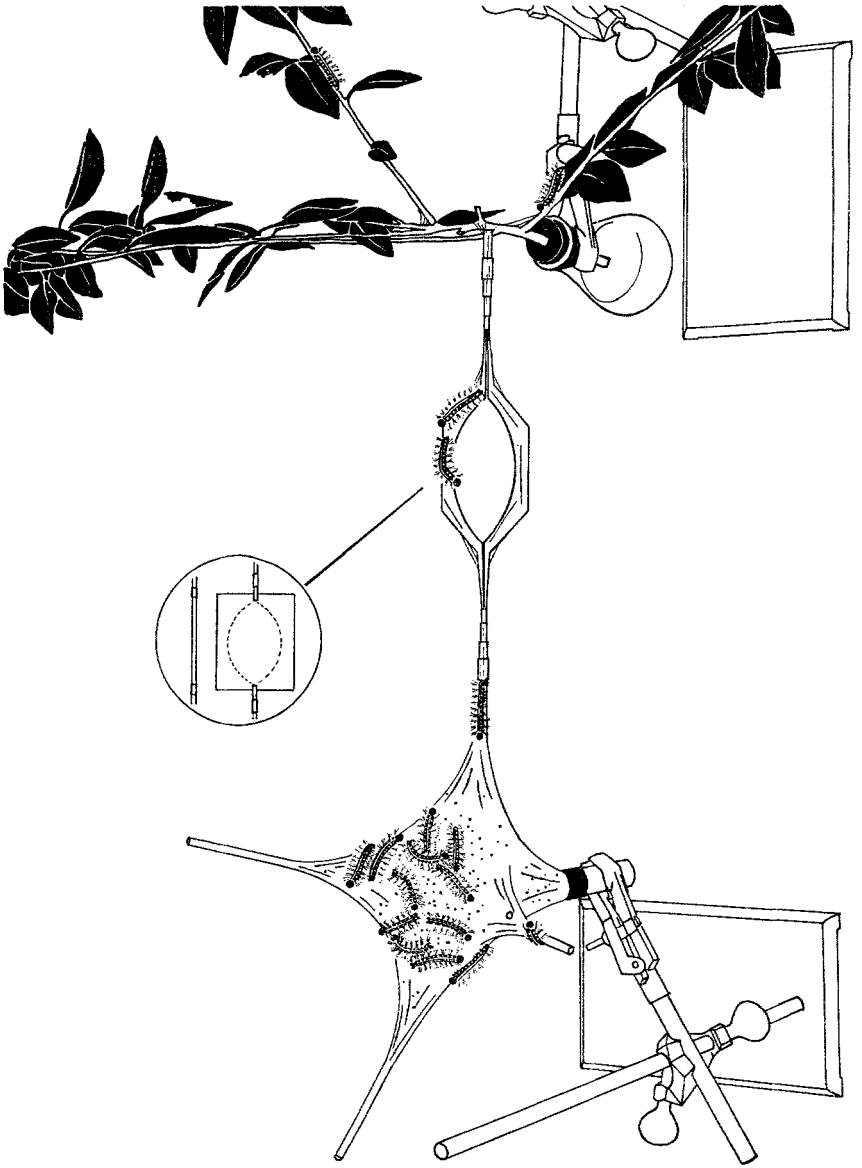

FIG. 1. Apparatus used to study the trail-following behavior of *Malacosoma americanum*. A double-ended Y-maze is shown attached to the bridge. The method of attaching a glass rod or plate in place of the maze is also shown. Dashed lines on the plate indicate the placement of solvent extract and control trails. Illustration based on a photograph.

trails that were no longer attractive to the caterpillars to determine if attractancy could be restored. Silk extract was also laid out in narrow curved or looping trails, 8–15 cm long on glass, cardboard, or phenolic plastic plates to determine if the extract would elicit trail following in the absence of a silk substrate. Plates were attached to the apparatus so that the extract trail was continuous with the previously silked tent and tree sections of the bridge (Figure 1). The movement of the larvae over the plate and the placement of their new silk trail was recorded relative to the location of the extract trail and a similar solvent control trail.

In another study, colonies of tent caterpillars were allowed to choose between a washed silk trail and a trail prepared from a solvent extract of tent or trail silk. A plate was attached to the bridge allowing the caterpillars to construct a silk trail across it. The plate was then removed, washed in methylene chloride, and replaced. A small quantity of silk was extracted in methylene chloride as previously described and the extract laid out in a narrow trail in an alternate location on the plate. The response of the colony to the two trails was observed during their next activity period.

Y-Maze Study

A study was conducted to determine if caterpillars could distinguish between new and aged silk trails spun on the arms of a double-ended Y-maze (Figure 1). One section of the two-piece maze was attached to the bridge until it was covered with silk. The section was then detached and allowed to age in the open at room temperature. A second section was attached to the bridge, allowing the larvae to construct a new trail over it. The section with the aged trail was reattached to the bridge opposite the newly silked section to form the Y-maze. The pieces were adjusted so that no gaps in the silk strands occurred at the bridge–maze juncture. A potential silk strand continuity factor and positional effect was precluded by detaching and reattaching the newly reinforced section prior to the start of each test and by shifting the sections to opposite sides 2–4 times during each test. The dimensions of the maze were varied to accommodate the growing larvae and to assure that the heads of the insects swept across both sections of the maze before the arms diverged.

The aged section of the Y-maze was not attached to the bridge until at least part of the colony was already on the tree. The response of individual larvae was then recorded as they approached the choice point from either the tent or tree end during their normal activity period. Colonies were observed for up to 3 h after the onset of foraging. Larvae active during that period crossed the maze once or twice.

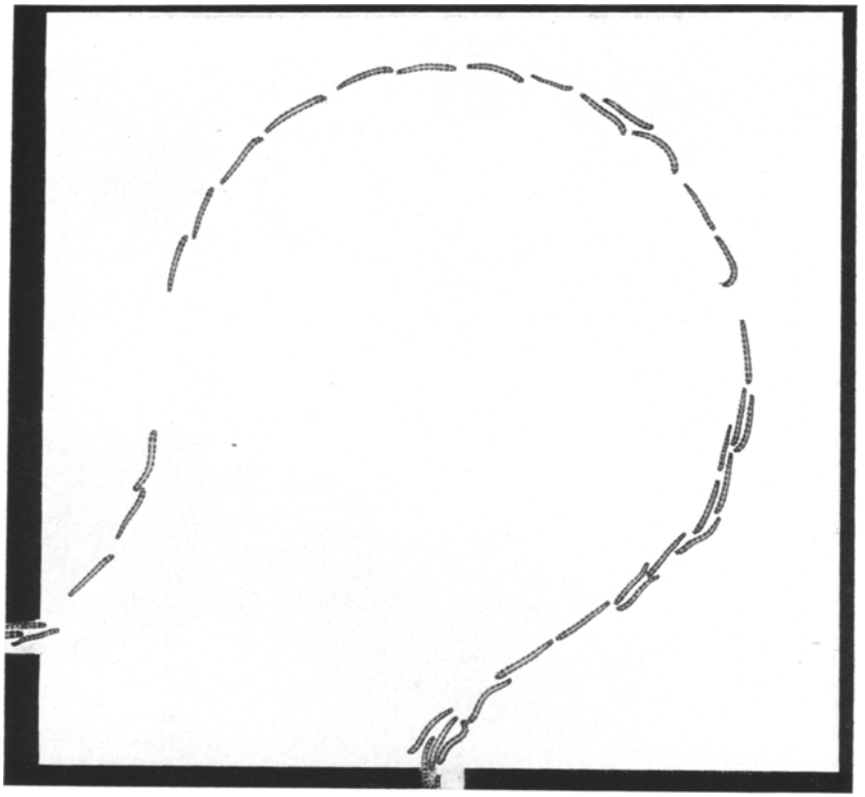

FIG. 2. Third instar caterpillars of *Malacosoma americanum* following a new silk trail laid down over a chemical trail prepared from a solvent extract of their silk. The trail is located on an elevated plate situated between bridge sections leading from a host tree (lower left) to their tent (bottom center). (Reduced 45% for reproduction.)

(*Facing p.* 191)

RESULTS AND DISCUSSION

Response of Caterpillars to Washed Silk Trails and to Silk Extract

In all of 14 tests involving five colonies, tent caterpillars initially failed to cross sections of silk trails they had laid down on glass rods when the sections were washed in methylene chloride. Larvae moving to feeding sites or returning to the tent stopped abruptly upon encountering the extracted section separating their tent from the tree. The caterpillars commonly lifted and swung the anterior portion of their bodies from side to side at the juncture of the rod before turning back. The larvae repeatedly returned to the extracted section and gradually extended the trail by laying down a few strands of silk at the proximal end of the rod. Small numbers of caterpillars eventually arrived simultaneously at the extracted section and hesitantly pushed en masse part way out onto the trail. The caterpillars continued in this manner until the entire section was reinforced with a new layer of silk. Colonies first succeeded in crossing the rods 12–135 min after initially encountering the extracted sections. The response of the caterpillars to these extracted silk trails showed that the tactile and visual components of extracted silk were not in themselves adequate to elicit the normal following response.

The attractancy of washed silk trails was restored when a solvent extract of silk was added to them. Solvent extract also elicited trail following by tent caterpillars in the absence of silk trails. In 36 of 40 tests (16 colonies) the caterpillars formed new silk trails directly over solvent extract trails laid down on plates from several minutes until up to 10 h before the onset of the colony's activity period. The larvae were also induced to follow more elaborate trails consisting of multiple "s" bends or broad circular loops laid out on sheets of cardboard connected to the bridge (Figure 2). Response of the caterpillars to these chemical trails ranged from immediate acceptance to marked hesitancy. In the latter case, the caterpillars backed up on the tent or tree, then slowly pushed en masse over the chemical trail, frequently turning back before bridging the section. Variation in response of colonies to these chemical trails can be attributed to differences in the initial strength of the extract, short-term changes in the readiness of larvae to follow trails other than those most recently reinforced (see below), inherent differences between colonies, and possibly to variable aging of the trails prior to the arrival of the larvae.

When allowed to choose between washed silk trails and chemical extract trails, the caterpillars clearly preferred the chemical trails. In 10 of 13 tests (seven colonies), caterpillars abandoned the washed silk trails they had followed across plates up to the time of washing in favor of chemical trails laid out in other locations on the plates. In one of the other three tests, the caterpillars followed only the washed silk trail, and in the other two instances, they

initially followed both trails, but subsequently followed only the washed trail. The preference of the caterpillars for the washed silk trails in these three cases is likely attributable to incomplete washing of the original trail, the preparation of a relatively weak silk extract, or a combination of both. A separate bioassay of silk extract showed that a chemical capable of eliciting a weak following response still remained in silk that had been washed with as many as four separate changes of methylene chloride over a 1-h period. The response of the eastern tent caterpillar in these tests indicated that one or more chemical components of the trail is essential to the elicitation of the trail-following response.

Y-Maze Study

Caterpillars responded to the alternate pathways of the Y-maze by favoring the newly reinforced trails over those not used during the previous one or more activity periods (trails aged 11 or more hours, Table 1). Caterpillars returning to the tent from the tree did not, however, discriminate between new trails laid down on their way out to the tree, and trails reinforced during their previous activity period (trails aged 5–8 h, Table 1).

In tests involving trails aged 11 or more hours, the caterpillars often showed marked hesitation at the choice point, swinging their heads from trail to trail before moving out onto one of the arms. Larvae crossing the arm covered with aged silk moved more slowly than those crossing the newly reinforced section. Over all, 38.6% of the caterpillars starting out on these older trails turned back before reaching the other side as compared to 1.3% of the larvae that started out on the newly reinforced trails.

The ability of larvae to discriminate trail age most likely occurs in response to the gradual breakdown or loss through volatilization of a chemical trail factor. The failure of larvae to discriminate between trails consisting

TABLE 1. RESPONSE OF SECOND THROUGH FIFTH INSTAR *Malacosoma americanum* CATERPILLARS TO NEW AND AGED SILK TRAILS SPUN ON THE ARMS OF A Y-MAZE

Number of tests	Total number of larval responses	Hours since aged trail last used		Percentage of caterpillars choosing new trail
		Mean	Range	
5	398	6.2	5– 8	48.5
5	329	13.0	11–15	91.8
15	851	24.2	21–30	94.9

wholly of new silk and those aged 5–8 h may be attributed to the fact that at the start of the tests the new trails were rather sparsely silked as compared to the aged trails, the latter having been reinforced several times during the previous activity periods. Gradual loss of attractancy of the older trails due to aging may have been compensated for by the larger quantities of silk, hence attractant, in these trails. Differences in the quantity of silk on trails aged over 11 h and the newly reinforced trails they were compared with were sometimes noticed, but had little apparent effect on larval response. Newly reinforced trails were invariably favored by the colonies even when they were composed of noticeably less silk than the older trails with which they were paired.

This preliminary study of the eastern tent caterpillar indicates that the silk trail of the insect contains one or more chemical components which play a dominant role in eliciting the trail following response of the larva. Breakdown or volatilization of a chemical component of the trail may further serve the colonies by enabling them to distinguish between new trails leading to current feeding sites and abandoned trails leading to exhausted feeding sites. At the present time, the literature contains little detailed information on the foraging behavior of this insect, and additional studies, both in the laboratory and field, will be needed before a complete understanding of its trail system is possible.

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BARK BEETLE PHEROMONES: PRODUCTION OF VERBENONE BY A MYCANGIAL FUNGUS OF *Dendroctonus frontalis*[1,2]

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Abstract—A mycangial symbiotic fungus of *Dendroctonus frontalis* is capable of oxidizing *trans*-verbenol to verbenone. Both of these compounds are known to be important behavioral chemicals for this species, and it is suggested that development of the fungus in the plant host may play a role in influencing the behavior of the beetle to a successfully colonized tree.

Key Words—pheromones, *Dendroctonus frontalis*, bark beetles, *trans*-verbenol, verbenone, microorganisms.

INTRODUCTION

The possibility that organisms within the gut of an insect might be capable of transforming dietary substrates into substances that may serve as pheromones was demonstrated by isolating a bacterium from the gut of *Ips paraconfusus* capable of oxidizing α-pinene to *cis*- and *trans*-verbenol and myrtenol (Brand et al., 1975). All three of these oxidation products occur in substantial amounts in the hindgut of various bark beetle species after exposure of individuals to α-pinene vapors (Hughes, 1973a; Hughes, 1973b;

[1] Supported in part by a cooperative agreement with the USDA Forest Service, Southern Forest Experiment Station, Pineville, Louisiana 71360.
[2] The use of a trade name in this paper does not constitute an official endorsement or approval by the USDA.

Renwick et al., 1973; Hughes, 1975). Hughes (1973b) suggested that these oxidation products might be formed in a region of the beetle other than the alimentary canal. In contrast, Brand et al. (1975) suggested that they may be formed where they occur in the alimentary canal by microbial action on α-pinene. It must be emphasized that neither the results of Hughes (1973b) nor those of Brand et al. (1975) are based on conclusive evidence, and it is quite possible that both the beetle's enzymatic repertoire and that of the gut microorganisms are capable of these transformations. One of our main goals in this general study is to ascertain to what extent both the insect and the microorganisms associated with it are capable of synthesizing and metabolizing certain pheromones.

SJB-133 is one of two filamentous fungi usually present as yeast phases in the mycangium of female *D. frontalis* (Barras and Perry, 1972; Barras and Taylor, 1973). It occurs as a *Sporothrix* imperfect form in the beetle gallery with the perfect form being produced only rarely in vitro. In addition, two true yeasts are often associated with the mycangium. The microorganisms of the mycangium are introduced into host pines upon attack by the insect and result in rapid invasion of the gallery system and surrounding phloem. Barras (1973) has shown that growth of the total mycangial complex in the gallery system is important in insect development as the absence of the complex causes greater than 50% reduction in the number of insect progeny and greatly extends the larval generation time. In addition to the mycangial complex, other microorganisms present on the exoskeleton will also colonize the gallery system (Howe et al., 1971; Barras and Marler, unpublished data). Therefore it is clear that many organisms have ample opportunity to transform and metabolize an array of host plant substances during their development in the phloem.

In transformation experiments with liquid cultures of the fungus SJB-133, similar to those previously described (Brand et al., 1975) but using a rather impure sample of DL-α-pinene, *trans*-verbenol was consumed and verbenone accumulated. This finding led us to suspect that this fungus could transform *trans*-verbenol into verbenone. In addition, as *cis*- and *trans*-verbenol may be produced from α-pinene by other microorganisms (Prema and Bhattacharyya, 1962; Brand et al., 1975) it also suggests that a more complex system may be operative in the natural environment of bark beetles in which the products accumulated by one microorganism may be the substrates of another, thereby leading to sequential transformations.

In order to investigate the possible production of verbenone from *trans*-verbenol we did the following experiment. The fungus, SJB-133, isolated by one of us (S.J.B.), was grown in stationary 1 l cultures of Sabouraud's dextrose for 7 days. The mycelial growth was then filtered on a Buchner funnel. Twenty-five milligrams of the damp mycelial mat were added to 2.5 ml

of phosphate buffer (0.05 M, pH 7.5) containing 10 μmoles of *trans*-verbenol (10% solution in ethanol). Either *trans*-verbenol or the fungus was omitted from the control flasks. After incubation of all flasks for 4 h at 30°C on a Dubnoff metabolic shaker, each solution was extracted with 6 ml *n*-pentane containing 1-octadecene as an internal standard. The pentane extracts were concentrated to 0.2 ml and 1-μl aliquots were injected into the gas chromatograph (10% SP-1000, 1.85 m × 3.25 mm stainless steel, 160°C).

The various chromatograms obtained showed that SJB-133 will readily convert *trans*-verbenol to verbenone under these experimental conditions. It appears that SJB-133 cannot oxidize verbenone further as the yield of verbenone was equal to the amount of *trans*-verbenol added. Similar experiments showed that SJB-133 will convert *cis*-verbenol quantitatively to verbenone and, also, that 3-methyl-2-cyclohexen-1-ol will be converted to the corresponding ketone in a yield of approximately 75%. The identity of the ketones was confirmed by mass spectrometry.

The various pheromones and chemicals identified from the Scolytidae have been summarized by Borden (1974). If one may be permitted to generalize from the table presented by Borden, it seems that the two ketones, verbenone and 3-methyl-2-cyclohexen-1-one, may both be involved in nullifying the attractive properties of various substances to certain *Dendroctonus* species. Verbenone is present as a major volatile component of the hindgut of emergent *D. frontalis* (Renwick, 1967) and is known to be an important substance in rivalry behavior between males of this species (Rudinsky and Michael, 1974). An increase in the concentration of verbenone released from a successfully colonized tree has been proposed as an important factor in inhibiting further attacks on the tree by both *D. frontalis* and *D. brevicomis* (Renwick and Vité, 1970). In addition, 3-methyl-2-cyclohexen-1-one has been termed an antiaggregative pheromone of *D. pseudotsugae* (Rudinsky et al., 1973; Rudinsky et al., 1974).

Our results show that both verbenone and 3-methyl-2-cyclohexen-1-one can be produced from their respective alcohols by SJB-133. With these results in mind, Fonken and Johnson (1972, pp. 89–93) give a number of examples illustrating that fungi and bacteria are able to oxidize monoterpene hydrocarbons and, in most cases, if the alcohol is formed then the corresponding ketone also appears. It is interesting to speculate that the termination of attack by *D. frontalis* because of an increase in verbenone concentration (Renwick and Vité, 1970) is related to the development of microorganisms that can produce it.

Our initial presentation of the possibility that microorganisms associated with the gut of bark beetles may be producing at least some of their pheromones (Brand et al., 1975) must now be extended. It is likely that SJB-133 growing in the phloem could produce verbenone from verbenol in situ, and,

if this is the case, then a microorganism external to the beetle would be responsible for part of the production of at least one of its behavioral chemicals. The proof of this hypothesis constitutes a part of our continuing investigations.

These transformation experiments have added additional evidence to biological studies that have shown a complex symbiotic relationship between the Southern Pine Beetle, associated microorganisms, and host tree components. The elaborate glandular mycangium in the female (Happ et al., 1971) indicates that such a high degree of symbiosis is not left to chance. Although the conclusion is somewhat speculative at this point, it appears that at least one fungus (SJB-133) of the mycangial complex, which is important in the beetle's nutritional regime, could play a significant role in regulating response to the plant host.

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2,6-DICHLOROPHENOL, THE SEX PHEROMONE OF THE ROCKY MOUNTAIN WOOD TICK, *Dermacentor andersoni* STILES AND THE AMERICAN DOG TICK, *Dermacentor variabilis* (SAY)[1]

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Abstract—2,6-Dichlorophenol is the only active sex attractant component detected in the extracts of the Rocky Mountain wood tick, *Dermacentor andersoni* Stiles and the American dog tick, *Dermacentor variabilis* (Say). It elicits from the male of each species a hierarchy of responses culminating in copulation. This compound probably occurs generally throughout the metastriate Ixodidae. 2,6-Dibromophenol, an artifact, also elicits the same sexual responses from the wood tick, but phenol and *p*-cresol do not.

Key Words—Rocky Mountain wood tick, dog tick, *Dermacentor andersoni*, *Dermacentor variabilis*, sex pheromone, 2,6-dichlorophenol.

INTRODUCTION

Several workers (Gladney et al., 1971; Gladney et al., 1974a, b; Berger et al., 1971; Berger, 1972; Sonenshine et al., 1974; Wood et al., 1975) have pre-

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sented evidence for a sex pheromone in species of ixodid ticks. Berger (1972) reported the isolation and identification of 2,6-dichlorophenol from the female lone star tick, *Amblyomma americanum* (L.). He concluded that it is a sex pheromone because feeding male lone star ticks responded to this compound by detaching, leg-waving, and general excitation. No other tests of the sex attractant potential of this compound were done, but incorporation of $Na^{36}Cl$ into 2,6-dichlorophenol was subsequently reported (Berger, 1974). Wood et al. (1975) isolated and identified phenol and *p*-cresol from fed females of five species of ixodid ticks; three of these species also contained 2,6-dichlorophenol, and four species contained salicylaldehyde. Evidence of sex pheromone activity was based on a bioassay in which the preference for either arm of a T-tube was recorded. The response of males of two species, *Rhipicephalus appendiculatus* Neumann and *R. pulchellus* Neumann, to phenol and *p*-cresol was described. *R. appendiculatus* also responded to 2,6-dichlorophenol, although this compound could not be detected in the washings from females of either *R. appendiculatus* or *R. pulchellus*. Chow et al. (1975), using Berger's bioassay, designated 2,6-dichlorophenol as the sex pheromone of the brown dog tick *Rhipicephalus sanguineus*.

In our experience with the Rocky Mountain wood tick, *Dermacentor andersoni* Stiles and the American dog tick, *D. variabilis* (Say), we found that the responses described by Berger are elicited by a number of artifacts such as an airstream, heat, or agitation. Consequently, we developed a bioassay involving a hierarchy of responses culminating in copulation of sexually active males with test objects (Sonenshine et al., 1974). The bioassays described here were done on a host.

We report that 2,6-dichlorophenol is the sex pheromone of the ticks, *D. andersoni* and *D. variabilis*. The significance of this finding in relation to reports of its presence in other metastriate Ixodidae is discussed.

METHODS AND MATERIALS

Rearing. Laboratory colonies of *D. variabilis*, started from field specimens collected near Richmond, Virginia, and *D. andersoni*, originally from the Rocky Mountain Laboratory, Hamilton, Montana, were maintained as a source of specimens. Mass rearing of adult ticks for extraction was done as described by Sonenshine et al. (1974). Approximately 1300 *D. andersoni* and 2700 *D. variabilis* females, in numerous batches, were used to extract the sex pheromone.

Extraction. Females attached to the host for 5 days were forcibly detached and placed in pentane or hexane in long-stem bulbs, which were sealed and subjected to three cycles of sonicate–freeze–thaw. The solution

was decanted, the residue rinsed, and the combined solution was concentrated to about 5 ml or less by distillation through a small packed column. Bioassays of the distillates were negative.

To determine if the pheromone was phenolic, samples of the concentrated solution of both species were extracted with 0.5 M NaOH, washed with distilled water, and dried (Na_2SO_4). The NaOH solution was saturated with CO_2 to regenerate phenolic compounds and extracted with hexane. Persistent emulsions prevented complete recovery of material.

Chemicals and apparatus. Solvents were doubly distilled through packed fractionating columns and monitored by glc for impurities. 2,6-Dichlorophenol, 2,6-dibromophenol, *p*-cresol, phenol, and formic acid were reagent grade; the phenols were purified by gas–liquid chromatography (GLC). A Varian Aerograph model 2740 GLC apparatus (flame ionization detector) was used for GLC fractionation. A Varian electron-capture detector (tritium–titanium foil) was used for detection and quantitation of halogenated compounds.

Two glass GLC columns, 3.34 m by 6.35 mm OD, were utilized. One was packed with 4% Carbowax 20M on Chromosorb G 60/80 mesh, 180° oven, 60 ml N_2/min. The second column was packed with 4% OV-17 on Chromosorb G 60/80 mesh, 175° oven, 120 ml N_2/min. In flame ionization detection, a glass column, 6.15 m by 6.35 mm OD, packed with 4% Carbowax 20M on Chromosorb G 60/80 mesh, was used at various temperatures between 140° and 185°, 55 ml He/min. The sonication devices were a Bransonic II ultrasonicator and a Varian ultrasonic cleaner. Liquid N_2 or liquid air was used for freezing.

Mass spectral electron impact (70 eV) data were obtained from a Finnigan GLC 9500 fitted with a 1.5 m by 3.2 mm glass column packed with 3% OV-101 on Chromosorb W-HP, 80/100 mesh, interfacing a Finnigan 3300 mass spectrometer. A System Industries 150 unit provided the printout.

Bioassays. Extracts, GLC total collections, GLC fractions, recombined GLC fractions, and authentic chemical compounds were diluted in hexane, and 100 μl aliquots of the resulting solutions were used for the bioassay. Concentrations of material from ticks were in the range of 0.1–33 tick equivalents (TE) per 100 μl.

All bioassays were done with a sequence of 10 sexually active males released individually near (2–3 cm) a spot consisting of 100 μl of the test sample or control (solvent) on the shaved skin of a laboratory rabbit after the method of Gladney et al. (1971). A 2-min period was allowed for solvent evaporation. Sample spots representing several different concentrations and the control (i.e., solvent only) were placed in series along the anterior–posterior axis, test samples on one side, controls on the other. The samples were spaced at intervals of 3–4 cm and the test samples and controls were

distributed randomly. At least one control was included with any combination of test samples.

The bioassay procedure was modified slightly in later tests by the addition of a preserved female tick attached to the host skin adjacent to each of the sample spots and another adjacent to the control spot to allow copulatory attempts. Each bioassay was performed with 10 sexually active males released at the posterior end of the body. At first, the males were released singly and each was allowed three trials, which required as much as 1 h. Later, males were released in groups of five, thus reducing the bioassay time to 10 or 15 min. Orientation, attachment, and copulatory attempts (when preserved females were available) were determined as the percentage of all of the test males.

A test was considered positive when two or more of these types of responses were noted or when the percentage of orientation exceeded that observed with the controls. No instance of attachment or copulatory attempts was observed in the presence of control spots.

Sexual readiness of the test males was assured by selecting them from a population in which more than 95% of the individuals were attracted to sexually active females. In addition, the males used in the bioassay were exposed to sexually active females after the test was completed to verify their capacity to respond to this stimulus. Bioassays were discarded if the sexual potency of the test males was in doubt.

RESULTS

Identification of the Sex Pheromone in Tick Extracts. Sex pheromone activity was found in the extracts of fed females of both species of ticks (Table 1). *D. andersoni* hexane solutions were further subjected to extraction with 0.5 M NaOH, which removed the activity; saturating the basic solution with CO_2 and extracting with hexane gave an active hexane solution containing the phenolic compounds. Sequential fractionation, of both the original extract and the "phenolic" solution, monitored by the bioassay, gave a single active fraction and a single active peak in this fraction. Moreover, the recombined material from which the active peak had been removed failed to attract sexually active males. The hierarchical responses of sexually active male *D. andersoni* are summarized in Table 1. Those of sexually active male *D. variabilis* are summarized in Table 2. The active peak was identified as 2,6-dichlorophenol by retention times (coinjection with an authentic sample) on two GLC columns, by a mass spectrum (see Fig. 1) congruent with that of an authentic sample, and by the intense response of an electron-capture GLC detector. The retention time of 2,6-dichlorophenol was clearly different

TABLE 1. DEMONSTRATION OF SEX PHEROMONE ACTIVITY IN VARIOUS FRACTIONS FROM *Dermacentor andersoni* TICKS

Sample	Number of tests	Number of TE	(Av.)[a] % orientation	(Av.)[a] % attachment	Evaluation[b]
Total extract	7	1.0	(46)	(38)	++
(not dried)	3	0.75	(73)	(73)	++
	3	0.50	0	0	−
Total extract	2	1.0	(70)	(57)	++
(anhydrous)	2	0.9	(50)	(45)	++
	2	0.8	(2)	0	−
Total GLC	2	1.0	(50)	(62)	++
collection	1	0.75	0	0	−
GLC peak identified	1	1.0	35	30	++
as 2,6-dichlorophenol	1	2.0	40	30	++
	1	3.0	30	15	+

[a] (Av.) used only when multiple tests of the same concentration were done.
[b] Definition of symbols: + means positive response, ++ means strong positive response, and − means negative response.

from those of its five isomers. We obtained approximately 2 ng of the active compound per tick from each of the two species, *D. andersoni* and *D. variabilis*. Activity in the extract and active fraction was tested at concentrations between 1.0 and 0.5 tick equivalents (TE) per 100 μl of solvent. These responses

TABLE 2. DEMONSTRATION OF SEX PHEROMONE ACTIVITY IN GLC FRACTIONS OBTAINED FROM *D. variabilis* EXTRACTS

Sample	Number of tests	Number of TE	(Av.)[a] % orientation	(Av.)[a] % attachment	Evaluation[b]
Total extract	1	1.0	50	40	++
	1	0.9	27	33	++
	1	0.8	20	7	+
GLC fraction including	1	1.5	27	30	++
2,6-dichlorophenol	2	1.0	(13)	(17)	+
	3	0.8	0	0	−

[a] (Av.) used only when multiple tests of the same concentration were done.
[b] Definition of symbols: + means positive, ++ means strong positive, and − means negative.

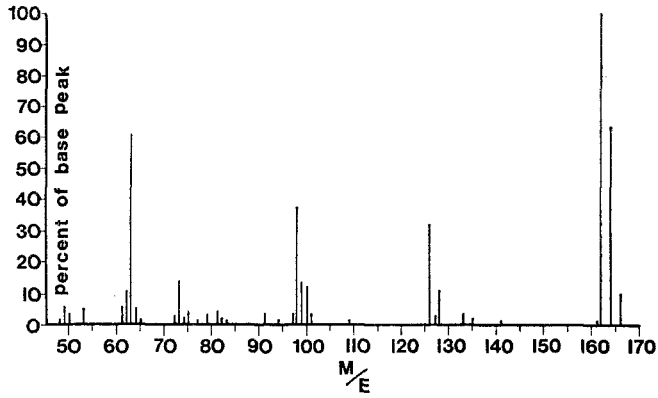

FIG. 1. Mass spectrum of 2,6-dichlorophenol. Finnigan 3300, 70 eV.

included orientation and, frequently, attachment adjacent to the preserved female (Tables 1 and 2); copulation with the preserved female was observed on several occasions. When males were allowed to remain *in copula*, they deposited spermatophores in the genital aperture. Other males attempted to copulate with one another.

Responses of Fed Males to Authentic Compounds. Authentic 2,6-dichlorophenol elicited the same hierarchy of responses in the bioassays at the same or similar concentrations as the compound isolated in the tick extracts (Tables 3 and 4). Positive responses were obtained when sexually active *D. andersoni* males were exposed to as little as 0.5 ng. Males responded to amounts as great as 200 ng; *D. variabilis* males were tested at, and responded to, amounts of 2 and 20 ng. Samples containing hexane, formic acid, phenol, and *p*-cresol failed to elicit any responses from *D. andersoni*, but 2,6-dibromophenol did elicit response.

Responses of Females and Unfed Males. No responses to extracts of fed females or to 2,6-dichlorophenol was elicited from unfed males or from fed or unfed females (Table 5). Thus, the pheromone does not appear to function as a general aggregation pheromone (Gladney et al., 1974a); it appears to be a true sex pheromone that elicits characteristic sexual responses from sexually active males.

DISCUSSION

Berger's (1972) pioneering work in this area met with some reservations because a compound such as 2,6-dichlorophenol, in Berger's own words, "is not easily accepted as being that of a natural product of an arthropod." Our own reservations were enhanced by the nonspecific bioassay used by Berger.

TABLE 3. BIOASSAY RESPONSES OF SEXUALLY ACTIVE *D. andersoni* MALES TO PURE COMPOUNDS RELEASED ONTO THE SKIN OF A RABBIT HOST

Type compound	Number of tests	Nanograms in sample	(Av.)[a] % orient.	(Av.)[a] % attach.	(Av.)[a] % copul.	Evaluation[b]
Hexane	3	—	(16)	0	0	−
Formic acid	1	1000	4	0	0	−
	1	1000	4	0	0	−
Phenol	1	2	16	0	0	−
	2	20	(3)	0	0	−
	2	200	0	0	0	−
P-Cresol	1	0.5	3	0	0	−
	1	5.0	0	0	0	−
	1	50.0	0	0	0	−
2,6-Dichlorophenol	1	0.1	0	0	0	−
	1	0.25	15	0	0	−
	1	0.5	60	20	40	++
	1	1.0	70	40	70	++
	5	2.0	(35)	(10)	(25)	++
	1	15.0	70	25	70	++
	6	20.0	(37)	(16)	(15)	++
	1	50.0	65	45	50	++
	1	80.0	55	35	50	++
	1	119.0	50	10	0	++
	1	150.0	80	65	20	++
	6	200.0	(48)	(27)	(40)	++
2,6-Dibromophenol	1	2.0	25	10	25	++
	1	20.0	25	15	25	++
	1	200.0	25	25	25	++

[a] (Av.) used only when multiple tests of the same concentration were done.
[b] Definition of symbols: + means positive ++ means strong positive − means negative.

TABLE 4. BIOASSAY RESPONSES OF SEXUALLY ACTIVE *D. variabilis* MALES TO PURE COMPOUNDS RELEASED ONTO THE SKIN OF A RABBIT HOST

Type of extract	Number of tests	Nanograms in sample	(Av.)[a] % orient.	(Av.)[a] % attach.	(Av.)[a] % copul.	Evaluation[b]
2,6-dichlorophenol	2	20	(5)	(2)	(5)	+
	2	2	(27)	(2)	(25)	++

[a] (Av.) used only when multiple tests of the same concentration were done.
[b] Definition of symbols: + means positive and ++ means strong positive.

TABLE 5. BIOASSAY RESPONSES OF *D. andersoni* UNFED FEMALES, PARTIALLY FED FEMALES, AND UNFED MALES TO KNOWN COMPOUNDS RELEASED ONTO THE SKIN OF A RABBIT HOST

Type of compound	Life stage	Number of tests	Nanograms in sample	(Av.)[a] % orient.	(Av.)[a] % attach.	(Av.)[a] % copul.	Evaluation[b]
2,6-Dichlorophenol	unf ♂	2	200	0	0	0	–
		2	20	(18)	0	0	–
		2	2	(5)	0	0	–
		1	0.25	0	0	0	–
		1	0.1	0	0	0	–
	unf ♀	2	200	0	0	0	–
		1	20	5	0	0	–
		1	2	25	5	0	–
		1	0.25	0	0	0	–
		1	0.1	0	0	0	–
	fed ♀	1	200	5	0	0	–
		1	2	20	0	0	–
Phenol	unf ♀	1	200	0	0	0	–
	fed ♀	1	200	10	0	0	–

[a] (Av.) used only when multiple tests of the same concentration were done.
[b] Definition of symbols: – means negative.

Another complicating factor is the small quantity produced by the ticks, which in our initial attempts resulted in failure to obtain observable activity from GLC fractions (Sonenshine et al., 1974).

On the basis of this study, it is reasonable to assume that 2,6-dichlorophenol, which Berger originally identified from fed females of the lone star tick, *Amblyomma americanum* (L.), functions as a sex pheromone in that species also, but confirmatory bioassays are needed. The same compound may be widespread throughout the metastriate Ixodidae. Observation of interspecific mating between *D. variabilis* and *D. andersoni*, and intergeneric mating between *D. variabilis* and the brown dog tick *Rhipicephalus sanguineus* (Latreille) tends to support this hypothesis (Sonenshine et al., 1974). Wood et al. (1975) speculate that sexual activity in ticks rests broadly on a phenolic basis.

2,6-Dichlorophenol is the only compound isolated that shows activity, and it is both necessary and sufficient to elicit the stereotyped sequence of orientation and copulation; it elicits these responses at concentrations in the same range in which it occurs in the tick extract. However, contributions of other compounds in the total mate-finding process cannot be excluded.

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TERMINOLOGY OF CHEMICAL RELEASING STIMULI IN INTRASPECIFIC AND INTERSPECIFIC INTERACTIONS[1,2]

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Abstract—The terminology of chemical releasing stimuli is examined in an attempt to reduce some apparent confusion. Two new classes of interspecific chemical signals, synomone and apneumone, are proposed.

Key Words—chemical ecology, hormone, pheromone, allomone, kairomone, synomone, apneumone, chemical communication.

INTRODUCTION

Numerous chemicals are produced or acquired by plants and animals that serve as releasing stimuli within or between organisms. The study of these chemicals and the interactions they mediate is a rapidly growing field within the relatively new area of chemical ecology (Sondheimer and Simeone, 1970; Whittaker and Feeny, 1971).

Since the terminology describing these releasing stimuli has been criticized and perhaps misunderstood, we have examined it with the hope of effecting some clarification. Rather than present an exhaustive list and discussion of the chemicals and the interactions they mediate, we will discuss only some of the more interesting and pertinent examples. Also, we have limited the discussion of hormones and pheromones because they have been thoroughly reviewed previously by others and appear to be well understood.

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[2] Mention of a proprietary product in this paper does not constitute endorsement of this product by the USDA.

Chemical agents are classified according to their function or effect in specific interactions (Whittaker and Feeny, 1971), and these functions are *not* mutually exclusive (Brown et al., 1970). It is not at all uncommon for a chemical to function in two or three different types of interactions.

We do not believe that definitions of "communication" should affect the validity of the terminology used for these releasing stimuli. For example, according to the definition of Burghardt (1970), chemical agents classified as pheromones involve true communication, whereas kairomones and many allomones would not. However, we do not feel that the classification implies that the terms are equated communicatively, a concern expressed by Blum (1974). Indeed, as noted by Otte (1974), some of the releasing stimuli discussed are signals that are fashioned or maintained by natural selection because they convey information related to survival, while other signals serve as incidental cues or signs in a particular relationship but their effect in this relationship is not their raison d'etre.

HORMONES

Hormones are one of the two major types of chemicals that mediate behavior or physiological interactions; semiochemicals are the other. Hormones are chemical agents produced by tissues or endocrine glands that cause specific reactions within the producing organism. Important processes in plants and animals that are controlled by hormones include: phototropism and geotropism in plants, diapause, growth and development, digestion, sexual maturation, oestrous and menstrual cycles, lactation, and pregnancy. Hormones are generally classified on the basis of the process they control such as growth hormone, molting hormone, and sex hormone. Various aspects of hormones have been reviewed by Gorbman (1959), Gorbman and Bern (1962), Pincus and Thimann (1948–64), Young (1961), Novak (1966), Gabe (1966), van der Kloot (1960), Andus (1959), Meyer et al. (1960), Siddal (1970), Engelmann (1968), and Williams (1970).

SEMIOCHEMICALS

The term semiochemical (Gk. *simeon*, a mark or signal) was proposed in 1971 by Law and Regnier to describe the chemicals involved in the chemical interactions between individual organisms. The semiochemicals are subdivided into two major groups, pheromones and allelochemics, depending on whether the interaction is intraspecific or interspecific, respectively.

Pheromones. Pheromones have been known to exist since at least 1609

when Charles Butler described how lone bees are attracted and provoked to mass sting by a substance released by a single sting. But it was not until 1961 that the first isolation and identification of a pheromone were reported (Butenandt et al., 1961), and the sting pheromone of the honey bee, *Apis millifera* L., was not identified until 1962 (Boch et al., 1962). Since 1961, with the improvements in chemical technology, literally hundreds of pheromones, components of pheromones, and other semiochemicals have been isolated and identified. Pheromones have been demonstrated in organisms ranging from algae (Starr, 1968; Siegel and Cohen, 1962; Mueller et al., 1971) to primates (Michael et al., 1971; Curtis et al., 1971), and they have been postulated in humans (Comfort, 1971; Michael et al., 1974).

The term ectohormone was proposed in 1932 by Beth to describe the chemicals involved in intraspecific interactions, but the term was a contradiction in itself (Karlson and Butenandt, 1959) so it was replaced by the term pheromone (Gk. *phereum*, to carry, and *horman*, to excite or to stimulate, Karlson and Butenandt, 1959; Karlson and Luscher, 1959). Pheromone was originally defined as a substance secreted by an animal to the outside that causes a specific reaction in another member or members of the same species. Some more common types are trail-marking or trail-following pheromones, alarm pheromones, dispersants, territoriality pheromones, synchronization pheromones, species aggregation pheromones, and sex pheromones (Wilson, 1963, 1965, 1970; Wilson and Bossert, 1963; Beroza, 1970; Jacobson and Beroza, 1964; Jacobson, 1965, 1966; Butler, 1964, 1967, 1970; Regnier and Law, 1968; Blum, 1969; Blum and Brandt, 1972). A single pheromone may have more than one pheromonal function or effect (Morse, 1972). Perception of a pheromone may result in an immediate behavioral response (releaser effect) or a complex set of physiological responses that are simply set in motion by the initial perception (primer effect) (Wilson and Bossert, 1963). These effects are also applicable to allelochemics and will be discussed later.

Allelochemics. The term allelochemic was proposed by Whittaker (1970a, b) to describe the chemicals involved in interspecific interactions. It was originally defined as a chemical significant to organisms of a species different from its source, for reasons other than food as such. The term xenomone was proposed by Chernin (1970) and has essentially the same meaning. The term allelochemic appears to be in more general use and will be used here. Allelochemics are herein divided into four subgroups: allomones, kairomones, synomones, and apneumones; the division is based on whether the emitter, the receiver, or both benefit in the interaction.

An allomone is an allelochemic originally defined as a chemical substance, produced or acquired by an organism, which, when it contacts an individual of another species in the natural context, evokes in the receiver a behavioral or physiological reaction adaptively favorable to the emitter

(Brown, 1968). The term was derived from alloiohormone, which has essentially the same meaning and was proposed by Beth (1932). Allomones include a wide variety of substances ranging from venoms and repellent secretions in animals and plants to growth inhibitors such as allelopathic substances and some antibiotics. As with pheromones, interactions mediated by allomones may involve a complex set of physiological changes (primer effect) or an immediate behavioral response (releaser effect). Some important reviews of allomones and their effects are those of Whittaker (1970a), Went (1970), Dethier (1970), Williams (1970), Eisner (1970), Whittaker and Feeny (1971), Happ (1973), and Bucherl et al. (1968–71).

Another type of allelochemic is the kairomone. The term kairomone (Gk. *kairos*, opportunistic) was proposed by Brown et al. (1970) and was originally defined as a chemical substance, produced or acquired by an organism, which, when it contacts an individual or another species in the natural context, evokes in the receiver a behavioral or physiological reaction adaptivity favorable to the receiver but not to the emitter. Thus, kairomones are maladaptive or at least nonadaptive to the individual emitter, but, as Brown et al. (1970) pointed out, it is possible for a kairomone to be adaptively favorable to the population as a whole by helping regulate the population dynamics in a way that is favorable in the long term. For example, Gilbert (1966) presents data showing that a predaceous rotifer produces a substance that causes the uncleaved eggs of its prey to produce spined individuals which are difficult for the predator to eat. This kairomone acts to maintain the population of the predator at a level that will not destroy its food source.

These chemicals may be incidental compounds utilized as cues by the receiver or signal emissions intended as pheromones, allomones, or hormones for the legitimate receiver, but which are exploited by illegitimate receivers. For these reasons there has been considerable controversy about this term. Blum (1974) argues that "the so-called" kairomones appear to be pheromones and allomones that have "evolutionarily back-fired" and as such do not "represent a class of chemical signals distinct from allomones and pheromones." Because each specific interaction must be considered separately and these interactions are not mutually exclusive, we do not concur with this reasoning. The fact that some phagostimulants, which stimulate a limited number of herbivores to feed on a plant also function, and probably originally evolved, to deter a vast multitude of herbivorous species from feeding on the same plant (Frankel, 1959) does not prevent the term kairomone from serving as meaningful terminology. In the interactions in which a compound is a phagostimulant, it is a kairomone and in interactions in which it is a feeding deterrent, it is an allomone. Even a hormone can act as a kairomone as in the case of corticosteroids of the rabbit and the rabbit flea, *Spilopsyllus cuniculi* (Dale) (Rothschild, 1965).

In order to remove some of the ambiguity present in some of the previous classifications, we would like to propose an additional division of allelochemics for mutualistic interactions that have generally been regarded as allomones (Brown et al., 1970) or as either allomones or kairomones (Whittaker and Feeny, 1971). Synomone (Gk. *syn*, with or jointly) is here defined as a chemical substance produced or acquired by an organism, which, when it contacts an individual of another species, in the natural context, evokes in the receiver a behavioral or physiological response adaptively favorable to both the emitter and the receiver. This group of allelochemics would include floral scents and nectars that attract insects and other pollinators and substances that play an important but often subtle role in symbiotic relationships (Henry, 1966; Nutman and Mosse, 1963; and others). For example, to survive on a diet of wood, the wood-eating cockroach, *Cryptocercus punctulatus* Scudder, requires wood-digesting protozoa in its gut. The hormone ecdysone, which regulates molting in the cockroach, also acts as a synomone that induces the sexual cycle of some of these protozoa (Cleveland, 1959), allowing them to reproduce. This is an example of a substance functioning as a hormone and as a synomone with a primer effect.

Multiple Species Interactions. Clarification of terminology is also needed for interactions involving three or more species, e.g., interactions involving a host plant, a phytophagous insect feeding on the plant, and its insect parasitoid. As previously discussed, phagostimulants that attract and stimulate the phytophagous species are kairomones. It is also known that some parasitoids utilize chemical cues emitted by the host's food plant to locate the host's habitat (Arthur, 1962; Monteith, 1958; Ullyett, 1953; and others) even in the absence of the host. We consider these cues to be synomones in this interaction, since they benefit the receiving organism in locating its host, and they benefit the emitter by reducing the damage caused by the phytophagous insect. The chemical serving as a kairomone for the phytophagous species may also serve as the synomone utilized by the parasitoid. Stimuli released from the phytophagous species that act as cues for the parasitoid are clearly kairomones. Vinson (1975) shows that the parasitoid *Cardiochiles nigriceps* Viereck is attracted to tobacco plants that have been damaged and that they are stimulated into an intensive search of the surrounding plant tissue if the damage was caused by the feeding of *Heliothis virescens* (F.). The proper classification of these cues is not clear. We suggest that those chemicals originating from the plant only as a result of the activities of, or only in combination with substances from, the phytophagous species that act as cues to the parasitoid should be considered kairomones. These same considerations can be applied to other multispecies interactions.

There are also three-way interactions in which an organism, such as a parasite or predator, is attracted to nonliving substances in which it may

find another organism, its host or prey, by chemical cues released from the nonliving substance, even in the absence of the host or prey. Thorpe and Jones (1937), for example, found that the ichneumonid parasite *Venturia canescens* (Gravenharst) is attracted to the odor of its hosts' food, i.e., fresh oatmeal. Also, Laing (1937) demonstrated that the braconid *Alysia manducator* Panzer and the chalcid *Nasonia* (= *Mormoniella*) *vitripennis* (Walker) are attracted to meat even though it never contained their dipteran hosts. These chemicals cannot be classified according to the previously described system for classifying chemical agents in three-way interactions. We here wish to propose the term apneumone (Gk. *a-pneum*, breathless or lifeless), which we define as a substance emitted by a nonliving material that evokes a behavioral or physiological reaction adaptively favorable to a receiving organism, but detrimental to an organism, of another species, that may be found in or on the nonliving material. Chemicals released from the nonliving material only as a result of activities of the potential host or prey, or only in conjunction with chemicals from the potential host or prey that are adaptively favorable to the receiver, would still be classified as kairomones.

TABLE 1. DEFINITIONS OF CHEMICAL MEDIATORS IMPORTANT IN CHEMICAL INTERACTIONS AMONG ORGANISMS

A. Hormone—A chemical agent, produced by tissue or endocrine glands, that controls various physiological processes within an organism.
B. Semiochemical—A chemical involved in the chemical interaction between organisms.
   1. Pheromone—Substance that is secreted by an animal or plant to the outside that cause a specific reaction in a receiving individual of the same species.
   2. Allelochemic—Chemical significant to organisms of a species different from their source, for reasons other than food as such.
      a. Allomone—A substance, produced or acquired by an organism, which, when it contacts an individual of another species in the natural context, evokes in the receiver a behavioral or physiological reaction adaptively favorable to the emitter but not to the receiver.
      b. Kairomone—A substance, produced, acquired by, or released as a result of the activities of an organism, which, when it contacts an individual of another species in the natural context, evokes in the receiver a behavioral or physiological reaction adaptively favorable to the receiver but not to the emitter.
      c. Synomone—A substance produced or acquired by an organism which, when it contacts an individual of another species in the natural context, evokes in the receiver a behavioral or physiological reaction adaptively favorable to both emitter and receiver.
      d. Apneumone—A substance emitted by a nonliving material that evokes a behavioral or physiological reaction adaptively favorable to a receiving organism, but detrimental to an organism, of another species, which may be found in or on the nonliving material.

There may be other chemical-mediated interactions involving nonliving material and various other organisms, but further study is needed before they can be classified. At the present time, for the sake of simplicity, we are considering apneumones as a subgroup of allelochemics; but if more interactions involving nonliving materials are found, it may be beneficial to reclassify them under another major group.

CONCLUSIONS

A classification of chemical mediators that serve as releasing stimuli is presented in Table 1. This classification is based on the function or effect of the chemical in each specific interaction. These functions are not mutually exclusive, and a chemical may have a function in each of two or three different types of interactions. Some of the original definitions have been altered slightly to accommodate interactions not previously considered and to eliminate the ambiguity of some terms.

The study of the chemical agents has great potential not only from the academic standpoint of increasing our understanding of behavior and evolution, but also from a practical standpoint, e.g., for controlling the behavior of various animals to our advantage. Much work has been done along this line, particularly with pheromones (see previous references) and kairomones (Lewis et al., 1975).

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BEHAVIOR OF *Tetranychus urticae* TOWARD ESSENTIAL OIL MIXTURES FROM STRAWBERRY FOLIAGE

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Abstract—A standard essential oil mixture (SEOM) was formulated containing volatile compounds in the relative proportions found in the essential oil of the foliage of "Citation," a strawberry cultivar relatively resistant to *Tetranychus urticae*. Other mixtures contained varied levels, relative to the SEOM, of *trans*-2-hexen-1-ol, nonanal, α-terpineol, and methyl salicylate. The behavior of *T. urticae* females in response to these mixtures at several concentrations in propylene glycol was studied in choice tube (preference) tests. Feeding effects were measured by incorporating the mixtures and individual components into sucrose with ^{32}P. In the choice tube tests, mites were generally attracted by mixtures at concentrations of 0.1% or below unless the level of methyl salicylate was below 0.5 × that in the SEOM, or the level of nonanal was above that of the SEOM. When mixtures were incorporated into food, methyl-salicylate-stimulated feeding and nonanal levels were inversely related to the amount of food ingested.

Key Words—strawberry resistance, *Tetranychus urticae*, essential oils, mite behavior, feeding attractants/deterrents.

INTRODUCTION

Volatile organic compounds (essential oils) occur widely in plant tissues and are thought to be secondary products of plant metabolism. These compounds frequently have very strong and characteristic odors that contribute greatly

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to the flavor of fruits and vegetables and in this way influence human preference or nonpreference for food products. Information on the influence of volatile compounds on preference or nonpreference of plant pests for their food supply is limited. In earlier studies we obtained information on the effects of several naturally occurring volatile compounds on spider mite behavior (Dabrowski and Rodriguez, 1971; Dabrowski et al., 1971; Rodriguez et al., 1971). The objective of the work reported herein was to study spider mite behavior, in particular, *Tetranychus urticae* Koch, in response to a mixture of volatile compounds and to determine the ability of mites to detect alterations in the composition of the mixture.

METHODS AND MATERIALS

The mixture of volatile compounds employed for these experiments was formulated from components identified in the essential oil of strawberry foliage by Kemp et al. (1968), except that *trans*-2-hexen-1-ol was substituted for *trans*-2-hexenal. It was recognized that the compounds in strawberry essential oil do not represent the totality of the volatile compounds in the intact strawberry foliage. However, strawberry essential oil served as a suitable model system; moreover, all of the volatile compounds included in our mixture have been reported widely as isolates from other plants.

The standard essential oil mixture (SEOM) contained the compounds in the relative proportions of "Citation" strawberry foliage essential oil,

TABLE 1. COMPOSITION OF THE STANDARD ESSENTIAL OILS MIXTURE (SEOM) COMPOUNDED AFTER STRAWBERRY FOLIAGE ESSENTIAL OIL

Component	Per cent
Trans-2-hexen-1-ol	5.2
Cis-3-hexen-1-ol	7.1
Furfuraldehyde	1.8
1-Octanol	1.5
Nonanal	36.0
Linalool	8.3
1-Nonanol	3.2
α-Terpineol	12.3
Methyl salicylate	20.9
2-Methylnaphthalene	2.8
Nonanoic acid	0.9

expressed on a percentage basis in Table 1. Other essential oil mixtures were formulated containing varied levels, relative to the SEOM, of the components *trans*-2-hexen-1-ol, nonanal, α-terpineol, and methyl salicylate. The first three were varied at levels 0.5, 1.0, 1.5, and 2.0 times the amount in the SEOM. Since the methyl salicylate concentration in the strawberry foliage had been found to vary greatly during the growing season, mixtures were formulated containing a wider range of levels of this compound: 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0 times the amount in the SEOM. In each concentration series, a mixture equivalent to the SEOM but without the appropriate component was also formulated.

The essential oil mixtures were used undiluted (100% concentration) or diluted in propylene glycol to concentrations of 0.01, 0.1, 1.0, and 10.0% of *trans*-2-hexen-1-ol, nonanal, and α-terpineol; to 0.0001, 0.001, 0.01, 0.1, 1.0, and 10.0% of methyl salicylate.

The effects of the essential oil mixtures on the behavior of *T. urticae* females involved two types of preference tests:

1. *Bioassay of Essential Oil Mixtures in Choice Tubes.* The choice tube technique used in this test was described in detail by Dabrowski and Rodriguez (1971). A choice was offered by placing the test material in the one microcup with propylene glycol as a control in the other cup. Four tubes of each combination were placed in a dark cabinet in order to eliminate the influence of light on mite behavior. The tubes were brought out at 5-min intervals during a 1-h period, read quickly, and replaced in the cabinet.

2. *Effect of Essential Oils Incorporated into Food on Ingestion.* According to Esau (1967), essential oils are widely distributed in the plant body. Hence, it was considered prudent to determine the effects on mite food intake of essential oils presented in a sucrose solution containing ^{32}P. Propylene glycol solutions of the four components individually, as well as the SEOM and the combinations with varied levels of the four components in the concentrations described above, were used. A 2% sucrose solution (9.85 ml) was dosed with 0.06 mCi of ^{32}P (in 0.1 ml H_3PO_4), and 0.05 ml of propylene glycol containing the appropriate essential oil mixture was added. The control treatment contained only the propylene glycol (0.05 ml) in the sucrose solution.

The system described by Ekka et al. (1971) utilizing a short glass tube covered on one end with a Parafilm sachet as a "feeding cell" was used here, with 10 teneral *T. urticae* females per cell. Three cells were placed in a 15-cm plastic dish, and this constituted one replicate. A treatment was composed of four such replicates. Three feeding cells were brought out at 4, 8, 12, and 24 h. At each time, the live and dead mites were separated and placed in nickel-plated planchets for radio-assay. Counting was done with a Baird Atomic Abacus Gm scaler model 123, and counts were adjusted for decay.

RESULTS

Bioassay of Essential Oil Mixtures in Choice Tubes

Changes in relative amounts of methyl salicylate, *trans*-2-hexen-1-ol, nonanal, and α-terpineol in the essential oil mixture elicited various behavior responses from the *T. urticae* females. Methyl salicylate produced the most notable effects. In the choice tube tests, the essential oil mixtures containing varied levels of methyl salicylate generally attracted *T. urticae* females at concentrations of 0.1% and below, provided that the level of methyl salicylate relative to the SEOM was above 0.5× (Table 2). The attractive effect was lessened with increasing concentration, and mixtures at concentrations above 1.0% were generally repellent. Within the 0.01% and 0.1% concentrations, there was a trend toward stronger preference at the higher levels of methyl salicylate. The mixtures containing 0 and 0.25× the standard methyl salicylate did not attract mites except weakly at the 0.1% concentration. Concentrations of 0.0001% and 100% were tested but were not included in Table 2 for the sake of brevity; the former exerted no effect on *T. urticae* females, and the latter exerted a strongly repellent effect, as did the 10% concentration.

In the choice tube tests where the levels of *trans*-2-hexen-1-ol, nonanal, and α-terpineol were varied as components of the essential oil mixtures, again the concentrations of 0.1% and below generally attracted the *T. urticae* females, while concentrations of 1.0% and above were generally repellent (Table 3). At the lower concentrations, the SEOM appeared to be the most

TABLE 2. RESPONSE OF *T. urticae* FEMALES IN CHOICE TUBES TO ESSENTIAL OIL MIXTURES AT VARIED CONCENTRATIONS AND WITH CHANGES IN METHYL SALICYLATE RELATIVE TO THE STANDARD ESSENTIAL OIL MIXTURE (SEOM)[a]

Relative amount of methyl salicylate in mixture	Per cent concentration of mixture in propylene glycol				
	0.001	0.01	0.1	1.0	10.0
2×	74.4***[b]	76.0***	69.2***	46.5*	16.5***
1.75×	76.8***	82.7***	71.9***	49.2	30.4***
1.25×	72.3***	80.8***	70.0***	36.5***	15.6***
SEOM 1×	72.8***	74.8***	65.2***	46.3*	34.2***
0.75×	73.5***	69.6***	62.5***	24.2***	17.7***
0.25×	50.8	52.9*	58.8**	41.5***	16.5***
0	52.1	52.9	55.8*	24.8***	18.6***

[a] Expressed as mean percentages attracted to the SEOM over propylene glycol blank; more than 50 denotes preference for SEOM, less than 50, nonpreference.
[b] *, **, ***: Significant from propylene glycol blank, at $P = 0.05$, $P = 0.01$, and $P = 0.001$, respectively.

TABLE 3. RESPONSE OF *T. urticae* FEMALES IN CHOICE TUBES TO ESSENTIAL OIL MIXTURES AT VARIED CONCENTRATIONS AND WITH CHANGES IN THREE COMPONENTS RELATIVE TO THE STANDARD ESSENTIAL OIL MIXTURE (SEOM)[a]

Relative amount of component	Per cent concentration in propylene glycol			
	0.01	0.1	1.0	10.0
SEOM 1×	74.8**[b]	65.2***	46.3*	34.2**
Trans-2-hexen-1-ol				
2×	56.3*	55.4	42.7*	32.2***
1.5×	70.4**	58.3**	44.4	34.1***
0.5×	72.1**	60.3**	47.7	40.4**
0	70.9**	55.8*	41.5**	43.2*
Nonanal				
2×	37.3**	41.9**	31.3**	30.4**
1.5×	44.0*	59.0**	46.0	33.3**
0.5	85.2**	54.2	55.6	31.7**
0	79.4**	50.2*	52.7	26.3**
α-Terpineol				
2×	66.0**	60.9**	48.2	33.1**
1.5×	68.8**	63.1**	49.1	33.3**
0.5×	71.7**	62.7**	45.2*	29.5**
0	62.1**	62.3**	45.8*	26.5**

[a] Mean percentages attracted to essential oil mixture; more than 50 denotes preference, less than 50, nonpreference.
[b] *, **, ***: Significant from propylene glycol blank at $P = 0.05$, $P = 0.01$, and $P = 0.001$, respectively.

attractive mixture in the series where *trans*-2-hexen-1-ol and α-terpineol were varied. The mixture containing no *trans*-2-hexen-1-ol was as attractive to the mites as were those containing 0.5, and 1.5× and more attractive than the mixture containing 2.0× the level of the SEOM. When nonanal was varied, at the 0.01% concentration, the mixtures containing 0 and 0.5× the level of the SEOM, as well as the SEOM itself, attracted the mites, while the mixtures containing 1.5 and 2.0× the level of the SEOM repelled the mites. At the 0.1% concentration, the SEOM appeared to be slightly more attractive to the mites than the mixtures containing nonanal at levels either above or below the SEOM (Table 3).

Effect of Essential Oils Incorporated into Food on Ingestion

When *T. urticae* females were fed for 24 h on a 2% sucrose solution

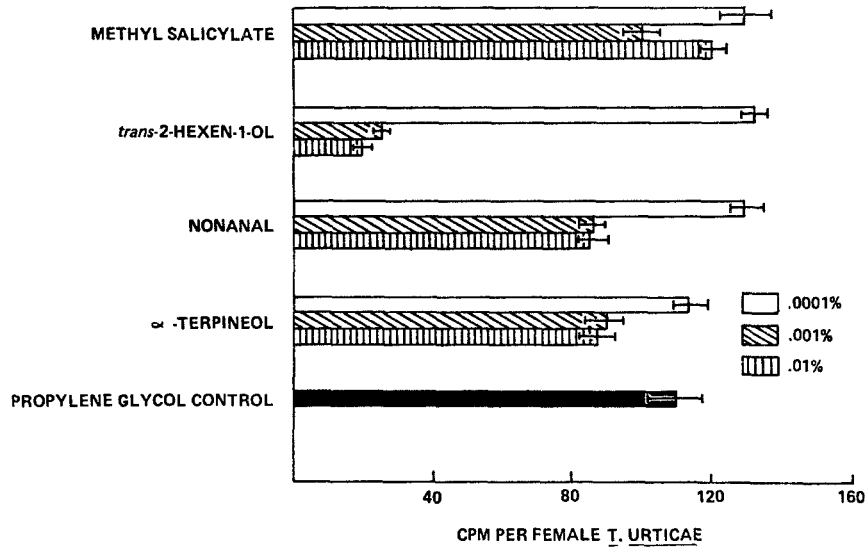

FIG. 1. Comparative ingestion of four (strawberry foliage) essential oils as measured by radioactivity of *T. urticae* females fed for 24 h on 2% sucrose solution containing ^{32}P and the individual essential oils at 0.0001, 0.001, and 0.01% in propylene glycol.

containing ^{32}P in propylene glycol (the control), the resultant feeding level, as indicated by radioactivity, was 110 counts/min (cpm) per female mite (Figure 1). Three of the essential oils tested individually at 0.0001% in the same solution significantly stimulated feeding: the females that fed on methyl salicylate, *trans*-2-hexen-1-ol, and nonanal contained 132, 135, and 130 cpm/mite, respectively. The feeding levels on 0.001% and 0.01% methyl salicylate did not differ significantly from that of the propylene glycol control. Concentrations of 0.001% and 0.01% of *trans*-2-hexen-1-ol, nonanal, and α-terpineol significantly decreased mite feeding (Figure 1). The most effective feeding deterrent was *trans*-2-hexen-1-ol, which at 0.001% reduced the radioactivity of *T. urticae* females to 27 cpm/mite. These females appeared to be intoxicated and 80% were dead after 24 h.

When the essential oil mixtures at 0.01% in sucrose solutions with ^{32}P were offered to *T. urticae* females, various feeding intakes were noted with varied levels of methyl salicylate, *trans*-2-hexen-1-ol, nonanal, and α-terpineol (Figure 2). After 24 h of feeding on the SEOM, a level of radioactivity of 108 cpm/mite was found. Looking first at methyl salicylate, it is seen that all the mixtures containing this component stimulated ingestion to levels which were significantly higher than that of the mixture containing no methyl salicylate, but lower than that of the SEOM. [In a test with varied methyl

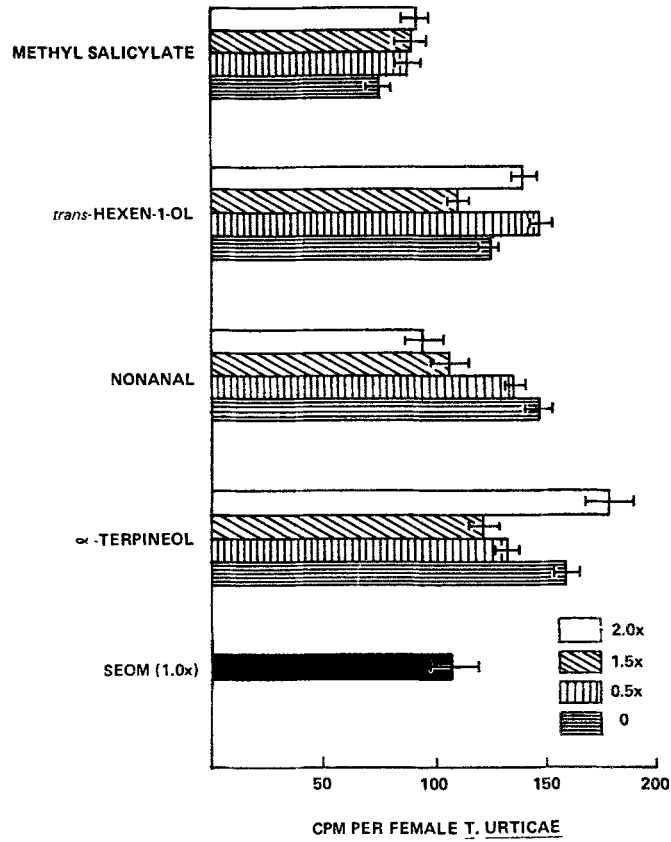

FIG. 2. Comparative ingestion of essential oils mixtures as measured by radioactivity of *T. urticae* females fed for 24 h on 2% sucrose solution containing ^{32}P and the mixtures at 0.01%. Levels of methyl salicylate, *trans*-2-hexen-1-ol, nonanal, and α-terpineol relative to the standard essential oil mixture (SEOM) varied as indicated.

salicylate levels in a SEOM at 0.1%, (not shown in Figure 2) the food intake of the mites was decreased to an amount about one-third of that of the 0.01% mixtures, and no significant differences in feeding occurred as a result of changing the levels of methyl salicylate.]

Feeding response of mites to changes in levels of *trans*-2-hexen-1-ol and α-terpineol were variable (Figure 2). The mixtures containing no *trans*-2-hexen-1-ol and 1.5 × the SEOM resulted in a level of ingestion not significantly different from that of the SEOM. The mixtures containing 0.5 × and 2.0 × the level in the SEOM effected increased ingestion to levels higher than those

on the other mixtures. The mixtures containing no α-terpineol and 2.0 × the level of the SEOM resulted in significantly higher ingestion than the SEOM, while the mixtures containing 0.5 × and 1.5 × the SEOM resulted in ingestion levels close to that of the SEOM (Figure 2).

Increasing the nonanal levels in the mixtures tended to depress ingestion. The mixtures containing no nonyl aldehyde and 0.5 × the SEOM resulted in levels of ingestion significantly higher than those of the SEOM or the mixtures containing 1.5 × and 2.0 × the nonanal of the SEOM.

DISCUSSION

Kemp et al. (1968) found that the total amount of essential oil isolated from strawberry foliage was of the order of magnitude of 0.01% (fresh foliage wt), with the quantity of any single component being much lower. In the choice tube tests reported here, *T. urticae* females were generally attracted to essential oil mixtures presented at 0.01% concentration in propylene glycol. Two exceptions were noted: in the cases where the mixtures contained no methyl salicylate or 0.25 × the amount in the SEOM, the attractive effect was lost; and where the mixtures contained nonanal at 1.5 × or 2.0 × the level of the SEOM, the mites were repelled. The mixtures presented at 0.001% and 0.1% in the methyl salicylate variation series attracted the mites. Concentrations lower than 0.01% were not used in the variation series of nonanal, α-terpineol, or *trans*-2-hexen-1-ol; here again the mixtures at 0.01% were generally attractive except where the level of nonanal was 2.0 × that of the SEOM. In virtually all of the choice tube tests, concentrations of essential oil mixtures at 1.0% or higher manifested repellency. It may be concluded, then, that *T. urticae* reacted favorably to essential oil mixtures at concentrations within a tenfold range above and below the concentration found in "Citation" strawberry foliage essential oil. Within this range, there was the suggestion that methyl salicylate acted as an attractant and that nonanal at levels above that of the standard mixture acted as a repellent.

In the tests where the essential oils in propylene glycol solutions were incorporated individually or as mixtures into the sucrose solution containing ^{32}P, effects on ingestion—whether stimulatory or deterrent—could be due either to taste, odor, or a combination of the two. In the incorporation tests with individual components, feeding effects varied with concentration: at 0.0001%, the three components methyl salicylate, *trans*-2-hexen-1-ol, and nonanal all stimulated feeding, while α-terpineol did not; at 0.001% and 0.01 %, methyl salicylate had no significant effect, and all of the other three compounds individually reduced ingestion to a significant degree.

It is recognized that the exact quantitative relationships between the

composition of the strawberry essential oil and the composition of the volatile compounds present in intact strawberry foliage is not known. (Disruption of the plant tissues in the preparation of the volatile compound probably changed the quantitative relationship.) Nevertheless, of the studies reported here, the tests incorporating the essential oil mixtures at 0.01% in propylene glycol into the food most nearly simulated artificially the conditions that to the best of our knowledge existed in the foliage. In these tests, all the mixtures containing methyl salicylate stimulated feeding over that of the mixture without methyl salicylate, but the amount of this essential oil relative to the SEOM did not strongly affect the feeding level. This result generally agrees with the preference shown for methyl salicylate in the choice tube studies.

In the mixtures containing nonanal at varied levels the amounts of food ingested by the mites were inversely related to the levels of this compound incorporated into the food. The same was true when nonanal alone was incorporated into the food. Also, in the choice tube tests nonanal at levels above the SEOM tended to repel the mites. These results all point to a negative behavioral response of *T. urticae* to increasing levels of nonanal.

No pattern of response of *T. urticae* to α-terpineol and *trans*-2-hexen-1-ol is immediately discernible. Dabrowski and Rodriguez (1971) reporting a study where the essential oils had generally elicited nonpreference reactions in mites, found that *trans*-2-hexen-1-ol was an attractant or repellent, depending on concentration. In the incorporation studies, the higher concentrations of this compound were effective feeding deterrents, but no strong effects were noted that could be ascribed to varying amounts of either of these compounds in the mixtures employed in the incorporation tests or the choice tube tests. It is possible that in mixtures mite responses to other essential oil components override the responses to α-terpineol and *trans*-2-hexen-1-ol. In the study quoted above, Dabrowski and Rodriguez also found that a 0.1% solution of α-terpineol + methyl salicylate significantly attracted mites in choice tube tests although neither compound presented alone did so.

It is known that mite populations suffer a drastic reduction in numbers during the summer months, that part of the growth period which relates to the postharvest season of strawberries (Chaplin et al., 1968; Dabrowski et al., 1971; Poe, 1973; Shanks and Barrett, 1975). What is not fully understood, however, are the causes for these population crashes. The biochemistry of the plant changes, making the plants relatively resistant to mite attack. Plant nitrogen, for instance, has been shown to influence mite fecundity; a positive correlation existed between mite population and absorbed nitrogen (Rodriguez et al., 1970). In this same study, however, we showed that strawberry leaf disks detached and subjected to mite bioassay in July, did not offer attractive feeding stimuli, as determined by the high numbers of mites that perished in the sticky barrier ringing the leaf disks. The question arises as to what

influence the essential oils may have on this resistance. In the present study we have shown that essential oils can evoke an attracting or avoiding response in *T. urticae*; also, they can stimulate or depress feeding, depending on the concentration of the individual essential oil and the make-up of the essential oil milieu. It is thus reasonable to expect seasonal variation in essential oil in foliage to affect mite population development.

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HARDERIAN GLAND PHEROMONE IN THE MONGOLIAN GERBIL *Meriones unguiculatus*[1]

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Abstract—The Mongolian gerbil (*Meriones unguiculatus*) secretes an attractant pheromone from the Harderian gland during a facial groom. The material exits at the external nares and is spread over select areas of the face. Its presence stimulates investigation by conspecifics, as seen by video observations. The half-life of the fluorescence of the material on the face following a groom parallels the half-life of attraction. Shock-avoidance and taste-aversion conditioning indicate that animals can both smell and taste a chloroform extract of Harderian glands. They will also seek out and investigate the extract when presented alone. It is suggested that thermoregulatory grooming, social needs, and chemosignaling are intimately linked.

Key Words—Harderian gland, Mongolian gerbil, *Meriones unguiculatus*, attractant pheromone, thermoregulation, social behavior.

INTRODUCTION

Historically, work on the Harderian gland goes back to 1694 when J. Harder first described the gland in deer. The gland is a bilobular structure wrapped behind the eyeball. Phylogenetically and anatomically the gland is related to the presence of the nictitating membrane in all terrestrial vertebrates. It is especially prominent in rodents.

[1] This research was supported by grant No. MH 14076–08 awarded to Dr. D. D. Thiessen.

The function of the gland has remained an enigma despite decades of exploratory work on its comparative structure and ultrastructure and its innervation. Attention has focused on its pigments, porphyrins. In some species (e.g., many *Cricetidae*) protoporphyrins predominate, making it possible to index its physiological condition by noting the intensity of its fluorescence (UV 360 nm). Currently there are three viable hypotheses of its function: (1) combating bacterial invasions, (2) extraretinal photoreception, and (3) a cushion and lubricant for the eyeball. Some evidence exists to support each of these functions, although no definitive evidence exists for any hypothesis.

In this report we offer evidence that the Harderian secretion acts as a pheromone in social interactions. The Harderian gland of the Mongolian gerbil (*Meriones unguiculatus*) secretes a chloroform-soluble material that evokes investigation by conspecifics (Thiessen et al., 1976).[4] Ordinarily the material is released from the orbital gland down the Harderian–lacrimal tract and out the external nares during a facial groom. The material, which fluoresces bright red under long-wave UV illumination, is mixed with saliva during a groom and spread over heat-exchange areas of the body (e.g., nose, chin, and cheeks). A heat-labile component (perhaps an enzyme) in the saliva dissipates the fluorescence within a few minutes, suggesting that the pheromone has a short half-life.[5] The nature of the cue eliciting conspecific investigation and grooming is unclear. In the experiments to be described here we show that there is an interaction between the act of grooming, social investigation, and the longevity of the facial fluorescence. Evidence is given to indicate that both an olfactory and a gustatory cue could mediate social interactions.

METHODS AND MATERIALS

Our initial video tape observations were done on 20 pairs of adult males in a novel environment. Two days after initial pairing in home cages pairs were transferred to a Plexiglas observation cage and filmed with a Sony Videocorder. Each pair was filmed until one member groomed, and then for an additional 4–6 min. The number of facial investigations by conspecifics was counted during the groom (which averaged about 10 sec) plus 10 sec following the groom, and for every 20 sec thereafter until 70 sec had passed. A "nongroom period" of 20 sec was selected from the tape by editing 10 sec immediately prior to the groom and another 10 sec selected randomly from the tape during the paired encounters. This interval was designated as a control period of investigation.

As Figure 1 indicates, grooming elicited an investigation by the non-

[4] Unpublished observations are available by writing D. Thiessen.

[5] Gerbil saliva obtained by stimulating animals with pilocarpine or human saliva will dissipate the fluorescence in vitro. The reaction is blocked by boiling the saliva.

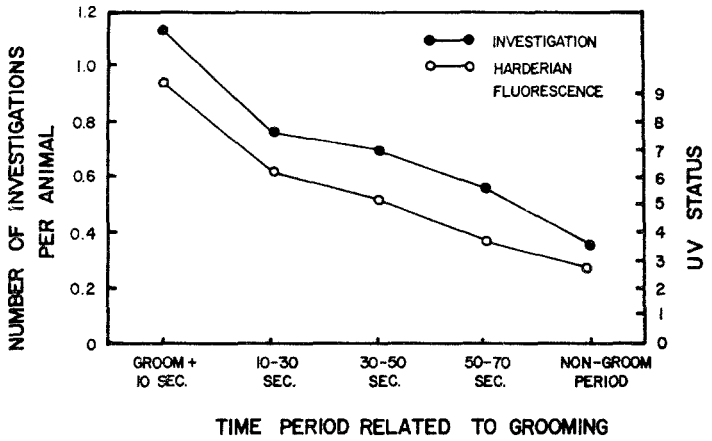

FIG. 1. Relationship of grooming to investigation by conspecifics and the spread of Harderian material on the nose, chin, and cheeks.

grooming animal. The frequency of investigation diminished toward control levels at about 50 to 70 sec. There was an investigation during or immediately following every groom; whereas between 50 and 70 sec the incidence was reduced by one half and during the control period only one third as many occurred. Typically, when one member of a pair facial-groomed, the second member stopped his activity, air-sniffed, and approached the groomer. Proximal investigation followed, involving sniffing and licking around the nose, chin, and cheek areas. As described earlier (Thiessen et al., 1976), grooming by one animal often appeared to stimulate grooming in the other.

Another eight adult animals were observed under UV light for intensity of facial fluorescence. The intensity of fluorescence was judged on a nine-point scale according to a procedure established earlier (Thiessen et al., 1976). Briefly, we rate the intensity of Harderian secretion from 0 to 3 for each of three body areas, the nose, the cheeks, and the chin. Thus an animal may have a secretory status (UV status) ranging from 0, indicating no secretion, to 9, indicating very copious secretion and spread of the material on all three body areas. The animals in this case were allowed to groom and immediately picked up for UV scanning. At other times, selected randomly, the animals were surveyed during nongroom periods. We have determined that fluorescence does not change over time simply because of UV illumination and only disappears when Harderian secretion is mixed with saliva. The change in UV status closely paralleled changes in social investigation, as seen in Figure 1. The pheromone may be the Harderian pigment, protoporphyrin, or its associated compounds, which are quickly broken down by some factor in the

saliva. In any case the presence of facial fluorescence is a correlate of social interaction and suggests the presence of an intraspecific attractant.

Both an olfactory and gustatory cue could be important in groom-induced investigation. In one experiment six adult food-deprived males were trained to bar-press for food reinforcement in a Skinner box on an FR-6 schedule, a reinforcement schedule that enables an animal to obtain a food pellet on every sixth bar-press response. The apparatus and general procedure have been described (Thiessen et al., 1974). After responding had stabilized, nine daily sessions of discrimination training were run in which animals were required to repress responding to the presence of Harderian gland extract odor in the air stream (the CS+ condition). The animals, however, responded freely for food during the presentation of a control odor (the CS-) and in the absence of specific odor stimulation (B condition). A failure to repress in the presence of CS+ resulted in a 1-sec or less 1.2 ma foot shock. Equal numbers of 1-min-long (CS+) and (CS−) stimulus trials were randomized during the 20-min session and were alternated with baseline trials (B). All trials were interspersed by brief time-out periods of approximately 10 sec. Harderian material was prepared by homogenizing glands in chloroform (3 ml per pair of glands), centrifuging, and retaining the supernatant to be used as CS+. It consisted of 0.75 ml of air-dried extract rubbed on a cotton swab and posi-

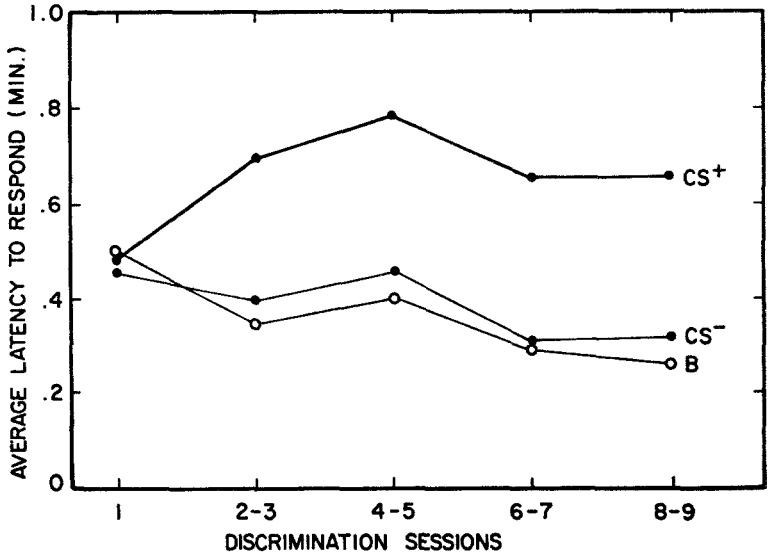

FIG. 2. Latency to lever-press for food to Harderian extract (SC+) in airstream, or during control (CS−) or baseline periods (B), when CS+ is associated with foot shock.

tioned in the air stream entering the apparatus. CS− was prepared in the same manner but without the Harderian material. B trials had nothing placed in the air stream. Latency to first response was recorded during every trial.

Figure 2 illustrates response latencies under the various stimulus conditions. Friedman two-way analysis of variance (Siegal, 1956) indicated a significant difference among conditions ($\chi r^2 = 10.33$, $k = 3$, $N = 6$, $p<0.002$). Additional comparisons showed that CS+ latencies differed from both CS− and B ($t = 6.81$, $p<0.001$; and $t = 6.92$, $p<0.001$) but the CS− and B did not differ ($t = 1.13$). Gerbils are therefore able to detect Harderian material by its odor.

In another experiment adult male gerbils were tested for their ability to taste Harderian material using a taste aversion test similar to those described by Garcia et al. (1974). Essentially, animals ($N = 6$) were food-deprived, habituated to the daily presentation of 20 Noyes food pellets, allowed to consume Harderian-smeared pellets on the conditioning trial, and immediately injected with semitoxic doses of a saturated lithium carbonate solution,

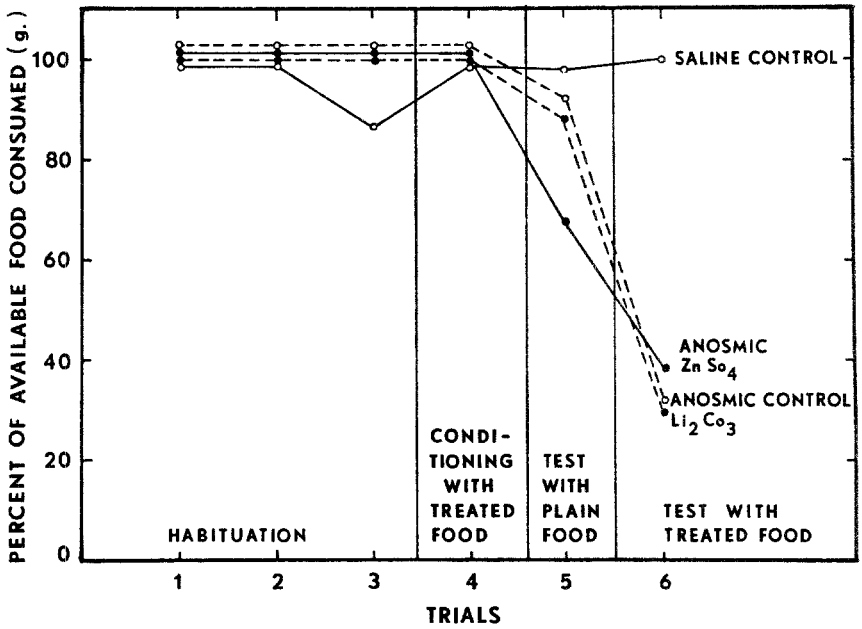

FIG. 3. Food consumed under various habituation and testing conditions. Trials 1–3, last three days of habituation on Noyes pellets; trials 4–5, consumption of Harderian-smeared or plain food prior to differential conditioning; trial 6, consumption of intact or anosmic animals after saline or Li_2CO_3 injections.

Li_2CO_3 (1% body wt I.P.), in order to induce sickness. They were then retested for ingestion of plain and Harderian-smeared food pellets in a counterbalanced order. Control animals ($N = 8$) were handled in a similar way except that they were injected with equal volumes of isotonic saline. Other animals were treated with Li_2CO_3 on the conditioning trial after either being made anosmic by flushing the nasal cavity with a 5% zinc sulfate ($ZnSO_4$) solution (Alberts and Galef, 1971) ($N = 12$) or having been administered an isotonic saline solution as a control ($N = 8$). Tests were spaced one day apart. Testing for taste aversion was conducted by measuring the consumption of both a Harderian treated and nontreated samples of Noyes pellets in two separate tests with a single flavor at a time. Lithium-treated animals were reconditioned if they failed to show an aversion response to Harderian smeared pellets.

The results are shown in Figure 3. Three of the six intact Li_2CO_3 animals showed Harderian aversion after one conditioning trial, two after two trials, and the last after a third trial. No control animal showed an aversion. The Li_2CO_3 and control groups differed significantly in consumption of Harderian treated food ($t = 5.91$, $df = 12$, $p < 0.002$) but not plain food ($t = 0.63$). Because of sickness or death in 4 $ZnSO_4$ animals the Wilcoxin Matched-Pairs Signed-Ranked test was applied to changes in consumption in anosmic and anosmic control animals. Six of the eight remaining $ZnSO_4$ animals showed a specific aversion to Harderian-treated pellets ($T = 4$, $N = 8$, $p = 0.05$). Similarly, all anosmic control animals showed a significant aversion ($T = 0$, $N = 8$, $P = 0.01$). Anosmia was verified following these tests by hiding a sunflower seed under the sawdust of the animal's cage. Although all gerbils would readily eat when presented with sunflower seeds, only two of the eight $ZnSO_4$-treated animals found the seed in 10 min compared with every control subject (Mann-Whitney $U = 1$, $N = 8$ & 8, $p < 0.001$). Apparently animals can react to Harderian material by taste, even when olfactory capabilities are eliminated.

Finally, it was of importance to demonstrate that olfactory cues from Harderian material could direct an animal's investigation even in the absence of another animal. Twelve adult male gerbils were tested singly for displayed interest in Harderian material during a 15-min test in an open field. The Harderian extract, prepared as before, was rubbed on a cotton swab and hidden beneath a treadle in the floor. A second treadle on the opposite side of the box hid a control swab that had been treated in the same way but without Harderian material. The position of the stimulus was randomized between treadles. Each treadle tripped a separate timer, allowing a determination of the animal's time near the Harderian or control swab. Time spent on one or the other side of the box was also recorded. In addition, the number of sniffs directed at each treadle was recorded, as well as the number of full facial

grooms and partial grooms (momentary head swipes) on each side of the box.

Time and groom measures proved statistically unreliable because of large individual differences. The number of sniffs directed toward the treadle covering the Harderian material was significantly more frequent than toward the opposing treadle ($\bar{X}$ $H = 3.42 \pm 0.79$, $\bar{X}$ $C = 1.42 \pm 0.38$, $t = 2.45$, $df = 11$, $p < 0.025$). Apparently olfactory cues can elicit investigation independent of the presence of an animal and without gustatory cues.

RESULTS AND DISCUSSION

Coupled with earlier observations (Thiessen et al., 1976), these data indicate that grooming is an important aspect of social interactions and is responsible for the spread of an attractant pheromone from the Harderian glands. Olfactory cues stimulate a conspecific to investigate and lick the areas of the face where the Harderian material is spread. Recent work in our laboratory appears to suggest that grooming is not accompanied by auditory or ultrasonic emissions, and that animals cannot detect Harderian material visually. The significance of this unique system of communication remains unknown, although we know from other data that dominant males more actively spread the pheromone than subordinates, and that Harderianectomized males are at a social disadvantage (Thiessen et al., 1976). Social interactions increase body temperature and stimulate thermoregulatory grooming and hence signaling (unpublished observation), suggesting a tight relationship between metabolic requirements, behavioral drives, and chemosignaling. We have seen a similar relationship between grooming and the spread of Harderian material in *Mystromys albicaudatus*, *Mesocricetus auratus*, *Meriones tristrami*, *M. libycus*, and *M. shawi*. Other species certainly have Harderian glands and no doubt spread the material with grooming, but in many species the Harderian secretion lacks the fluorescent pigment protoporphyrin and cannot be detected with UV light. Nevertheless, the extensive use of grooming in numerous species suggests that grooming pheromones may be extremely important in the regulation of social organizations.

The observations, in addition, suggest a physiological interpretation of displacement grooming found in conflict situations. Behavioral arousal, as it occurs during conflict, perhaps increases body temperature and stimulates thermoregulatory grooming and evaporative cooling. Harderian secretion released simultaneously provides a pheromonal signal of arousal. Conspecifics could then use the signal to identify the sender and assess his physiological state. It appears that social interactions, physiological arousal, thermoregulatory responses, and olfactory communication may have evolved as an integrated system.

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SPECIES-CHARACTERISTIC RESPONSES TO CATNIP BY UNDOMESTICATED FELIDS[1]

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Abstract—Thirty-three large felids belonging to six different species were exposed to catnip and catnip extract. The species-characteristic response to catnip and the sensitivity of the response to various concentrations of catnip were examined. Putative relationships between catnip sensitivity, species range, age, estrous cycle, and behavioral complexity are discussed. The behavioral response to catnip shown by the domestic cat is seen in several different large felids. Lions and jaguars were extremely sensitive to catnip compared to tigers, cougars, and bobcats, who gave little or no response. Both males and females of the same species tested alike. Reproductive-age adults were more sensitive than either aged or immature animals. It was quantitatively demonstrated that catnip responsiveness is not limited to the domestic cat, that it is not limited to the female, and that it varies dramatically between species and age of felids.

Key Words—catnip, olfaction, behavior, threshold response, felines, sensitivity.

INTRODUCTION

The first record of the domestic cat's peculiar response to catnip (*Nepeta cataria*) is lost in time. It is well known, however, that catnip stimulates an "innate releasing mechanism" (IRM) in domestic cats that elicits a predictably

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"playful" behavior pattern apparently independent of experience and learning (Ewer, 1973 and Leyhausen, 1975). *Cis, trans*-nepetalactone (Waller *et al.*, 1969; McElvain et al., 1942; McElvain and Eisenbraun, 1955) is reported to produce this behavioral response and to be metabolized by cats. Other reports assign activity to *trans, cis* nepetalactone (Bates and Siegel, 1963). It has been verified by Todd (1963) that the response to catnip is mediated by olfactory and not gustatory stimuli. Catnip sensitivity has been related to a dominant autosomal gene and to the estrous cycle by Todd (1962). These reports indicated that catnip elicits certain behavioral responses in some of the domesticated as well as undomesticated cats, but these responses have not been shown to be species specific, typical of, or limited to the female sex. In addition, it has been suggested (Todd, 1963) that catnip mimics a pheromone found in cat urine; however, evidence supporting this theory is scant.

An unpublished attempt to relate the catnip response to different felids (Todd, 1963) utilized catnip leaves and recorded stereotyped behavioral responses. There are shortcomings in this approach. Qualitative descriptions of behavior are often inadequate criteria by which to assess sensitivity to catnip.

The present experiments were designed to investigate the qualitative and quantitative aspects of catnip sensitivity by testing undomesticated felids with respect to control and experimental (catnip-containing) objects. In addition, catnip extracts sprayed onto targets objects were employed in order to carefully control the amount of stimulating material and study felid sensitivity to reduced amounts of material.

The types of behavior which occur as positive responses to catnip were first identified and described. Two experiments were then performed to determine species differences in the response to catnip. Finally a procedure for demonstrating sensitivity thresholds was developed.

EXPERIMENT 1: THE RESPONSE OF FELIDS TO CATNIP-FILLED BOXES

Animals were presented with stimuli attached to the outside of their cage in a way that allowed them to approach the experimental and control stimuli but eliminated touching or tasting the sample.

Methods and Materials

The behavioral response and sensitivity to catnip of the undomesticated felids at the Knoxville Zoological Park was examined in thirty-three cats of various ages maintained in cages (Table 1). All experimental animals had been born and raised in captivity. The cats were tested during the months of March

TABLE 1. LIST OF FELIDAE[a] INCLUDED IN THE SURVEILLANCE FOR CATNIP SENSITIVITY[b]

Scientific name	Common name	Male number (Age in months)	Female number (Age in months)
Lynx rufus	Bobcat	1 (Adult)	1 (Adult)
Panthera leo	Young lion	2 (10)	3 (10)
Panthera leo	Lion	2 (36, 50)	
Panthera leo	Lioness		5 (24–36)
Panthera leo	Lion cub	1 (6)	
Panthera onca	Jaguar	1 (39)	2 (39, 41)
Panthera pardus	Spotted leopard	1 (32)	1 (27)
Panthera pardus	Spotted leopard cubs	1 (8)	1 (6)
Panthera pardus	Spotted leopard	1 (120–180)	1 (120–180)
Panthera pardus	Black spotted leopard	1 (21)	1 (26)
Panthera tigris	Tiger	1 (62)	1 (55)
Panthera tigris	Tiger		2 (26, 27)
Panthera tigris	Tiger cub		1 (6)
Puma concolor	Cougar	1 (24)	1 (11)
Puma concolor	Cougar	1 (25)	1 (25)

[a] The term "felid" will be used routinely in this study in order to include all living *Felidae* (Ewer, 1973) including the three subfamilies *Pantherinae* (the so-called greater cats: lion, tiger, leopard, jaguar, etc.), *Acynonychinae* (the cheetas), and the Felinaes (the so-called lesser cats: lynx, cougar, bobcats, domestic cats, etc.).

[b] Ages are expressed where known and approximated by ranges in other cases. Animals are listed on the basis of enclosure.

through June early in the morning and after closing hours to avoid visitor distractions. Attempts to involve two adult cheetas in the survey were unproductive, since they never approached catnip or control objects.

Two Plexiglass panels were constructed approximately 28 cm square by 1 cm thick. Four holes (5 cm diameter) were drilled through each piece of plexiglass. The holes of the experimental panel were filled with dried catnip leaves (Hartz Mountain) and the control panel was left empty. Cardboard was taped to each side to contain the catnip. Both the control and experimental panels were then wrapped in black electrical tape, and small slits were cut in the tape and cardboard so that the catnip scent could easily be released. Metal hasps were attached with wire to each end of the panel so it could be attached to the cage.

A behavioral check-list was devised for characterizing the responses of the cats to the experimental versus the control panels. The list was initially based on observations made on the domestic cat (Todd, 1962; Palen and Goddard, 1966; Hatch, 1972) and was altered as necessary during the first exposure of

TABLE 2. BEHAVIORAL RESPONSE TO CATNIP[a]

A. Behavioural responses to catnip-containing boxes

	1. Biting box	2. Biting cage	3. Licking box	4. Pawing	5. Reaching through cage	6. Both paws on cage	7. Scratching floor in front of box	8. Sniffing	9. Rubbing body to box	10. Pacing	11. Lying/sitting	12. Rolling over	13. Total specific responses
Female lions (G. L.)	B	B	+	+			+	+	+		+		6
Female lions (J)	+		B	B	+			+					3
Female jaguars	B	+	B	+	+	+		+	+	B	+	+	8
Lion (J)	B		B	B	+		+	+					3
Jaguar (S)			+	+		+		+	+	+			6
Young lions	B	B		B				+			+	+	3
Spotted leopards	B	B		B	B	+		+				+	3
Male tiger								+					1
Spotted leopard cubs	B	B	B				+	+				+	3
Black spotted leopards	B	B	B				+	+				+	3
Female tiger	B	B			+			+					1
Spotted leopards (old)								+					1
Female cougar	B	B	B	+	B								1
Tigers (field)			+					+					2
Lion cub (male)	+	+	+					+			+	+	6
Tiger cub (female)	B	B	B					+					1
Cougars (paired)								+					1
Male cougar	B	B	B		+			+			+		3
Bobcats (paired)			+					B					1

B. Behavioral response to rocks sprayed with catnip

	1. Biting	2. Carrying in mouth	3. Licking	4. Holding between paws	5. Carrying in paws	6. Pouncing on	7. Rubbing face on rock	8. Kicking and pursuing	9. Play (one cat jumping the other, who rolls over)	10. Lying/sitting beside	11. Rolling over	12. Total specific responses
Female lions (G.L.)	+	+	+	+	+	+		+	+	+	+	10
Female lions (J)	+	+	+	+	+			+	+	+	+	9
Female jaguars	+		B	+	+	+	+	B		+	+	7
Lion (J)			+	+	+			+		+		5
Jaguar (S)	+		B	+	+					+		4
Young lions			B	+		+			+		+	4
Spotted leopards	+		+	+				B			+	4
Male tiger			+	+						+		3
Spotted leopard cubs			B			+	+	B				2
Black spotted leopards			B			+	+	B				2
Female tiger												
Spotted leopards (old)			B					+				1
Female cougar			+					B				1
Tigers (field)			B									
Lion cub (male)			+					+				1
Tiger cub (female)			+									1
Cougars (paired)												
Male cougar												
Bobcats (paired)												

[a] The length of the test period was 10 min. Behavior observed to the experimental box only (+). Behavior observed to both experimental and control (B). Both experimental and control were presented simultaneously.

each animal to catnip. This first test consisted of a 15-min exposure to the experimental box alone. Any new type of behavior was recorded and added to the check-list. Several days later each animal was presented with both control and experimental panels simultaneously. The panels were attached to the outside of the cages approximately 45 cm above ground level and about 70 cm apart. The test period was reduced to 10 min since most animals either lost interest in the first 10 min or remained interested for the full 15 min.

Types of behavior toward control and experimental panels were recorded on check-lists for individual animals. The total time with each panel was recorded. When more than one animal was present in a cage, the response of the first animal to be attracted to either panel was recorded.

Results

Table 2 lists the behaviors that were recorded during the test periods for each species. Certain types of behavior can be seen to occur in response to both panels in almost every animal. Thus, these types of behavior were not specifically elicited by catnip. Other types of behavior occur almost exclusively in response to the experimental panel but are seen on occasion in response to the control panel (Table 2).

It is noticeable that some animals perform several different types of behavior in response to the experimental stimulus, while other animals respond with only one or two types (Table 2: A6–A12 and B4–B7 and B9–B11). Animals can be ranked according to total number of different behavior patterns performed in response to the experimental treatments (Table 2: A13 and B12).

A relative index was calculated weighing the difference between experimental and control conditions by normalizing scores in terms of the time spent elsewhere (Table 3, E). This relative index rates the animals with respect to the length of their behavioral response to catnip. The relative index is defined as equal to [% (time at catnip minus times at control)/% elsewhere] × 100. The percentage of time spent elsewhere was limited to a minimum of 0.1%; thus, the range for the relative index becomes 0–999. This relative index can distinguish between an increased general arousal and an attraction to catnip, since it normalizes in terms of time spent at an activity other than time spent at the catnip-containing panels. It is clear that certain felids (i.e., lions and jaguars) are attracted more easily than others to catnip-containing panels (Table 3, A and B), as shown by the time spent during exposure to catnip versus control boxes.

The two adult male lions (housed with different prides) gave variable responses. The oldest lion (G.L.) scored low (Table 3) on several occasions. This older lion was housed in a very large enclosure, which may have encour-

TABLE 3. DURATION OF RESPONSE TO CATNIP AND CONTROL PANEL[a]

	A	B	C	Δ	E
	Catnip time (sec)	Control time (sec)	Time elsewhere (sec)	Delta	Relative index
Positive responders					
Female lions (J, 2)	540	60	0	80.0	800
Female jaguars (2)	538	62	0	79.4	794
Female lions (G.L., 3)	355	21	124	55.7	269
Male jaguar (S)	310	174	116	22.7	117.6
Partial responders					
Spotted leopards (paired)	251	11	338	40.0	71.1
Young lions (2 male, 3 female)	182	25	393	21.6	39.8
Spotted leopard cubs (paired)	128	40	432	20.6	28.6
Lion cub (male)	143	73	384	11.6	18.1
Nonresponders					
Spotted leopards (old pair)	28	12	560	2.7	2.9
Tigers (field, 2)	95	86	419	1.5	2.2
Female tiger	4	0	596	0.7	0.7
Male tiger	2	0	598	0.3	0.3
Bobcats (paired)	2	2	596	0	—
Cougars (paired)	0	0	600	0	−0.2
Male cougar	37	37	524	−0.3	−0.3
Black spotted leopards (paired)	161	169	270	−1.4	−1.4
Female tiger cub	42	81	477	−6.5	−6.5
Male lion (G.L.)	140	210	250	−11.7	−28.1
Female cougar	155	325	120	−28.4	−142.0

[a] Each test lasted 600 sec. Delta = (time at catnip–time control)% The percentage of time spent elsewhere is limited to a minimum of 0.1%; thus, the range of the relative index is 0 to 999. Relative index = (delta/% elsewhere) × 100. Animals are listed on the basis of the decreasing relative index scores in column E.

aged his indifference to events on the exterior. In checking zoo records, however, it was also found that he had a recent history of illness and medication which might also explain his lack of responsiveness during these tests. The young male lion (J) scored high on the first tests (Table 2 and 4) but was frequently distracted by his pride during other tests.

EXPERIMENT 2: THE FELID RESPONSE TO CATNIP EXTRACT

Since catnip leaves mounted on the outside of enclosures may generate

behaviors different from the unrestrained situation, experiments were included in which the felids were permitted access to experimental and control objects. It was necessary to use a catnip extract applied to indestructible objects since all attempts to build a sturdy catnip recaptacle proved futile.

Tests were performed in which cats were permitted access to experimental and control objects placed inside their enclosure and their behavior was recorded.

Methods and Materials

"Catnip Extract" (Hartz Mountain), an aerosol spray made from filtered catnip leaves, was used in order to allow sprayed objects to be placed inside the cage with the animals. Spraying was conducted in the same manner each time by "brushing" the aerosol on the object (moving can at 15–20 in. distance) until an opaque film covered the surface.

River rocks were selected, sprayed, and placed in the cages. The rocks were generally prolate (8 cm × 10 cm) with a thickness of about 2 or 3 cm.

As in Experiment 1, each animal received an initial 15-min exposure to only the catnip-sprayed rock. Types of behavior were recorded on separate check-lists. Several days later each animal was given a 10-min simultaneous test period with a catnip rock and control rock placed inside the cage approximately 3 ft apart. Data similar to that of Experiment 1 were recorded. A detailed determination of half-life of the volatile attractant was not made, however, for lions the catnip rock was still identifiable 7 days later.

Results

Table 2 (B) shows the types of behavior recorded and how each animal responded when it had access to a catnip-sprayed rock.

A variety of behaviors were elicited in addition to behavior that involves rolling. Subsequent actions by the animal were directed toward maintaining contact with the catnip-sprayed rock. Such action paralleled the results of Experiment 1 with the animal positioning itself and its activity near the catnip-containing receptacles. While sniffing and licking behavior were demonstrated towards both fixed catnip panels and catnip-sprayed rocks, biting was only occasionally demonstrated toward the rocks. Biting actions may represent attempts to gain access to foreign objects on the exterior. Aggressive behavior was rarely observed and when displayed was of extremely short duration. A lack of biting/chewing and aggression was reported by Palen and Goddard (1966) for the domestic cat. The total time spent with each rock as well as the relative index, are recorded in Table 4.

The animals were ranked on the basis of their attraction to the catnip

TABLE 4. DURATION OF RESPONSE TO ROCKS SPRAYED WITH CATNIP EXTRACT[a]

	A	B	C	Δ	E
	Catnip time (sec)	Control time (sec)	Time elsewhere (sec)	Delta	Relative index
Positive responders					
Female jaguars (2)	550	50	0	83.1	831
Male jaguar (S)	420	2	168	69.7	248.9
Female lions (G.L., 3)	290	0	310	48.3	93.4
Partial responders					
Male lion (J)	245	4	351	40.1	68.6
Young lions (2 male, 3 female)	197	10	393	31.1	47.5
Black spotted leopards (paired)	170	20	350	25.0	42.9
Spotted leopards (paired)	130	30	440	21.2	28.9
Lion cub (male)	116	49	435	11.1	15.3
Male tiger	65	0	535	10.8	12.1
Nonresponders					
Spotted leopard cubs (paired)	56	3	541	8.8	9.8
Female cougar	72	36	492	6.0	7.3
Tiger cub (female)	52	43	505	1.5	1.8
Spotted leopards (old paired)	15	10	575	0.8	0.8
Female tiger	3	0	597	0.5	0.5
Cougars (paired)	0	0	600	0	0
Male cougar	0	0	600	0	0
Bobcats (paired)	0	0	600	0	0
Tigers (field, 2)	5	16	519	−1.9	−2.2

[a] Each test period lasted 500 sec. Terminology and rank ordering are as described in Table 3.

rock versus control as expressed in the relative index (column E). When the animals are divided into arbitrary categories as responders (E = 80 or above), partial responders (E = 10 or above), and nonresponder, it is apparent that most of the individual animals fall into the same category as in Experiment 1, Table 3.

EXPERIMENT 3: SENSITIVITY TO CATNIP

Two sets of behavior for the animals and two relative indices have been developed to rank felid response to catnip. The frequency and intensity of behavioral responses to catnip, which have already been described, may not

necessarily reflect the olfactory threshold or sensitivity to catnip. The final experiment was designed to distinguish the level of olfactory "sensitivity."

Methods and Materials

Six identical metal rods were used (25.5 mm diameter). These objects were selected because they were indestructible, their surface area could be easily calculated, and thus the area sprayed with catnip extract could easily be varied. Masking tape was used to cover the parts of the rod not being sprayed.

Four rods were sprayed with catnip extract to give different total areas of experimental stimuli. The remaining two rods were used as controls. One-half of the first rod was sprayed with catnip extract to give an exposed surface area of approximately 20 cm^2. Other rods had sprayed surface areas of approximately 10, 4, and 0.25 cm^2 each. All animals were exposed to the three larger doses. The fourth dose (rod area = 0.25 cm^2) was only used with the adult African lions.

The sensitivity experiment was performed in the evenings after the zoo closed. The animals were distracted by the calls of a keeper while the rods were placed in the cage at the opposite end. One experimental rod with a small identifying mark and two control rods were used for each test. The rods were placed side by side on the floor of the cage approximately 3 ft apart. Four data records were made: (1) length of time to find the catnip rod, (2) length of time first attraction to the catnip rod lasted, (3) total time at the catnip rod, (4) total time at either control rod. The test period was again 10 min. All the animals were first tested with dose 1. Several days later they were tested by dose 3 and then by dose 2. All animals were tested with one dose before any were tested with the next dose.

Results

There is a general decrease in response to decreasing doses of catnip with the exception of the lionesses in the G.L. pride (Figure 1). Lions consistently exhibited sensitivity to catnip greater than any other species tested even at a dosage that was 16 times lower (0.25 cm^2 area) than the next lower dose (4.0 cm^2 area) responded to by any other species (Figure 1).

With regard to time spent at the catnip, the relative index was selected as the most valid means of ranking the animals, since it distinguishes animals that demonstrate attraction to catnip from animals with a high general activity level, which may give spuriously high attraction to catnip. The lists in Tables 3 and 4 were ranked according to the relative index. For these rankings, the animals were divided into three arbitrary groups. A relative index of 80 or greater was taken as a clear positive response, while less than 10 was considered

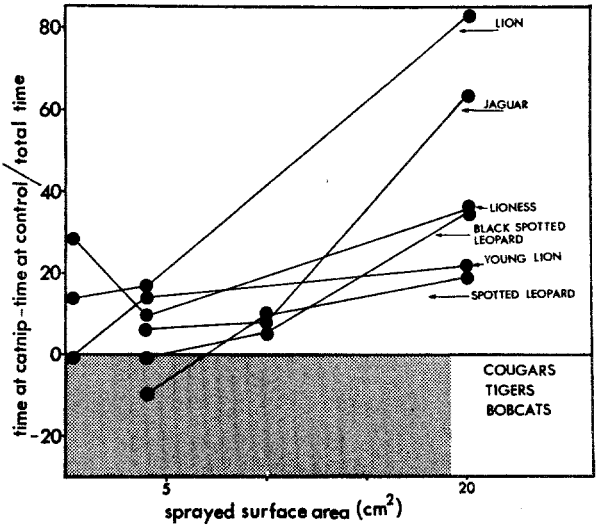

FIG. 1. Sensitivity to decreasing doses of catnip. The length of each test period was 600 sec. Values are representative of single animals. The shaded area corresponds to negative responses. Each individual animal of a species in the shaded area tested negatively on all three doses (5, 10, and 20 cm^2).

equivalent to no response. It can be seen that most animals are in the same group in both tables. Sensitivity and performance can be ranked visually in Figure 1. Thus, five different methods of ranking have scored the species response to catnip with the result that only a small variation in hierarchy occurs.

The lions (except for a cub, Little Josh) and the jaguars were the most sensitive (Figure 1). The adult spotted leopards, the lion cubs, the spotted leopard cubs, and the black spotted leopards comprised a "borderline" response group. The tigers, cougars, bobcats, and old spotted leopards were all at or below zero.

In Figure 1 it appears that the adult lions (male and female, but not young lions) are sensitive to a dose of catnip (0.25 cm^2 surface area) 16 times lower than that responded to by any other species. Since lions were used as a reference for dose effectiveness, every catnip bar dose was first checked against the lions. Other animals demonstrated a sensitivity to a sprayed surface area of 4 cm^2, which was lower than the sensitivity of lions and jaguars.

The dose sensitivity of the young lions, along with the fact that the lion cub consistently responded less positively than the aged lions, could perhaps be correlated with reproductive maturation. Young lions experienced a decreased response at a higher catnip concentration than adult lions.

Age might also account for differences in sensitivity in the spotted leopard (Table 4). Leopard cubs responded less positively than the adults, who were more positive than very old adult leopards. Studies on young adults castrated at an early age might be able to distinguish whether or not catnip sensitivity is related to sexual maturity at the neural or hormonal level. Castration after sexual maturity did not block the response to catnip in domesticated cats (Palen and Goddard, 1966).

GENERAL DISCUSSION

Responsiveness to catnip in undomesticated felids involves sets of behavior in such a way that the intensity of the behavioral response is only a general indicator of sensitivity. Adult lions and jaguars routinely responded positively to catnip, with the adult lions (male and female) showing the greatest sensitivity to low doses. Tigers, cougars, and bobcats routinely gave very low to zero response to catnip. Therefore the olfactory attractant in catnip is differentially effective and species specific in large fields.

Palen and Goddard (1966) observed that in the domestic cat "rolling over" behavior was associated with the response to catnip as well as with behavior during hormonally induced estrus. In the present experiments the male jaguar that was found sensitive to catnip never demonstrated a copulatory "after reaction" such as the routine "rolling over" that is seen with the lions (Pavlik, unpublished observations). Positive responses to catnip were observed in naive, reproductively immature animals (young lions, lionesses, and black-spotted leopard) as well as in pregnant lionesses and in male lions. "Rolling over" behavior occurred in many undomesticated felids that were either positive responders or nonresponders to catnip. It was even observed in response to the control boxes (Table 2, black leopard). A correlation was not found between "rolling over" behavior to catnip and "rolling over" behavior by large felids in estrus.

The results indicate that no one specific behavior of felids can be considered the response to catnip. Most prominent in the set of behavioral events elicited by catnip were sniffing, licking, sustained contact, and often play (which is defined here as the interaction between individuals demonstrating such behaviors as biting, pawing, jumping and rolling over). The four types of catnip responses (I. sniffing, II. licking and chewing with head shaking, III. chin and cheek rubbing, IV. head-over rolls and body rubbing) described by Todd (1963) were also evident in these studies; however, the "head-over roll" was not often observed when the animal had access to the sprayed objects. The head-tuck behavior by jaguars reported by Todd (1963) was not observed in these studies. In addition, the catnip response in large felids could be seen

to continue almost indefinitely (once as long as 60 min.) without an observable period of decay. In general, an animal could lose interest in the object but would return within 1 or 2 min. In contrast, the domestic cat exhibits a response which rarely exceeds 15 min in duration, and it is accompanied by a 1-hr refractory period (Todd, 1963).

With the exception of a single male lion, G.L. (a nonresponder, Table 3) no sexual differences within a species were found. No differences were noted in animals caged alone compared with those caged in pairs or groups; however, this comparison is difficult to make since there were some species in which no members were caged individually. Todd (1963) reported that a tiger cub actively avoided the catnip and actually ran away from it. No such response was found in our observations. While the tiger cub in this study did not respond positively to catnip, he did not avoid it either.

The fact that some species would not encounter *Nepeta cataria* (Youngken, 1950; Slife, 1960) in their natural habitat might explain the species differences in response. Lions and jaguars would not ordinarily encounter catnip (which is endemic to North America and Europe, but not to the southern hemispheres) and yet they responded positively to it. Bobcats and cougars showed no response even though they would encounter catnip in their natural habitat. Within the limits of our survey, species sympatric with *Nepeta cataria* do not respond, while allopatric species do respond or partially respond (leopards) to catnip. These observations may not have any evolutionary significance; alternatively, sensory adaptation may have displaced this response. Such adaptation may occur in the domestic cat, where the response rarely exceeds 15 min in duration and is accompanied by a refractory period (Todd, 1963). This idea is in contrast to the hypothesis that *no* relationship exists between the distribution of *Nepeta* and catnip sensitivity (Todd, 1963).

Todd (1962) attributed the predisposition to respond to catnip to a dominant autosomal gene occurring in 65% of domestic cats. He classified subjects as responders or nonresponders according to whether they displayed "rolling-over" behavior. Our observations indicate that the response to catnip is comprised of sets of behavior. In species other than the domestic cat, "rolling-over" behavior may be an insufficient criteria for determining responsiveness to catnip since it ignores other frequently elicited behaviors as well as aspects of the response that are described in this paper by dose sensitivity. Since diverse complex responses describe sensitivity to catnip, it is likely that any complete response is polygenic and relies on genetic specificity determining type and number of sensory receptors, sensory afferent communication, central integration, and efferent response pathways.

Recently, Cambell et al. (1969) have shown that catnip can change the pattern of single-unit discharge in the hypothalamic ventromedial nucleus.

The catnip response appears to be linked to peripheral nicotinic mediation with central muscarinic and seratonergic facilitation complete with a prominent voluntary component. Inhibition seems to involve central muscarinic and nicotinic mechanisms (Hatch, 1972). Thus, behavioral responsiveness to catnip requires that each participating process be completely functional. Age-dependent catnip sensitivity may originate from one or several undeveloped participating processes. Todd's behavioral genotype may in reality be a phenomenon comprising multiple integrated genes. Any one gene mutation might ultimately interrupt the total response by blocking a single essential component and thus suggest the operation of a single dominant gene.

The present study confirms Todd's observations (Todd, 1963) that responsiveness to catnip is not sexually dimorphic, and that felids can be categorized as "responders," "partial responders," and "nonresponders." In addition we have observed distinct species variation occurring with respect to the catnip behavioral response and olfactory sensitivity to it.

Finally, the catnip response is always *observationally* clear-cut. Our efforts to quantify this response in order to understand the relationships between behavioral intensity and olfactory threshold do not entirely reflect a numerical distinctiveness, and because of the limited numbers of each species they cannot have a statistical interpretation. These efforts should be regarded as descriptions of the extent of responses by the individual and as precedents by which animals can be grouped and tested in subsequent experiments involving replication. Lastly, this paper illustrates how zoo animals can be manipulated in the study of behavior.

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THE CHEMICAL BASIS OF ATTRACTION OF ITHOMIINE BUTTERFLIES TO PLANTS CONTAINING PYRROLIZIDINE ALKALOIDS

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Abstract—The plant *Heliotropium indicum* L. (Boraginaceae) contains pyrrolizidine alkaloids. When dried, it is a powerful attractant for male ithomiine and danaine butterflies, which congregate and feed at its dead shoots. The butterflies use alkaloids derived from the plants for the formation of chemicals with pheromone/allomone activity. Baiting with alkaloids and "esterifying acids," which form a part of the alkaloid molecules, indicates that a volatile product derived from the esterifying acids attracts males to the plants, where intact alkaloids then act as phagostimulants.

Key Words—pheromones, Lepidoptera, pyrrolizidines, Ithomiinae, Danainae, alkaloids.

INTRODUCTION

Various Angiosperm genera concentrated in the families Boraginaceae, Compositae, and Leguminosae produce 1,2-dehydropyrrolizidine alkaloids, compounds made of an amino–alcohol moiety esterified by a unique group of acids (Bull et al. 1968). In this report, the acids will be referred to as "esterifying acids." When dead and withered, many alkaloid-containing plants attract large numbers of Lepidoptera, which congregate on the dead shoots to feed (Fig. 1A) (Beebe 1955, Pliske 1975*a*). Among the butterflies, nearly all visitors are males belonging to the closely related nymphalid subfamilies Ithomiinae and Danainae. We have captured over 80 species in

South and Central America, and of over 3,000 individuals captured, 97% are males. In addition, the plants have attracted over 300 species of moths, mainly in the families Ctenuchidae and Arctiidae (Pliske 1975*a*).

Male Danainae have extrudable, brushlike "hairpencil" glands at the tip of the abdomen, which secrete and disseminate aphrodisiac pheromones during the courtship flight (Brower et al. 1965, Pliske and Eisner 1969, Pliske and Salpeter, 1971). In nearly all species so far analyzed, the hairpencils contain pyrrolizidine-derived compounds, dihydropyrrolizines, as a major component (Meinwald et al. 1966, 1969, 1971, 1974; Edgar et al. 1971, 1973; Edgar and Culvenor 1975). Furthermore, the works of Pliske and Eisner (1969), Edgar et al. (1973), Edgar and Culvenor (1975), and Schneider et al. (1975) give evidence that danaid males require an exogenous source of 1,2-dehydropyrrolizidines to produce dihydropyrrolizines. Males of several ithomiine species apparently use certain esterifying acids of 1,2-dehydropyrrolizidines to produce a sexual secretion that is disseminated from hairpencils on the costal margins of the hindwings (Fig. 1B) (Edgar et al. 1976). In many of the species studied, the major component of the secretion is the same, a lactone. The lactone has been shown to be a powerful repellent for males of species producing the compound, probably serving as a territorial marker and as a means of male-recognition in the termination of male–male homosexual courtship pursuits (Pliske 1975*b*, Edgar et al. 1976). Acting both intraspecifically and interspecifically, the lactone thus exhibits properties of both a pheromone and an allomone with respect to its effects on recipient male butterflies.

This report deals with the chemical basis of attraction and feeding at dead shoots of *Heliotropium indicum* L. (Boraginaceae), a common roadside weed throughout lowland tropical America and a powerful attractant for male ithomiines and danaines, as well as other Lepidoptera.

STUDY SITE, METHODS, AND MATERIALS

We carried out our observations at the Estación Rancho Grande, Estado Aragua, Venezuela, during June and July of 1974 and 1975. We observed butterflies' responses both to *H. indicum* baits and to samples of purified alkaloids and their derivatives. These included the alkaloids indicine (Fig. 2, structure I), the major alkaloid of *H. indicum*; heliotrine (III), a common alkaloid of other *Heliotropium* species; and two common esterifying acids of pyrrolizidine alkaloids, (+)-trachelanthic acid (II) and (−)-viridifloric acid (IV). The latter we considered to be likely precursors to the substance found in the costal fringes of male Ithomiinae (Edgar et al. 1976). Unfortu-

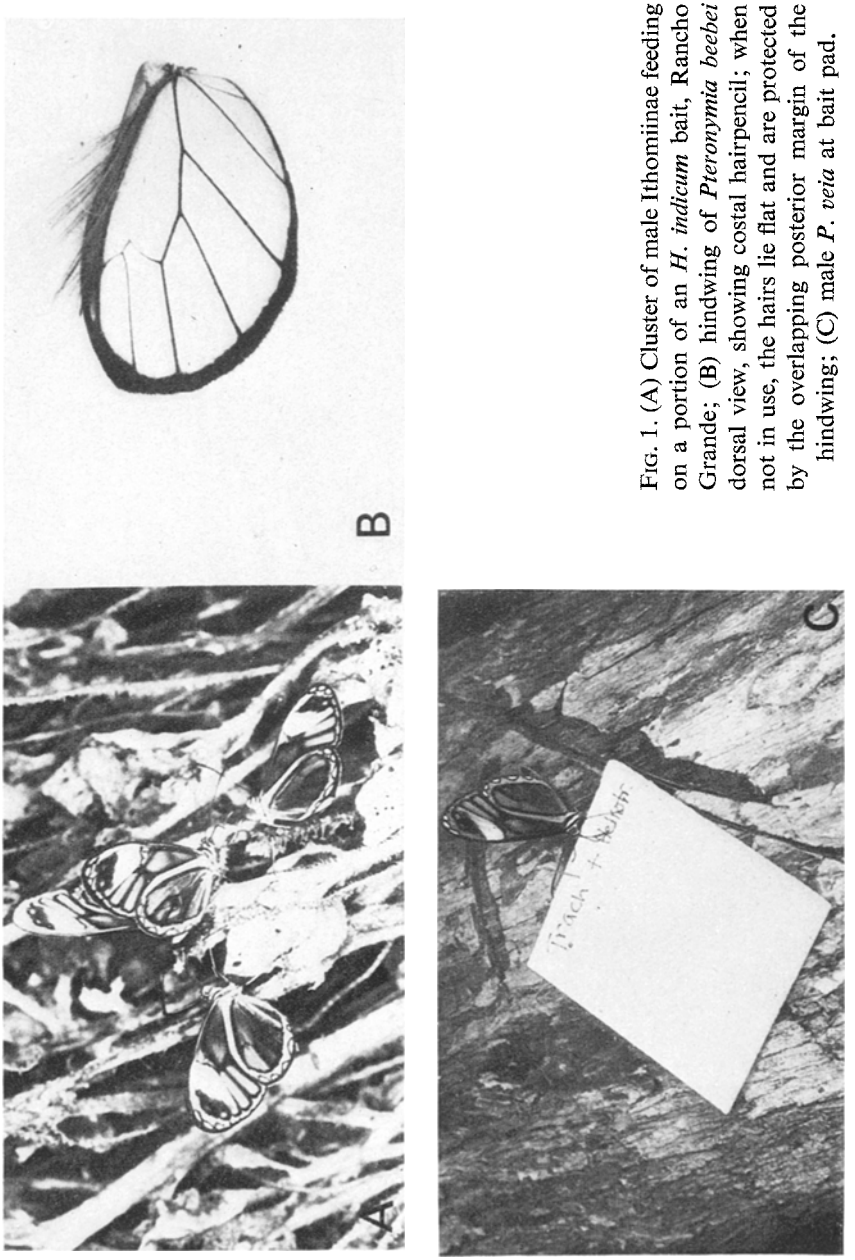

FIG. 1. (A) Cluster of male Ithomiinae feeding on a portion of an *H. indicum* bait, Rancho Grande; (B) hindwing of *Pteronymia beebei* dorsal view, showing costal hairpencil; when not in use, the hairs lie flat and are protected by the overlapping posterior margin of the hindwing; (C) male *P. veia* at bait pad.

(*Facing p.* 256)

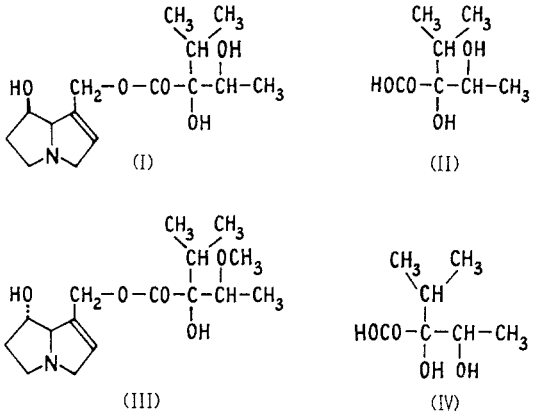

FIG. 2. Structural formulas of compounds used in baiting: (I) indicine; (II) (+)-trachelanthic acid; (III) heliotrine; (IV) (−)-viridifloric acid.

nately, (−)-trachelanthic acid, the esterifying acid of indicine, and heliotric acid, the acid of heliotrine, were unavailable at the time of the field bioassays.

Two lots of esterifying acid were bioassayed at Rancho Grande. The first lot was prepared as pure crystals in 1973 and tested during the summer of 1974. At the time of testing, both trachelanthic and viridifloric acids had pronounced floral odors. Suspecting that a decomposition product was present, we prepared fresh samples of both compounds in the spring of 1975, sealed in ampuls and sent to the senior author just prior to field testing. When these were prepared for bioassays, they were odorless.

We prepared *H. indicum* baits for testing by uprooting whole plants, bundling several stalks together, and thoroughly crushing the leaves and stems. After storing the bundles outdoors overnight, we then suspended them along trails in the surrounding cloud forest (elevation 1,100 m) at chest height. We prepared chemical baits by dissolving approximately 50 mg of each compound in 2 ml ethanol and then impregnating 5 × 5-cm. white blotter pads with the entire solution. We pinned the dry pads to branches along forest trails, making sure they were spaced at least 1 m apart. Ithomiinae were abundant in the area, since we had placed several attractive *H. indicum* plants nearby to ensure their presence. We replaced the bait pads every 2 days, and in the case of the 1975 bioassay of esterifying acids, we replaced the pads as soon as they had any detectable odor. We observed and recorded the behavior of butterflies approaching and landing at baits, and noted the species composition of the sample collected at each bait. The data from the 1974 bioassays (see Table 1) were collected between 10:00 a.m. and 5:00

p.m. EST, July 11–14. A single representative collection was made from *H. indicum* on the morning of July 14. The 1975 bioassay of esterifying acids was conducted in an identical manner and in the same location on July 5–8. Resident species and the categories of their behavioral response are as follows:

Type A: Approached bait, hovered, and departed without landing.

Type B: Approached, landed, and probed bait with proboscis, leaving either without feeding or having fed for less than 30 sec.

Type C: Approached, landed, and fed for at least 30 sec.

OBSERVATIONS, RESULTS, AND DISCUSSION

The typical behavior of male Ithomiinae toward *H. indicum* was to approach the bait from downwind, land, walk about while exploring the surface of the twigs with extended proboscis and antennae, and finally settle to feed. Dried inflorescences were the most attractive parts of the plant, drawing 90% of the feeding butterflies. If the plant was moist, the butterflies drank copious quantities of surface droplets, but if the plant was dry, they regurgitated droplets of liquid, spread them over the surface of the twigs with their probosces, and reimbibed. While feeding, males were docile and could easily be picked up (and sometimes replaced on the plant) without any attempt to escape. On moist plants, butterflies became so engorged that they could barely fly, and would remain on attractive baits for as long as 6 h. Recruitment to new baits was extremely rapid. Often, males would follow and cluster around the investigator while he carried plants from the laboratory to the forest. Dried *H. indicum* loses its potency after about 2 weeks (longer in dry weather). Two to three days before the plants lose all attraction, ithomiines will approach plants, land, and explore, but often leave without sustained feeding.

Responses of butterflies to chemical baits (Table 1) indicate two sorts of biologically active compounds acting synergistically to produce the behavior observed at *H. indicum*. During the 1974 tests (Table 1), the florally scented samples of esterifying acids consistently elicited upwind searching, landing, and exploration of pads, but little sustained feeding. Purified alkaloids on their own elicited almost no responses. We interpret the initial 30 min of the indicine attraction, during which Type B and C responses occurred, to be the result of impurities [possibly related to dissociated (−)-trachelanthic acid], since no further responses occurred thereafter, even in the presence of abundant male ithomiines. Only when alkaloids were mixed with esterifying acids did we observe behavior identical to that shown toward *H. indicum* (see Fig. 1C). Compared with results using scented esterifying acids alone,

males made many more Type C responses to the mixtures, with a corresponding decrease in the number of Type B responses. Males would not remain on pads impregnated with mixtures (max 35 min) as long as on fresh *H. indicum* baits. This behavior is almost certainly the result of their failure to imbibe anything from the pads; regurgitated droplets were always absorbed by the porous paper.

Whereas "odorous" trachelanthic and viridifloric acids appear to be equipotent attractants for male Ithomiinae, the 1975 bioassays of odorless pure samples under the same conditions gave essentially negative results. The trachelanthic acid pads elicited Type A responses from single male *Hymenitis andromica*, *Pteronymia veia*, and *P. nubivaga*. Viridifloric acid pads elicited Type A responses from 2 male *H. andromica* and from single males of *Ithomia iphianassa*, *P. nubivaga*, *P. veia*, and *P. beebei*; 2 male *P. nubivaga* gave Type B responses. These results suggest rather strongly that the unidentified floral odor is associated with attraction, and not with the pure esterifying acids themselves.

In an earlier report (Edgar et al. 1974), we suggested that the origin of the attraction of male Ithomiinae and Danainae to pyrrolizidine alkaloid–containing plants rests in a past association with larval food plants containing these chemicals. We further proposed that volatile chemicals associated with pyrrolizidines were important cues for ancestral butterflies, both males and females, to locate larval host plants. Pyrrolizidine-related volatiles may have induced receptivity in females, so that mating originally took place near the source of larval food. Accordingly, the ancestral host plants may have experienced strong selection to eliminate any odors that attracted herbivores. The host plants of most modern ithomiines (Solanaceae) and Danainae (Asclepiadaceae and Apocynaceae) do not contain pyrrolizidines. However, female *Danaus gilippus* (Brower et al. 1965, Pliske and Eisner 1969) and *D. chrysippus* (Seibt et al. 1972) retain responses associated with earlier larval food plants. Thus, they, and presumably many other danaines and ithomiines as well, can be induced to copulate only when their mates disseminate a pyrrolizidine alkaloid–derived pheromone.

An exceptional situation that may help reveal the origin of this intriguing relationship between plants and butterflies exists in the primitive ithomiine genera *Tithorea* (Neotropics) and *Tellervo* (Indo-Australian). These genera use species of *Fernaldia* (*Tithorea*) and *Parsonsia* (*Tellervo*) in the Apocynaceae as larval food plants (Edgar et al. 1974, Edgar 1975, Common and Waterhouse 1972). *Parsonsia* species have recently been shown to contain pyrrolizidine alkaloids (Edgar and Culvenor 1975), and there is also evidence for the presence of pyrrolizidines in *Fernaldia* (Edgar 1975), so that these two butterfly/plant associations may reflect aspects of the proposed ancestral situation. Very little is known of the behavior of *Tithorea*, except that, unlike

TABLE 1. OBSERVATIONS OF MALE ITHOMIINAE RESPONDING TO PLANT AND CHEMICAL BAITS—RANCHO GRANDE, VENEZUELA, JULY 1974[a]

	Responses					
	Type A		Type B		Type C	
Bait	Species[b]	No.[c]	Species	No.	Species	No.
H. indicum			*Pv*	2	*Pv*	15
					Pa	1
					Pn	3
					Pb	4
					Hd	1
					Om	1
					Op	2
					Ii	4
TOTALS:		0		2		31
(−)-Viridifloric acid	*Pb*	1	*Pv*	6	*Pb*	1
	Om	1	*Pn*	2		
			Pb	1		
			Gk	1		
			Ii	1		
TOTALS:		2		11		1
(+)-Trachelanthic acid			*Pv*	4	*Pv*	2
			Pb	1		
			Hd	1		
			Ha	1		
			Ii	1		
TOTALS:		0		8		2
Heliotrine TOTALS:		0		0		0
Indicine	*Pn*	1	*Pv*	2	*Pv*	5
			Hd	1		
TOTALS:		1		3		5
Heliotrine and	*Pn*	1	*Pb*	1	*Pv*	10
(+)-Trachelanthic acid			*Hd*	1	*Pb*	1
			Ha	1	*Hd*	2
TOTALS:		1		3		13
Heliotrine and	*Pv*	1	*Pv*	4	*Pv*	4
(−)-Viridifloric acid	*Pn*	1				
TOTALS:		2		4		4
Indicine and	*Pn*	1	*Pv*	1	*Pv*	16[d]
(−)-Viridifloric acid	*Ha*	1	*Hd*	1	*Pn*	4
					Gk	1
					Om	3
					Op	1
					Ii	1
TOTALS:		2		2		26

other ithomiine genera, approximately equal numbers of male and female *Tithorea* species are drawn to *H. indicum* baits (Edgar et al. 1974; Pliske 1975*a*). Unfortunately, nothing at all is known of the behavior of *Tellervo*.

While it is apparent that males of most ithomiines and danaines must feed at pyrrolizidine sources to acquire compounds for sex pheromones, it is important to note that a small number of females are also usually attracted, and that females feed in the same manner as males (Pliske 1975*a*). There is nothing to suggest that females have a specific pheromone requirement. Moreover, with *Tithorea* species, the behavior of which is most likely to resemble ancestral butterflies, both males and females are equally attracted and both feed. It is therefore likely that adult feeding at pyrrolizidine plants originally showed no sexual bias, and was perhaps initiated by a nutritional requirement, rather than a need for pheromone or allomone. Support for this hypothesis can be found in Pliske's (1975*a*) observations that *Heliotropium* and other pyrrolizidine plants attract a variety of Coleoptera, Diptera, Orthoptera, and Dictyoptera, which also feed on the decaying leaves, flowers and shoots.

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[a] Several diurnal ctenuchid moths also visited the baits. Numbers of individuals are given in parentheses. All visitors were males. *Euagra cerymica* Druce—Response A: (1) to indicine/viridifloric acid, (1) to heliotrine; Response C; (2) to indicine/viridifloric acid, (1) to heliotrine/viridifloric acid, (3) to heliotrine, and (1) to heliotrine/trachelanthic acid. *Aethria stipata* Wlk.—Response C: (1) to heliotrine and (1) to heliotrine/viridifloric acid. *Myrmecopsis crabronis* Druce—Response A: (1) to heliotrine/viridifloric acid. *Sphecosoma* sp.—Response C: (1) to heliotrine/viridifloric acid.

[b] *Pv*) *Pteronymia veia* Hewitson; *Pa*) *P. asopo* Felder; *Pn*) *P. nubivaga* Fox and Fox; *Pb*) *P. beebei* Fox and Fox; *Hd*) *Hymenitis dercetis* Herrich-Schaeffer; *Gk*) *Godyris kedema* Hewitson; *Ha*) *Hymenitis andromica* Hewitson; *Om*) *Oleria makrena* Hewitson; *Op*) *phemonoë* Doubleday and Hewitson; *Ii*) *Ithomia iphianassa* Doubleday and Hewitson.

[c] An approximate ranking of the relative species abundance in the area based on several collections at *H. indicum* was $Pv > > > > Pn > Ii = Pb > Ha = Gk = Ha = Om > Op > Pa$ (1974 bioassays).

[d] Sample includes one female.

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ISOLATION OF A LACTONE, STRUCTURALLY RELATED TO THE ESTERIFYING ACIDS OF PYRROLIZIDINE ALKALOIDS, FROM THE COSTAL FRINGES OF MALE ITHOMIINAE

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Abstract—Adult male Ithomiinae feed at plants containing pyrrolizidine alkaloids. Certain species secrete on hairs of the "costal fringe," a specialized organ on their hindwings, a novel γ-lactone structurally related to the unusual branched-chain acids that are unique to these alkaloids.

Key Words—Pyrrolizidine alkaloids, costal fringe, male Ithomiinae.

INTRODUCTION

Male butterflies of the closely related nymphalid subfamilies Ithomiinae and Danainae are attracted to and feed on the surface of dead, withering, or damaged plants containing pyrrolizidine alkaloids such as indicine (Fig. 1, structure I) (for references, see Pliske 1975, Edgar 1975). Male danaines use the aminoalcohol portion of the alkaloids to produce dihydropyrrolizidine derivatives (Fig. 1, structures II–IV), which they secrete on extrudable brush-like organs (hairpencils) (Meinwald et al. 1966, 1969, 1971, 1974; Edgar et al 1971, 1973; Edgar and Culvenor 1974; Edgar 1975). The hairpencils are used during courtship to subdue and seduce females (Brower et al. 1965). In the most extensively studied species, *Danaus gilippus berenice* Cramer, the dihydropyrrolizine component (II) of the hairpencil secretion has been

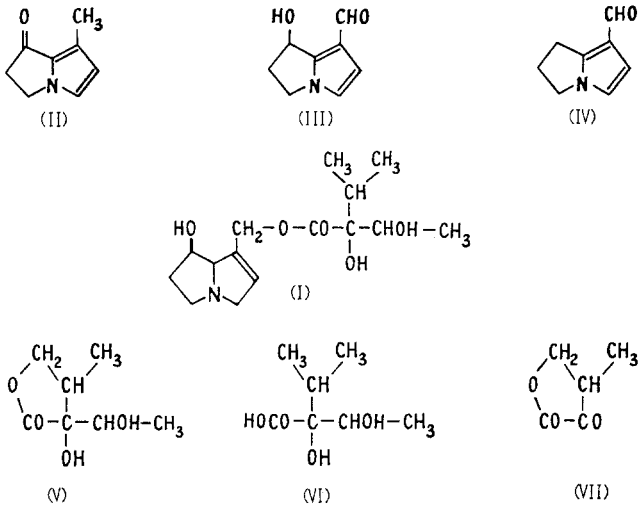

FIG. 1. Structural formulas of alkaloids: (I) indicine; (II–IV) dihydropyrrolizine derivatives; (V) lactone; (VI) trache-lanthic and viridifloric acids; (VII) α-keto-β-methyl-γ-lactone.

established as an aphrodisiac that facilitates mating (Pliske and Eisner 1969; Schneider and Seibt 1969, Myers and Brower 1969).

Male ithomiines, with the exception of the primitive genus *Tellervo*, possess an erectile fringe of hairs on the costal margin of their hindwings (costal fringe), thus raising the possibility that they visit and feed at pyrrolizidine alkaloid–containing plants to acquire alkaloid precursors of costal fringe chemicals. We describe here the results of analysis of the costal fringe secretions of 10 species of Venezuelan Ithomiinae.

METHODS AND MATERIALS

Male butterflies of the species listed in Table 1 were captured at the Estación Biologia Rancho Grande, Estado Aragua, Venezuela, during the summer of 1972, and methylene chloride extracts of the costal fringes, combined for each species, were sent by airmail in sealed ampuls to Melbourne for chemical analysis.

Gas chromatography was performed on a Varian aerograph model 1440 gas chromatograph, and a Varian MAT 111 was employed for combined gas chromatography–mass spectrometry (GC-MS). All gas chromatography was carried out on 1.5 m × 2 mm (ID) glass columns packed with 2% OV17 on

Gaschrom Q, mesh size 80–100, with temperature programming from 70° to 230°C at 6° · min^{-1}. The carrier gas was nitrogen or helium flowing at 15 ml · min^{-1}. Nuclear magnetic resonance spectra were measured with a Varian HA100 spectrometer, and the infrared spectrum (micro KBr disc) was recorded on a Perkin–Elmer 577 infrared spectrometer.

Acetylations

Samples of 30 μg were treated with 30 μl acetyl bromide in a dry nitrogen atmosphere. After varying periods, the excess acetyl bromide was removed in a stream of dry nitrogen. The residue was taken up in 30 μl methanol, and the solution was reevaporated. The methanol treatment was repeated several times before 5 μl of the final methanol solution was examined by GC-MS.

Reaction with Thionyl Chloride

The sample (30 μg) was treated, at room temperature, with thionyl chloride (30 μl), and after 60 min, excess reagent was evaporated in a stream of nitrogen. The residue was several times taken up in methanol (30 μl) and reevaporated. A 5-μl aliquot of the final methanol solution was examined by GC-MS. The remaining methanol solution was evaporated and the residues treated, at room temperature, with 30 μl 0.1 N hydrochloric acid. After 30 min, the hydrochloric acid was removed in a stream of nitrogen, and the residues were taken up in methanol for examination by GC-MS.

Periodic Acid Oxidation

The oxidation was carried out on a 150-μg sample in dry diethyl ether (500 μl) containing 2 mg periodic acid. The progress of the reaction was followed by injecting aliquots (10 μl) of the reaction mixture onto the GC-MS.

RESULTS

A common volatile component, retention time (Rt) 8 min, subsequently identified as the lactone (see Fig. 1, structure V), was observed in fringe extracts of 7 ithomiine species (Table 1).

The lactone (V) was isolated as colorless plates mp 87–88°C, from the fringe extract of each of the 7 species by sublimation at 70°C and 10 mm Hg. The melting point was undepressed when crystals from each species were in turn mixed with those from *P. veia*. The lactone (V) isolated from each

TABLE 1. OCCURRENCE OF LACTONE (V) ON COSTAL FRINGES OF MALE ITHOMIINE BUTTERFLIES

Species	No. of butterflies	Amount[a] of lactone (V) (μg/butterfly)
Hymenitis dercetis Herrich-Schaeffer	241	25
H. andromica Hewitson	51	20
Godyris kedema Hewitson	218	11
Pteronymia beebei Fox and Fox	255	14
P. nubivaga Fox and Fox	31	6
P. veia Hewitson	200	24
Oleria phemonoe Doubleday and Hewitson	13	0
O. makrena Hewitson	173	0
Ithomia iphianassa Doubleday and Hewitson	106	11
Hyalaris cana–H. coeno complex	6	0

[a] Based on amount obtained on sublimation or estimated from gas chromatographic peak heights.

species gave a single peak on GC-MS corresponding in retention time and mass spectrum with the material observed in the crude extracts. Elemental analysis supported the formula $C_7H_{12}O_4$ (calculated: C, 52.5; H, 7.5; found: C, 52.9; H, 7.5; N, O). The mass spectrum, however, exhibited no appreciable molecular ion (M^+) at *m/e* 160, but there was a small $M^+ + 1$ ion at *m/e* 161. This mass spectral behavior is typical of both trachelanthic acid (see Fig. 1, structure VI) and its diastereomer, viridifloric acid, which are closely related to the lactone (V). The mass spectrum showed peaks at *m/e* (relative intensity) 161 (1), 145 (0.5), 143 (0.5), 127 (0.5, 125 (1.5), 116 (100), 101 (55), 71 (47), 70 (78), 55 (60), 45 (67), and 43 (50). After D_2O exchange, peaks were observed at *m/e* 164, 118, 103, 72, 71, 70, 56, 55, and 46, in agreement with the presence of 2 exchangeable protons and the addition of 1 deuterium to the lactone (V) molecule. The infrared spectrum of the fringe compound showed strong absorption bands at 1,750 cm^{-1} (γ-lactone carbonyl) and 3,340 cm^{-1} (OH).

Reactions of the Lactone (V)

Acetylation. Three clearly resolved GC peaks (Rt 12 min, 14 min, and 16 min) were observed after brief treatment (1 min) of the lactone (V) with acetyl bromide. They exhibited mass spectra corresponding respectively, to monoacetyl [*m/e* 203 (0.5) $M^+ + 1$, 185 (0.5), 158 (25), 116 (100), 101 (20), 98 (10), 70 (30), 56 (35), and 43 (99)], bromoacetyl [*m/e* 267, 265 (7) $M^+ + 1$, 224, 222 (2), 206, 204 (15), 185 (10), 184 (20), 180, 178 (25), 125 (35), 99 (100), 81 (98), 79 (40), and 43 (99)], and diacetyl [*m/e* 245 (2) $M^+ + 1$, 200

(6), 187 (4), 185 (5), 158 (90), 116 (100), 113 (35), 98 (95), 56 (90), and 43 (99)] derivatives of the lactone (V). Extension of the reaction time to 5 min or more resulted in complete conversion to the bromoacetyl and diacetyl derivatives, while acetylation with acetic anhydride produced only the monoacetyl derivative, even after prolonged reaction. Acetylation with acetyl bromide was performed on lactone (V) from all ithomiine species with identical results, using either purified lactone (V) or crude fringe extract residues.

Cyclic Sulfite Formation and Hydrolysis. Treatment of the lactone (V) with thionyl chloride gave one product (Rt 11 min). Its mass spectrum exhibited peaks at *m/e* 206 (3) M^+, 162 (6), 148 (8), 126 (6), 120 (2), 111 (4), 92 (97), 90 (15), 84 (20), 83 (6), 70 (10), 69 (16), 64 (20), 56 (100), and 43 (70), indicating reaction of the reagent with the vicinal diol grouping and formation of a cyclic sulfite derivative. Hydrolysis of the cyclic sulfite derivative with dilute acid resulted in almost quantitative reversion to the lactone (V), as ascertained by GC-MS.

Periodic Acid Oxidation. Cleavage of the vicinal diol grouping present in the lactone (V) occurred on periodic acid oxidation, with formation of the α-keto-β-methyl-γ-lactone (see Fig. 1, structure VII) identical in GC retention time (5.4 min) and giving the same mass spectrum [*m/e* 114 (32) M^+, 86 (10), 69 (100), 57 (20), 44 (40), and 41 (56)] as an authentic sample prepared by the method of Fleck et al. (1950).

Nuclear Magnetic Resonance Spectrum

The nuclear magnetic resonance (NMR) spectrum of the lactone (V) (Fig. 2) was measured in deuterochloroform by signal accumulation over 25 scans. Two 3-proton doublets, corresponding to the methyls in the CH_3-CH and CH_3-CHOH groupings, were observed at δ1.10 and 1.32. Near δ2.6, there was a 1-proton multiplet overlapping with a broad absorption, which was removed on exchange with D_2O. A subsequent 52-scan spectrum on a D_2O-exchanged solution in deuterobenzene showed conclusively that the multiplet, δ2.54, was made up of 6 lines with nearly equal spacings, *c* 0.7 Hz, consistent with the CH_2-$\underline{\underline{C}}\underline{\underline{H}}$-$CH_3$ grouping present in the lactone. A triplet at δ4.01, and near triplet at δ4.38, are assignable to the cyclic methylene group. The geminal coupling was −8.5 Hz, and the vicinal couplings to the ring CH were 8.0 and 7.5 Hz, respectively. The geminal coupling is within the range, −9.6 to −7.5 Hz, quoted for methylene groups adjacent to oxygen in 5-membered rings (Jackman and Sternhell 1969). An overlapping multiplet, δ *c.* 3.88, was sharpened to a quartet on D_2O-exchange, and is thus due to the $\underline{\underline{C}}\underline{\underline{H}}$OH-$CH_3$ hydrogen. Two broad singlets, δ1.58 and 3.37, which integrated for 1.5 and 1.0 H, respectively, were removed on D_2O

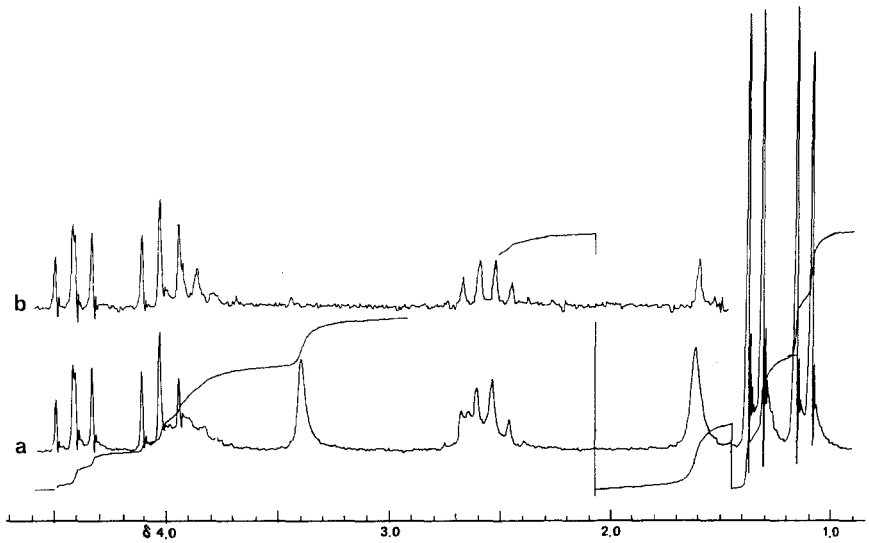

FIG. 2. (a) NMR spectrum of the costal fringe compound measured at 100 MHz in deuterochloroform; (b) NMR spectrum after brief shaking of the deuterochloroform solution with deuterium oxide.

exchange—that at $\delta 1.58$ more slowly than the other. Thus, there were 3 groups of signals due to exchangeable hydrogen, suggesting that an impurity, possibly water, may also be present.

DISCUSSION

The elemental analysis of the costal fringe compound supports the formula $C_7H_{12}O_4$ and a molecular weight of 160. The mass spectrum of the cyclic sulfite derivative has its highest mass ion at *m/e* 206, which is also consistent with a molecular weight of 160 for the costal fringe compound. The lack of nitrogen in the molecule (indicated by elemental analysis) confirms that the *m/e* 161 ion, observed as the ion of highest mass in the mass spectrum of the costal fringe compound, is not the molecular ion (M^+), but an $M^+ + 1$ ion. The presence of $M^+ + 1$ ions in the mass spectra of the lactone (V) and its mono-, di-, and bromoacetyl derivatives is in accord with the behavior of model compounds, including trachelanthic and viridifloric acids (VI), and also the closely related heliotric and latifolic acids (Edgar, unpublished).

The periodate oxidation product (VII), the structure of which was confirmed by synthesis, establishes most of the structural features of the lactone

(V) molecule. It incorporates the $-CH_2-CH-CH_3$ grouping indicated by the NMR spectrum, as well as the γ-lactone ring indicated by the infrared carbonyl absorption. The presence of a vicinal diol grouping (indicated by both periodate cleavage and cyclic sulfite formation) and a $-CHOH-CH_3$ grouping attached to a carbon lacking hydrogens (as shown by the NMR spectrum) establishes the rest of the structure of the costal fringe lactone.

The structure (V) is consistent with the formation of mono-, di-, and bromoacetyl derivatives following treatment with acetyl bromide. Clarification of the stereochemistry of this substance must, however, await further investigation.

Origin of the Lactone (V)

The lactone (V) can be derived from trachelanthic and viridifloric acids (VI) by hydroxylation and lactonization. These unusual branched-chain acids (VI) are unique in nature to alkaloids of the pyrrolizidine type (e.g., indicine [I]), and they, or their decomposition products, appear to be the attractants that draw male ithomiines to pyrrolizidine alkaloid–containing plants (Pliske et al. 1975). We have examined for alkaloids the bodies of two of the ithomiines (*H. dercetis* and *O. makrena*), the fringe extracts of which we report on here. They contained the pyrrolizidine alkaloids lycopsamine, indicine (I), and/or intermedine, which have as part of their structures viridifloric and trachelanthic acids (VI). The larval host plants of the Ithomiinae, in the family Solanaceae, are not known to contain pyrrolizidine alkaloids, and visits by male ithomiines to pyrrolizidine alkaloid–containing plants may therefore be related, at least in part, to a requirement for precursors of the lactone (V). This view is further supported by the finding that the fringes of male *I. iphianassa* raised in captivity on their normal larval host plant (*Witheringia solanacea* L'Herit), without access to a pyrrolizidine alkaloid source, are devoid of the lactone (V). Conclusive proof of the lactone's origin has not, however, been obtained, and attempts to produce lactone (V) on the fringes of deficient *I. iphianassa* males by providing them with access to possible precursors (trachelanthic and viridifloric acids, heliotrine, and aqueous infusions of *Heliotropium indicum* L.) have so far failed.

Role of the Costal Fringes and the Lactone (V)

Males of at least two lactone-containing ithomiine species (*Pteronymia beebei* and *P. nubivaga*) perch in open areas of the forest with their fringes displayed. Behavioral studies by one of us (Pliske, in preparation) have established that the fringes of these and other lactone-containing species (and the lactone itself) do not attract females, but act as interspecific male

repellents. Behavioral studies are continuing, and it is hoped that these studies will further clarify the role(s) of the costal fringes and the lactone (V).

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PLANTS—THE ORIGIN OF KAIROMONES UTILIZED BY PARASITOIDS OF PHYTOPHAGOUS INSECTS?

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Abstract—Chemicals previously identified as kairomones utilized by parasitoids have been found in significant quantities in food plants of host insects. Different plant species contain varying concentrations and ratios of these chemicals. Feeding studies with chemically labeled kairomones indicate that these cues are concentrated and released unaltered by the host insect. In this report, a theoretical framework consistent with these findings is considered.

Key Words—kairomones, parasitoids, phytophagous insects, *Heliothis zea* (corn earworm), *Phthorimaea operculella* (potato tuberworm), *Trichogramma evanescens* Westwood, *Orgilus lepidus* Muesebeck.

INTRODUCTION

Recent studies of interspecies chemical communication systems indicate that chemical signals may play an important role in determining the ecological balance between insect hosts and their parasites (Jones et al. 1973, Hendry et al. 1973). These chemical agents (kairomones [Brown et al. 1970, Whittaker and Feeny 1971]), normally emitted by the host insect, appear to serve one or more functions, e.g.: (1) to attract parasites to areas having infestations of the host insect; (2) to promote close-range searching of the parasite for the host; and (3) to initiate oviposition of the parasite in the host.

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This investigation was initiated to determine the origin of these chemical signals, which to our knowledge had not been elucidated. Two unrelated host–parasite communication systems were chosen for study: (1) the corn earworm (*Heleothis zea*) and the egg parasite, *Trichogramma evanescens* Westwood; (2) the potato tuberworm (PTW) (*Phthorimaea operculella* Zeller) and the pupal parasite, *Orgilus lepidus* Muesebeck.

The kairomone system of the corn earworm moth was previously shown by Lewis et al. (1975) to be composed of a series of normal hydrocarbons, C_{21}–C_{25}, associated with host eggs. These hydrocarbons, and in particular tricosane, $CH_3\ (CH_2)_{21}\ CH_3$, elicited intense host-searching responses from the parasite *T. evanescens*.

The kairomone system of the PTW was shown by Hendry et al. (1973) to be a mixture of normal aliphatic acids. The acids were identified in potato tuberworm frass, which is expelled by larvae as they mine exposed potatoes and foliage. The kairomones, and in particular heptanoic acid, induce host-searching behavior from a primary parasite of the tuberworm, *O. lepidus*.

Since our previous investigations of other insect chemical messenger systems suggested that chemical signals may originate in food plants (Hendry et al. 1975*b*), food sources of the host insects were analyzed for the presence of the kairomones.

We wish to report the identification of kairomones in food plants of both hosts. Initially, corn, a food source of the corn earworm, was analyzed for hydrocarbons C_{21}–C_{25}. A sample of 150 g fresh sweet corn was homogenized in a Waring blender with 400 ml redistilled pesticide-grade CH_2Cl_2. The CH_2Cl_2 layer was filtered, concentrated on a rotary evaporator, and chromatographed on silica gel thin-layer plates, using redistilled pesticide-grade hexanes as the developing solvent. Glass plates, 20×20 cm, were prepared using E. Merck silica gel H, layer thickness 0.75 mm. All plates were developed 3 times with hexane prior to use; the top 3 cm was eluted and discarded to remove hydrocarbon impurities. Final concentrations of hydrocarbons (see Table 1) were corrected for recovery from thin-layer plates. Recoveries were determined using hydrocarbon standards. A band having an R_f identical to standard hydrocarbons was eluted with hexanes and analyzed by preparative gas chromatography (GC) on a nonpolar 3% OV-1 column. A Hewlett–Packard 7610A High Efficiency Gas Chromatograph was used for this analysis; chromatographic conditions were: helium carrier gas 40 ml/min; isothermal oven temperature, 205°C; column, 183 cm × 4 mm ID glass U-tube packed with 3% OV-1 on 60/80 mesh Gas Chrom Q. A fraction having similar retention times to standard hydrocarbons, C_{21} through C_{25}, was collected and injected on a Finnigan 3200 gas chromatograph–mass spectrometer (GC-MS) with model 6000 computer data system

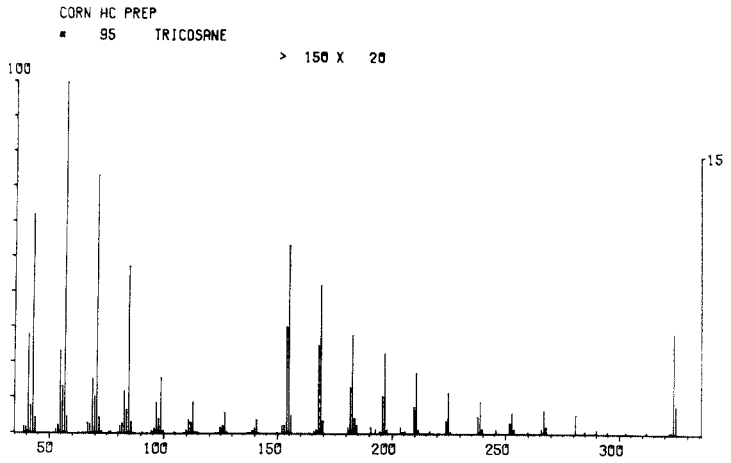
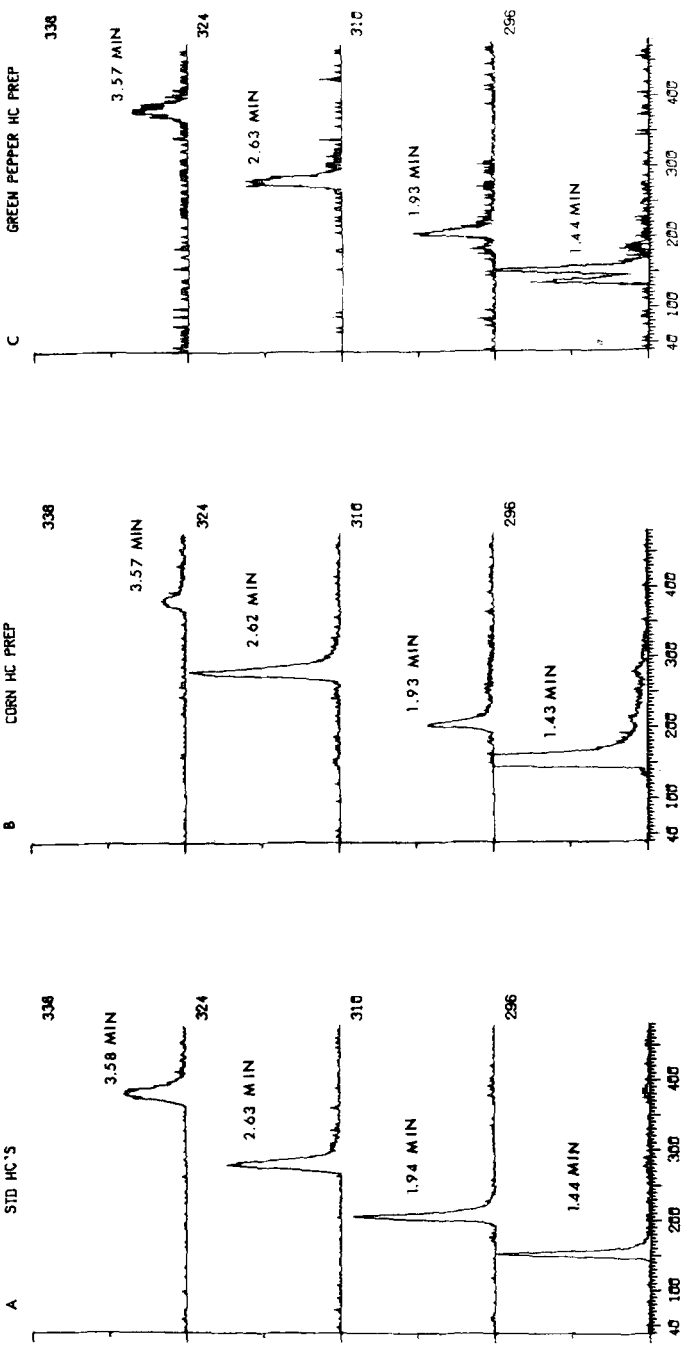


FIG. 1. Mass spectrum of corn extract component identified as tricosane.

(same GC conditions as above). Full mass spectra (35–400 amu) were recorded approximately every second. Comparison of GC retention times and mass spectra of the compounds in the corn isolate with authentic samples indicated the presence of a series of hydrocarbons C_{21} through C_{25}. All analyses were replicated 3 times; GC retention times were identical within 1–2%, and full mass spectra were quantitatively identical within 5% to authentic hydrocarbon standards. Fig. 1 is the mass spectrum of the component identified as tricosane.

Both relative and absolute quantities of these hydrocarbons in corn and other agricultural crops were determined. Rigorous chemical identifications using GC-MS retention times and full mass spectra were conducted as described above on celery, string beans, lettuce, and peppers. In addition, analyses were performed on all plants, using mass fragmentography. Mass fragmentography (Hendry et al. 1975*a,b*; Hendry and Hindenlang 1975) has been described previously in regard to the identification of insect chemical messengers and is an extremely powerful technique; very little sample (50–150 pg) is required for identification of candidate compounds, and it is amenable to accurate quantitative studies. In this case, the GC-MS was programmed to scan only for the molecular ions of hydrocarbons C_{21} through C_{25}: *m/e* 296, 310, 324, 338, 352. Two series of mass fragmentograms were recorded using *m/e* 296, 310, 324, 338, and *m/e* 310, 324, 338, 352, in order to examine 5 molecular ions, 4 at a time. Sample computer plots of the results are shown in Fig. 2. Figure 2F, a blank, was important in this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STD HC'S
338
3.58 MIN
324
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310
1.94 MIN
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1.44 MIN
40 100 200 300 400
B
CORN HC PREP
338
3.57 MIN
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310
1.93 MIN
296
1.43 MIN
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C
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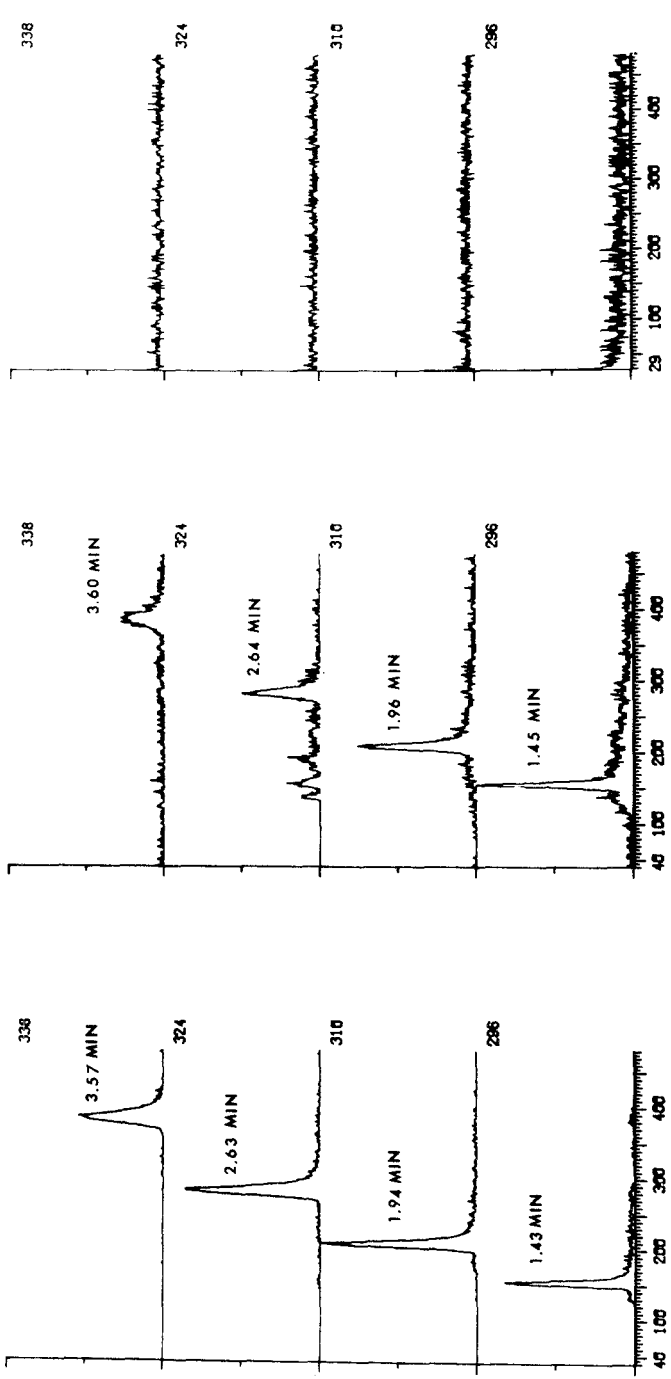


FIG. 2. Mass fragmentograms of plant isolates and authentic standards: (A) standard hydrocarbons C_{21} through C_{24}; (B) corn hydrocarbons; (C) green pepper hydrocarbons; (D) string bean hydrocarbons; (E) lettuce hydrocarbons; (F) blank. These plots represent only relative concentrations of hydrocarbons in each extract, and are not normalized with respect to each other. See Table 1.

TABLE 1. QUANTITIES (NG/G) OF HYDROCARBONS C_{21} THROUGH C_{25} PRESENT IN CERTAIN AGRICULTURAL CROPS

Hydrocarbon	Corn	Green pepper	String bean	Lettuce	Celery	Blank (pg)
C_{21}	452	21	14	9	16	1.2
C_{22}	29	8	24	10	18	0.7
C_{23}	153	32	50	13	24	0.5
C_{24}	28	31	59	12	30	0.3
C_{25}	74	82	74	16	32	0.1

study, since hydrocarbons might be isolated as artifacts. Analysis of a blank by mass fragmentography of *m/e* 296, 310, 324, 338 (Fig. 2F) evidenced no detectable peaks when GC-MS operating parameters were the same as for plant extracts (Figs. 2B–E). Quantitation of the amounts of hydrocarbons in the plant extracts was performed using hexadecyl acetate as an internal standard; these results are shown in Table 1. A precisely known quantity of hexadecyl acetate (30 ng/g plant) was added to each extract after thin-layer chromatography to follow accurately the efficiency of the analytical procedure. Quantities of hydrocarbons in plants were determined by GC-MS comparison to precisely known samples of authentic hydrocarbons and internal standard. The technique used was mass fragmentography of *m/e* 61, 116 (for hexadecyl acetate), 113, 127 (for the hydrocarbons). Quantities of hydrocarbons (Table 1) and aliphatic acids (Table 2) in the blanks were determined by conducting the entire plant extraction and analysis procedures; values obtained were expressed on a scale analogous to the plant extracts. The amounts reported for the blank are maximum values based on mass fragmentography at the limits of detectability of the instrument.

In a second series of experiments, chemical analyses were performed to determine the presence of aliphatic acids in potatoes, peppers, carrots, lettuce, celery, tomatoes, and frass of the potato tuberworm. Approximately 300 g of each plant and 10 g frass were separately homogenized with 250 ml purified methanol and filtered through a coarse sintered-glass filter. The filtrate was concentrated on a rotary evaporator, and 200 ml 5% aqueous sodium carbonate was added. The aqueous mixture was extracted with purified ether to remove nonacidic organic compounds, neutralized with HCl, and thoroughly extracted with ether. (Pesticide-grade methanol and anhydrous diethyl ether were redistilled over sodium carbonate to purify and remove

TABLE 2. QUANTITIES (NG/G) OF ALIPHATIC ACIDS PRESENT IN AGRICULTURAL CROPS AND IN PTW FRASS

Acid	Frass	Potato	Carrot	Lettuce	Pepper	Celery	Tomato	Blank
Hexanoic	800	9.3	1.4	2.5	3.3	3.8	5.4	<0.01
Heptanoic	150	2.3	0.7	0.3	0.1	0.6	1.0	<0.01
Octanoic	270	9.2	1.6	1.3	2.4	3.2	2.7	<0.01

trace acid contaminants.) The ether phase was dried with anhydrous sodium sulfate and concentrated on a rotary evaporator. Gas chromatographic–mass spectral analysis of the residue using a polar column incorporated with phosphoric acid showed that all extracts had components having the same GC retention times and mass spectra as standard aliphatic acids C_6–C_8. The GC conditions for all GC-MS acid analyses were: 40 ml/min He carrier gas; 160°C isothermal oven temperature; 5% DEGS PS on 100/120 mesh Supelcoport (Supelco, Inc., Bellefont, Pennsylvania); 2-mm ID 150-cm glass U-tube column. Quantitation of the exact amounts of acids in the extracts was performed using mass fragmentograms of ions *m/e* 41, 60, and 87 with cosane (*m/e* 282) as an internal standard. A precisely known quantity of cosane (5 ng/g plant, 250 ng/g frass) was added to each extract before GC-MS analysis. Amounts of aliphatic acids in the plants and frass were determined by comparison to authentic standards. The quantities (see Table 2) are minimal amounts, assuming complete recovery from the extraction procedures. Sample mass fragmentograms of these aliphatic acids are shown in Fig. 3. The amounts of acids expressed in nanograms per gram of fresh substrate are recorded in Table 2; a blank is also included.

To further elucidate the possible origin of aliphatic acids in the potato tuberworm frass, feeding studies were conducted. Larvae of the potato tuberworm were fed potatoes incorporated with 10 ng α-mono-deuterated heptanoic acid/g. Heptanoic acid was esterified using methanol and a catalytic amount of sulfuric acid. GC-pure methyl heptanoate was treated with an equivalent amount of lithium-2,2,6,6-tetramethylpiperidine, and the resulting anion quenched with D_2O. Mono-α-deutero-heptanoic acid was then obtained via aqueous HCl hydrolysis of the deuteroester. Frass was collected and extracted as described above. Labeled heptanoic acid was identified in the frass extracts by examining total mass spectra of the acidic products. Quantitative measurements of the amounts of incorporation of labeled heptanoic acid in the frass were made by mass fragmentography of ions *m/e*

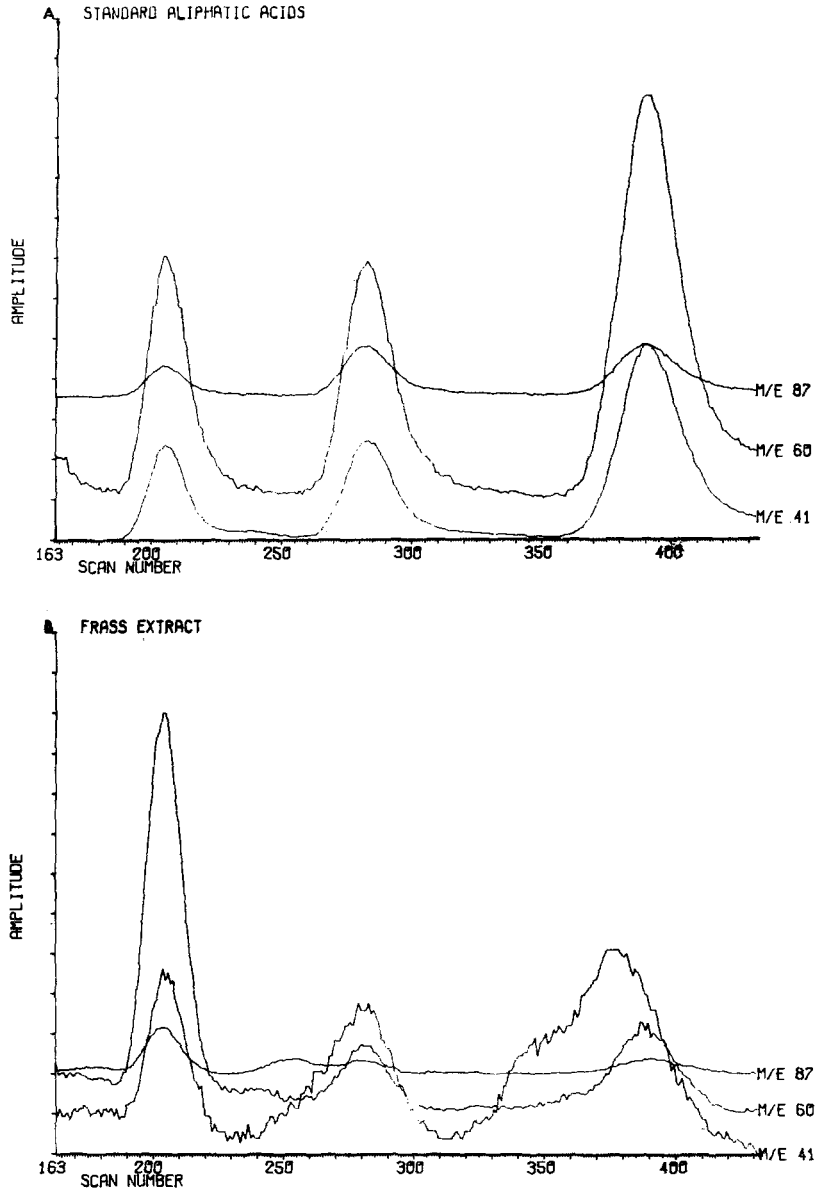
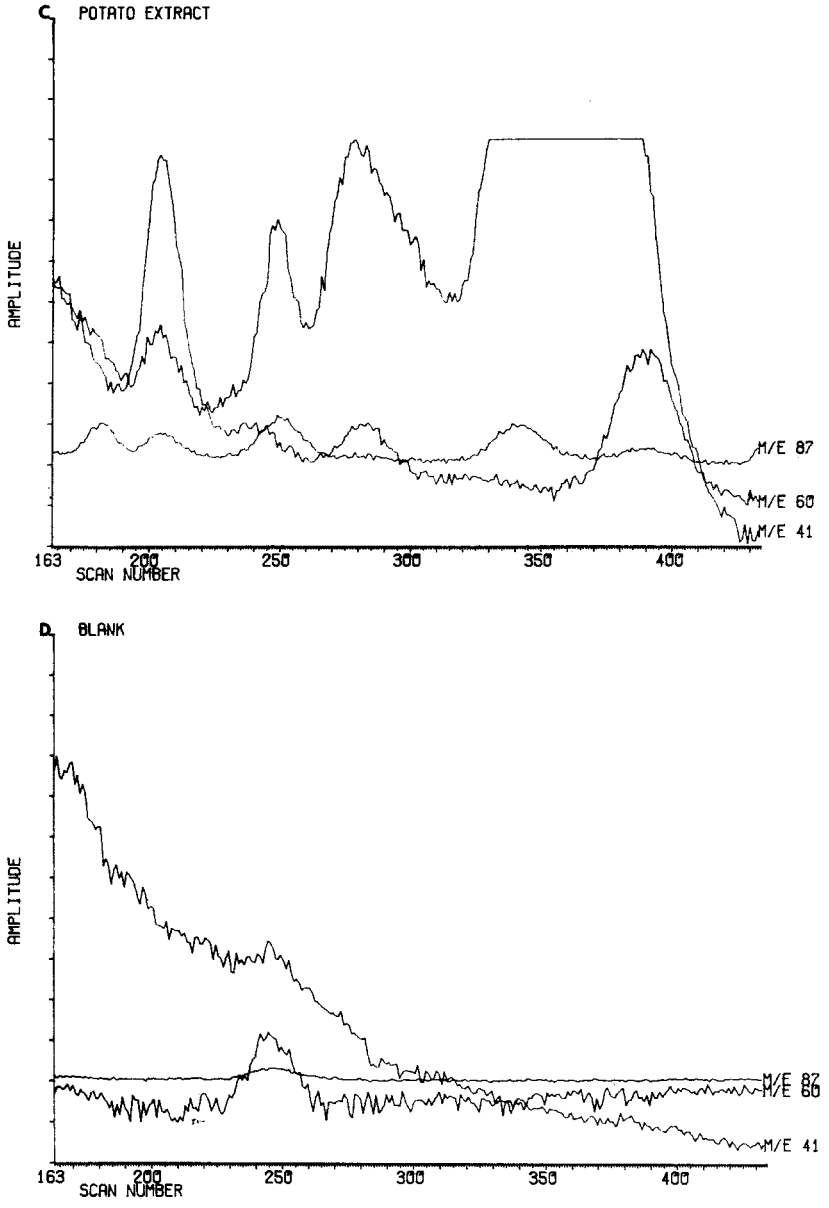
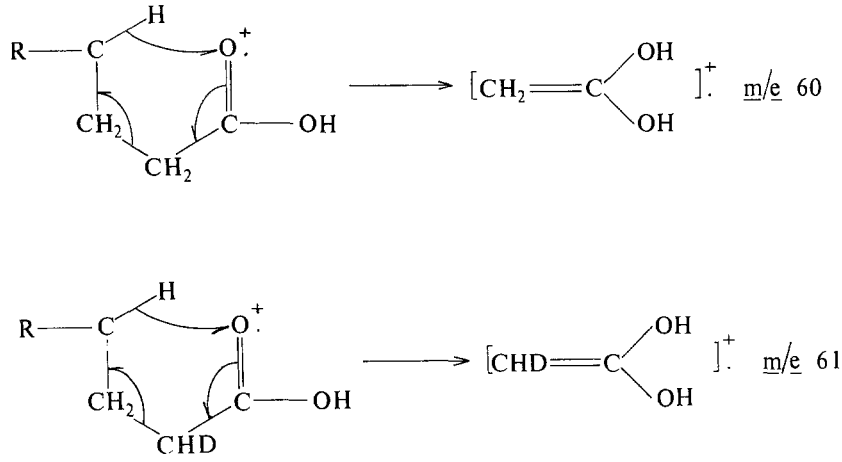


FIG. 3. Mass fragmentograms of extracts and authentic standards: (A, above) standard hexanoic, heptanoic, and octanoic acids; (B, above) PTW frass acids; (C, facing page) potato acids; (D, facing page) blank. These plots represent only relative concentrations of aliphatic acids in each extract, and are not normalized with respect to each other. See Table 2.


C
POTATO EXTRACT
AMPLITUDE
M/E 87
M/E 60
M/E 41
163
200
250
300
350
400
SCAN NUMBER
D
BLANK
AMPLITUDE
M/E 87
M/E 60
M/E 41
163
200
250
300
350
400
SCAN NUMBER

60 and 61, which arise by the McLafferty rearrangements shown below (Budzikiewicz et al. 1967):



In this experiment, the relative and absolute incorporation of α-deutero heptanoic acid in the frass was equivalent to the incorporation of naturally occurring heptanoic acid from the potato.

To our knowledge, this is the first report of the isolation and identification of host–parasite chemical signals in the food plants of the host insects. However, in the first case, despite the demonstrated importance of tricosane in the host-searching behavior of the parasite *T. evanescens* (Jones et al. 1973, Lewis et al. 1975), it is not surprising to find the identical hydrocarbon in corn, since the presence of saturated hydrocarbons in plants is almost ubiquitous (Eglinton and Hamilton 1963). Similarly, although the identification of aliphatic acids in potatoes may be important from a phytochemical standpoint, the results indicate that a broad spectrum of plants contain the same compounds. Nevertheless, the additional findings that, of the plants analyzed, corn has the greatest amount of all the hydrocarbons including tricosane, and that potatoes contain the greatest amount of the acids including heptanoic, suggest a very direct relationship between host–parasite signals and food-plant chemistry. Evidence for this relationship is strengthened by the finding that heptanoic acid is highly concentrated by potato tuberworm larvae as they feed on potatoes. This is the first definitive evidence via chemical labeling studies for the direct utilization of plant chemicals in an insect communication system.

From these results, we believe that tricosane, heptanoic acid, and other host-finding chemicals yet to be identified may provide a dual evolutionary purpose by initially attracting parasites to fields containing suitable foliage and subsequently to hosts that may have concentrated these agents from food

plants. It follows that certain host insects may be able to escape parasitism by transferring to alternate food sources having different chemical compositions (Ron Carroll, personal communication), e.g., in the cases studied, from corn to lettuce or from potatoes to peppers. Previous observations of intimate associations between insect parasites and various plants support these postulates (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). Whether the variation of hydrocarbons and aliphatic acids in plants is critical in these host–parasite communication systems awaits extensive experiments that include determining the exact amounts of the kairomones that are naturally evaporating from infested and noninfested plants.

Specificity of chemical language as previously reported in parasitic Hymenoptera (Jones et al. 1973; Hendry et al. 1973) may be due to particular chemical complexes (dialects) available in plants. Whether some insect parasites may be programmed or "imprinted" to chemical complexes in plants (a similar mechanism has been postulated in insect pheromone communication [Hendry et al., 1975*b*]), or whether host-finding is entirely genetically determined, is an interesting question. Whatever mechanisms are involved, there may be as yet undiscovered intimate chemical relationships between parasitic insects and plants—relationships that have important evolutionary consequence. It is intriguing to speculate that pressures for divergence and convergence of some parasitic species may be in part due to adaptation of insects to specific host plants via complexes of chemical messengers.

With this report, all major categories of chemical messengers have been found in plants: pheromones (Hendry et al. 1975*b*), allomones (Reichstein et al. 1968, Brower 1969, Brower et al. 1972, Eisner et al. 1974), kairomones, and hormones (De Souza et al. 1969, Slama 1969 and references therein). The overall significance of these findings in the evolution of insect behavior remains to be elucidated, but this report indicates that the origin of chemical signals is not irrelevant to our concepts and definitions in the study of chemical communication.

Acknowledgments—This project was supported by grants from Research Corporation (RC-Inhibitor 3733), the Environmental Protection Agency (EPA-Pheromones 7450), the USDA (USDA-CRA 4012), and the Rockefeller Foundation (RF-Insect 3726).

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CHEMICAL COMMUNICATION IN THE MATING BEHAVIOR OF *Trogoderma glabrum* (HERBST) (COLEOPTERA: DERMESTIDAE)

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Abstract—Male mating behavior of the stored product pest beetle *Trogoderma glabrum* (Herbst) was analyzed into three major phases: arousal/searching, preliminary recognition, and genital (copulatory). Airborne pheromone released by calling females elicits male arousal via antennal sensillae. Contact chemoreception via male mouthpart sensillae appears to be necessary for copulation to occur. A procedure was developed for quantitative bioassay of presumptive pheromone compounds in both airborne and contact chemoreception. (*E*)-14-methyl-8-hexadecenal, present in airborne pheromone, but not detectable in extracts of whole females, elicits both arousal and attempted copulation. The activity of (*E*)-14-methyl-8-hexadecenal is equal to that of the total airborne pheromone. Activity of additional possible pheromone component candidates [including (*E*)-14-methyl-8-hexadecen-1-ol, γ-caprolactone, *n*-hexanoic acid, and methyl (*Z*)-7-hexadecenoate] was investigated. Evidence for a behavioral role for *n*-hexanoic acid is presented.

Key Words—pheromone, mating, *Trogoderma*, Dermestidae, Coleoptera, behavior, airborne pheromone, calling, *Trogoderma glabrum*, (*E*)-14-methyl-8-hexadecenal.

INTRODUCTION

Within the genus *Trogoderma* (Coleoptera: Dermestidae) are several serious stored product pests of relatively cosmopolitan distribution. We report

here work on the biology of mating and the chemical communication system of *T. glabrum*, one aspect of the development of a pheromone trapping/disease dispersal control system for *Trogoderma* spp. (Schwalbe et al. 1974).

Females of *T. glabrum* produce a sex pheromone, released during calling in a circadian rhythm (Hammack and Burkholder 1976, Hammack et al. 1976). The pheromone will elicit arousal in conspecific males and attraction to the pheromone source (Burkholder and Dicke 1966). Compounds with pheromone-like activity have been isolated from whole females, characterized chemically, and assayed for biological activity in eliciting a relatively non-specific arousal response (Yarger et al. 1975).

Knowledge of the role of all the components of the pheromone complex will presumably allow more selective attraction of the species of *Trogoderma*, and also more efficient trapping over long distances. We therefore analyzed the mating behavior of *T. glabrum*, especially from the point of view of sensory structures and chemical signals involved in mediating changes in behavioral state. We observed mating behavior in normal insect pairs, and in pairs including males from which the antennae or mouthparts, or both, were surgically removed. A recently devised method for the simple and efficient collection of a relatively large quantity of airborne compounds (Byrne et al. 1975) allowed us to compare the chemistry and activity of released pheromone with active compounds isolated from extracts of macerated whole females.

METHODS AND MATERIALS

Insects were obtained from cultures that had been maintained for about 15 yr in the Stored Product Insects Laboratory, Madison, Wisconsin, and were reared as described in Hammack et al. (1973). Insects were removed from the culture as pupae and sexed on the basis of body size; females were returned to the rearing chamber, males were placed in a female-free incubator. Insects were maintained on a 16:8 light:dark photocycle, with lights-on at 7 a.m. CDT.

Sensory Ablation and Experimental Mating

Under light ether anesthesia, experimental males had antennae or maxillary palps, or both, removed mechanically. The appendages were grasped at the base with fine jewelers' forceps and pulled off. This procedure resulted in 100% survival. For insects treated in this way, control insects were of two types: those not operated on, and those with antennae or maxillary palps or both, removed unilaterally. Since the labial palps were too small and

protected to be conveniently removed by this method, the mouthparts of one group of males were cauterized by applying the tips of jewelers' forceps heated to incandescence in a gas flame to the mouth-part area for a second or two. This is a fairly crude procedure that does not allow for precise localization of tissue destruction. Survival for the cauterized males was about 90%. Control insects for this procedure were cauterized in the region of the labrum and clypeus, including maxillary palp destruction in one insect. After mating, randomly sampled males were observed either by dissecting microscope or as cleared whole mounts under the compound microscope to confirm loss of appendages. Males were 5–6 days post-emergence at the time of operation, and were returned to the incubator for 2 days of postoperative recuperation before being used in mating experiments.

Experimental matings were conducted in 5.5 ml shell vials filled to a depth of about 1 cm with rearing medium that was covered with 12.7 mm–diam untreated anti-bacterial assay disc (Schleicher and Schuell, Keene, New Hampshire).[3] One 7–12-day-postemergence virgin female was placed in each vial at about 12 noon CDT. After a few minutes, one male was introduced into each vial. After about 28 h, the males were removed and placed in separate marked vials in a 13°C holding incubator for subsequent observation. Vials containing females were returned to the rearing room. After approximately 20 days, when larvae could be easily observed in the medium, these vials were scored for successful mating. Number of larvae produced was not recorded.

Chemicals

Collection, extraction, purification, analysis, and characterization of pheromone are described elsewhere (Cross et al., in press). These methods provide us with total pheromone, samples of major components in proportion to their representation in the total extract, and the total extract from which selected components had been removed. Synthetic (*E*)- and (*Z*)-14-methyl-8-hexadecen-1-ol were obtained from Farchan Division, Story Chemical Corporation, Willoughby, Ohio, and oxidized to the corresponding aldehydes, using Collins' reagent (Cross et al., in press). *n*-Hexanoic acid was obtained from the Applied Science Laboratory, State College, Pennsylvania; γ-caprolactone and methyl (*Z*)-7-hexadecenoate were provided by R. Yarger, Monell Chemical Senses Center, University of Pennsylvania. Commercial compounds were purified by gas chromatography. The hexane was distilled through a fractionating column packed with glass beads. Chemicals were stored as hexane solutions at −4°C until use.

[3] Mention of a proprietary product in this paper does not constitute an endorsement of the product by the U.S. Department of Agriculture.

Bioassay

Assays were conducted in the early afternoon, the period that corresponds to the peak of female calling activity (Hammack et al. 1976) and to the peak of male responsiveness (Vick et al. 1973). Several hours before testing, 7–12-day-postemergence males were transferred to individual 5.5 ml shell vials. Males were used only once. Assays were conducted in a room reserved for that purpose, kept at a positive pressure with respect to the neighboring laboratory and maintained at 26°C and 40–60% relative humidity, with illumination provided by cool white fluorescent bulbs with an intensity of about 750 lx at the assay location.

The bioassay procedure that was developed allows for simultaneous determination of activity of test compounds in both the airborne and contact communication systems. A small paraffin chip (Eskar No. 40, 57°C),[3] about the size of the *T. glabrum* female, was treated with 10 μl of a hexane solution of the test compound. Then the treated chips were transferred with jewelers' forceps to filter paper, and the solvent was allowed to evaporate for 2 min. An undetermined quantity of test solution is absorbed by the filter paper or flows onto the substrate (a plastic petri dish) on which the chip is treated. Thus, the quantities indicated in this report represent maximum values of test compound actually applied. Finally, a single treated chip was dropped into a vial with a single male, and the behavior of the insect was observed for 60 sec. The assays were replicated in groups of 10 each. Hexane controls were run in all series, and chips were generally used only once. Positive arousal responses were scored if the male raised his antennae, head, and thorax, and began to walk about (Vick et al. 1970). Positive mating responses were scored if the male contacted the chip with his mouthparts, extended his aedeagus and, by reversing his orientation, brought it into contact with the chip. Positive responses in both cases were generally unambiguous.

RESULTS

Description of Mating Behavior

Our observations of mating led us to analyze the sequence into three stages: arousal/searching, preliminary recognition, and genital (copulatory) (see Figs. 1a–c).

Arousal may be elicited by the presence of a nearby female, by a female extract, or by any of several synthetic compounds (see below). Arousal may range from partial elevation of the head or antennae, or both, to full head/antennal elevation, with extension of the pro- and mesothoracic legs, followed by locomotion, sometimes apparently chaotic or zigzag, sometimes oriented directly to the pheromone source.

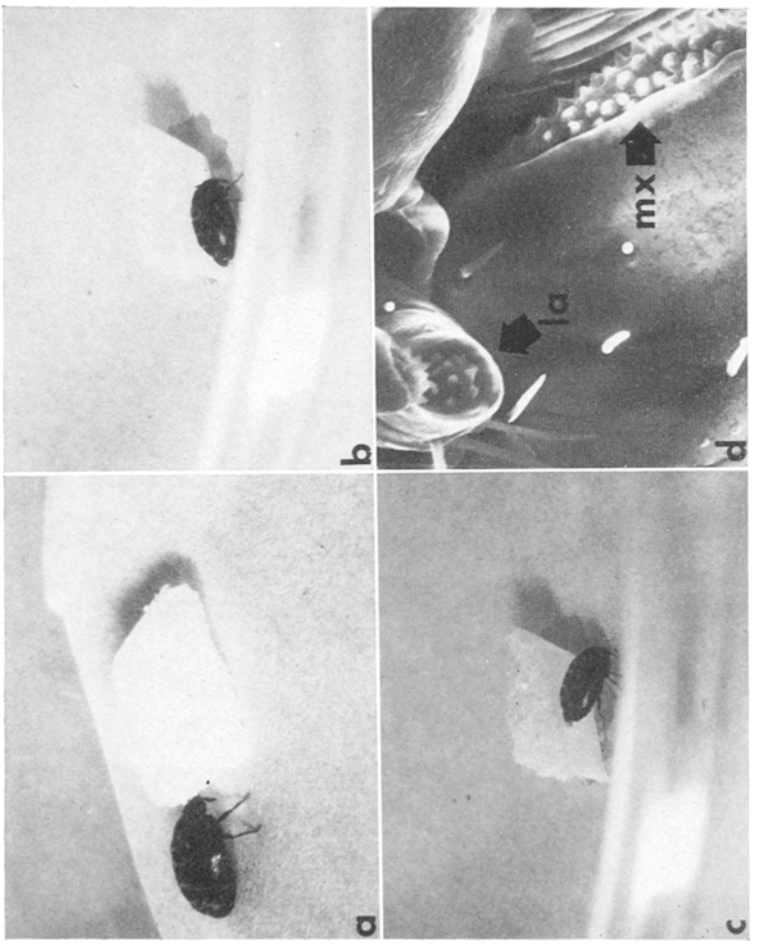

FIG. 1. (a) Male *T. glabrum* contacts (*E*)-14-methyl-8-hexadecenal–treated paraffin chip with extended maxillary palps (10 × life size); (b) male extends aedeagus and simultaneously reverses orientation (6 × life size); (c) male attempts copulation with treated model (6 × life size) (b and c from same series); (d) SEM photomicrograph of distal ends of maxillary (mx) and labial (la) palps of female *T. glabrum*, showing sensillae (1,800 × magnification).

(*Facing p.* 288)

The preliminary recognition phase begins when the male comes into contact with the female (or model). The male draws its antennae to either side of its head, and contacts the presumptive female with the distal ends of its maxillary palps, which are richly supplied with setae (see Fig. 1d). This behavior is often interspersed with "head butting"—pushing against the female with the top of the head. The preliminary recognition phase may last from 2 to 30 or more sec, during which the male will move along the female, its head frequently reaching the caudal end of the abdomen. If sufficient cues for recognition are present, the insect moves to the genital phase; if not, the male becomes quiescent, perhaps to begin again after a few seconds or minutes. The female apparently plays no active role in the recognition phase.

The genital phase begins with the extension of the male aedeagus and the more or less simultaneous turning of the male to bring its aedeagus roughly to the location that had just previously been explored by the palps. The male seems to continue to "explore" the area with its genitalia, and if this explora-

TABLE 1. EFFECTS OF REMOVAL OF SENSORY APPENDAGES ON MATING ABILITY OF *T. glabrum* MALES

Sensory structure(s) removed					
	Palp				
Antenna	Maxillary	Labial	Other head structures	No. of insects	Successful matings (%)
—	—	—	—	15	93.3
Unilateral, mechanical	—	—	—	6	100
—	Unilateral, mechanical	—	—	6	100
—	—	—	Clypeus/labrum, thermal	5	60
—	Bilateral, mechanical	—	—	19	95
Bilateral, mechanical	—	—	—	10	90
Bilateral, mechanical	Bilateral, mechanical	—	—	10	100
—	Bilateral, thermal	Bilateral, thermal	—	14	0
Bilateral, mechanical	Bilateral, thermal	Bilateral, thermal	—	10	0

tion leads to successful discovery of the female genital pore, and if the female is receptive, copulation ensues. Copulation is brief, generally lasting about 1 min. Successful copulation is followed by a sexually inactive period of undermined duration. With the exception of receptivity to intromission and pheromone release, the female is passive throughout. At no point does the male normally mount the female.

Ablation Experiments

The results of experimental matings are shown in Table 1. It can be seen that only those males lacking both maxillary and labial palps were unable to mate successfully, though males lacking these appendages but with intact antennae demonstrated apparently normal arousal behavior, searching, and head butting. Among the cauterized controls that mated successfully was one with its maxillary palps destroyed, but with apparently normal labial

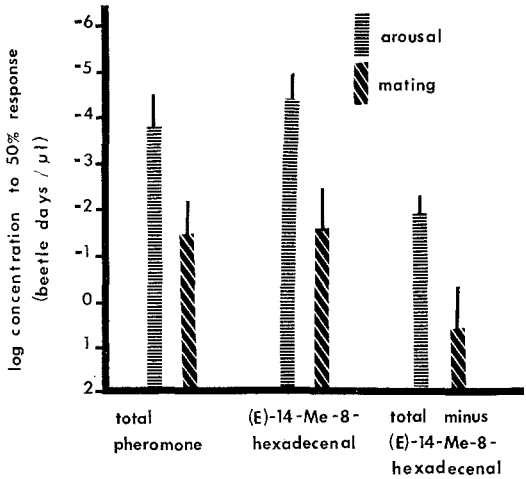

FIG. 2. Activity of total airborne pheromone, (*E*)-14-methyl-8-hexadecenal from pheromone, and total pheromone minus aldehyde in eliciting arousal and attempted mating. Activity is represented by the concentration required for 50% activity, as determined by the best linear fit to serial dilution bioassay data. Aliquots of 10 μl were used (see the Methods and Materials section). One beetle-day equals the production of 1 female in 24 h. Narrow bars represent 95% confidence intervals computed from pooled data.

palps. Bilaterial removal of the antennae did not interfere with normal mating under the experimental conditions (pairs confined to a very small area for over 24 h). However, the normal arousal/searching behavior was not observed in bilaterally antennectomized males. Bilateral maxillary palp removal did not block mating; presumably the intact labial palps substituted for the missing maxillae, which would normally be used.

Bioassay

Total and fractionated Porapak extracts of airborne natural pheromone, as well as synthetic compounds, were assayed for activity in eliciting both arousal and attempted copulation. The results for natural pheromone samples are shown in Fig. 2. The total pheromone was able to elicit the entire beha-

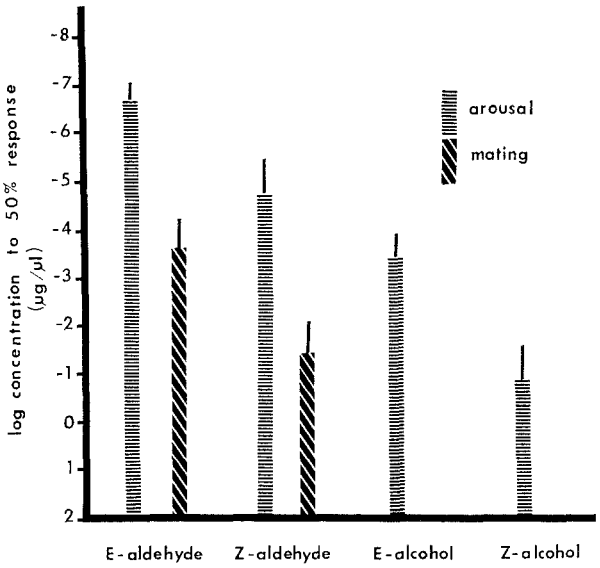

FIG. 3. Activity of synthetic (*E*)-14-methyl-8-hexadecenal, (*Z*)-14-methyl-8-hexadecenal, (*E*)-14-methyl-8-hexadecen-1-ol, and (*Z*)-14-methyl-8-hexadecen-1-ol in eliciting arousal and attempted mating. Aliquots of 10 µl were used (see the Methods and Materials section). Activity is measured by the concentration of the compound (measured in µg/µl) required for 50% activity, as determined by best linear fit to serial dilution bioassay data. Narrow bars represent 95% confidence intervals computed from pooled data. Neither alcohol was sufficiently active to determine a 50% mating response concentration.

vioral response, provided the male was able to come into direct contact with the paraffin model. No significant difference was noted between the 50% threshold concentration for the total mixture and that for its most active component, (*E*)-14-methyl-8-hexadecenal, in eliciting either arousal or attempted mating. The remainder of the Porapak extract [total less aldehyde, and containing (*E*)-14-methyl-8-hexadecen-1-ol, γ-caprolactone, *n*-hexanoic acid, methyl (*Z*)-7-hexadecenoate, and several as yet unidentified components] showed some activity in eliciting arousal and attempted copulation, though significantly less than either the total Porapak extract or (*E*)-14-methyl-8-hexadecenal isolated from the Porapak extract.

Assay data for synthetic alcohol and aldehyde are shown in Fig. 3. Among the compounds tested, (*E*)-14-methyl-8-hexadecenal had by far the highest activity in eliciting both arousal (50% response at 1.9×10^{-7} μg/μl) and attempted mating (50% response at 3.5×10^{-4} μg/μl) and was roughly 100-fold more active than the *Z*-isomer. At concentrations 100 times that of the respective 50% response level, either aldehyde elicited essentially 100% arousal or mating response. (*E*)-14-methyl-8-hexadecen-1-ol was significantly

TABLE 2. EFFECT OF *n*-HEXANOIC ACID ON ACTIVITY OF (*E*)-14-METHYL-8-HEXADECENAL AND (*E*)-14-METHYL-8-HEXADECEN-1-OL

Test compound and concentration (10-μl aliquots)	No. of replicates, 10 insects each	Arousal response (average %)	Mating response (average %)
(*E*)-14-methyl-8-hexadecen-1-ol, 5×10^{-4} μg/μl	8	60.0 (48.0, 71.2)[a]	7.5[b] (2.8, 15.2)
(*E*)-14-methyl-8-hexadecen-1-ol, 5×10^{-4} μg/μl,	16	57.5 (49.2, 65.5)	20.0[b] (13.9, 27.3)
+ *n*-Hexanoic acid, 5×10^{-2} μg/μl			
(*E*)-14-methyl-8-hexadecenal, 10^{-5} μg/μl	6	78.3 (67.0, 86.7)	13.3[c] (6.0, 24.3)
(*E*)-14-methyl-8-hexadecenal, 10^{-5} μg/μl,	8	73.5 (62.1, 83.1)	31.3[c] (21.1, 43.0)
+ *n*-Hexanoic acid, 5×10^{-2} μg/μl			
n-Hexanoic acid, 10^{-1} μg/μl	4	0	0

[a] Values in parentheses are exact 95% binomial confidence intervals.
[b,c] Difference of means within each pair significant $P < 0.05$.

less active (by over 3 orders of magnitude) in eliciting arousal, and while the *E*-alcohol in high concentrations occasionally elicited mating responses, it did not demonstrate sufficient activity for 50% response concentration to be determined. (*Z*)-14-methyl-8-hexadecen-1-ol was least active.

None of the other compounds tested had significant activity in eliciting the stereotyped behaviors when tested separately. Methyl (*Z*)-7-hexadecenoate and γ-caprolactone did not elicit mating at any concentration tested (up to 1 μg/μl and 2 μg/μl, respectively), and they were inconsistent in eliciting arousal, which seemed to be of a rather generalized character, lacking the stereo-typed qualities characteristic of the activity of (*E*)-14-methyl-8-hexadecenal. Neither methyl (*Z*)-7-hexadecenoate nor γ-caprolactone was tested as mixtures with other presumptive pheromone components. Methyl (*E*)-14-methyl-8-hexadecenoate was not available for bioassay.

n-Hexanoic acid at high concentrations (10–100 μg/μl) elicited an apparently anomalous response. An initial arousal phase would be followed by an "inappropriate" attempted copulation—e.g., with the glass wall of the test vial—even when the insect was prevented from making direct physical contact with the hexanoic acid source. Such behavior was rarely, if ever, observed with any of the other pheromone components, even at comparably high concentrations. When *n*-hexanoic acid, at a concentration that had no activity on its own (5×10^{-2} μg/μl), was added to (E)-14-methyl-8-hexadecenal or (E)-14-methyl-8-hexadecen-1-ol, there was an enhanced activity of the mixture in eliciting mating (see Table 2). No significant effect on arousal activity was observed.

DISCUSSION

The mating sequence in *T. glabrum* is relatively simple, and the transitions are mediated by sensory structures with apparently overlapping but non-identical chemical specificity. A generally similar sequence is observed in other coleopterans, though the details of behavior and gross sensory structures vary considerably (Wojcik 1969, Pinto 1975). Our behavioral observations are in general agreement with those of Wojcik (1969) on *T. glabrum* (though we believe that the significant role of airborne pheromone in the mating behavior is well demonstrated). Levinson and Bar Ilan (1970) have described a similar behavior sequence for *T. granarium*.

The data presented here support the conclusion that the primary component of the *T. glabrum* sex pheromone (in terms of biological activity) is (*E*)-14-methyl-8-hexadecenal. This aldehyde seems to be largely or solely responsible for antennal mediated arousal, and (in conjunction with hexanoic acid and perhaps other as yet unidentified compounds) responsible for the

palp-mediated mating response. The behavioral roles, if any, of other compounds remain to be demonstrated.

It is a notable feature of this system that the most active compound extracted from whole females, (*E*)-14-methyl-8-hexadecen-1-ol, is not the one released into the air, except in relatively low concentration; the most active airborne component, (*E*)-14-methyl-8-hexandecenal, is not found in female extracts (Yarger et al. 1975; Cross et al., in press). This finding suggests that the stored form of the pheromone, the alcohol (or the ester), may be converted to the more volatile aldehyde before or during release. An alternative possibility is that the aldehyde is enzymatically destroyed during the procedure of extracting whole insects after maceration. Hill et al. (1975) likewise identified an aldehyde as a major pheromone component from a Porapak (aeration) extract of the adult female orange tortrix, *Argyrotaenia citrana* (Fernald), but detected only small amounts in an extract of excised abdominal tips. Bierl et al. (1970) found only small amounts of the epoxide pheromone and larger amounts of the olefin precursor in the extract of abdominal tips of the female gypsy moth, *Porthetria dispar* (L.). We suggest that a reexamination of other pheromones with a suitable aeration procedure may be in order.

The difference in biological activity between (*E*)-14-methyl-8-hexadecenal and its corresponding alcohol can hardly be attributed solely or largely to differences in volatility or solubility in paraffin. Similar differences are observed when the compounds are assayed on filter paper discs (Shapas, personal communication). We have not directly measured the difference in volatility, but it would require a factor of 1,000 to explain the observed difference in activity between the two compounds.[4]

Comparison of the activity of the total pheromone with the activity of the (*E*)-14-methyl-8-hexadecenal from the extract shows no evidence for synergistic interactions between the aldehyde and other components in eliciting arousal. However, the activity of the remainder of the extract (total minus aldehyde) is greater than would be expected if essentially all the activity in this mixture could be attributed to the presence of (*E*)-14-methyl-8-hexadecen-1-ol alone. The alcohol is present in the airborne pheromone at about 2% of the level of the aldehyde (Cross et al., in press) and has about 3 orders of magnitude less activity. Yet instead of the decrease in

[4] While the difference in vapor pressure between equimolar hexane/paraffin solutions of (*E*)-14-methyl-8-hexadecenal and its corresponding alcohol is unknown, an estimate of the difference may be obtained by considering the pair decanal/decanol. From their measured boiling points (Hodgman 1959), their molar heats of vaporization may be estimated using the Nernst–Bingham approximation (Dean 1973), and from this a value for the vapor pressure of the pure substances at room temperature may be computed. Such calculations yield an estimate that decanal would have slightly less than 3 times the vapor pressure of decanol.

activity of more than 4 log units that would be expected to result, the decrement is only on the order of 2 log units. There are no other known components in the mixture that are present at high enough concentrations to account for this additional activity (if we assume additivity of activity of components). Incomplete removal of aldehyde might account for this discrepancy. Alternatively, the effect may be due to as yet unidentified components, or to as yet undetermined synergistic interactions between components.

Our data do suggest that there are synergistic interactions between the components of the pheromone that mediate contact recognition and elicit copulation. We have assigned a tentative role to *n*-hexanoic acid in this process, which apparently interacts in a nonlinear manner with (*E*)-14-methyl-8-hexadecenal. Under our bioassay conditions, the results are not striking, though they are apparently real and suggestive. [The experimentally determined sample means are expected to come from the same binomial distribution with $P<0.05$ by the test of Burr (1974).]

n-Hexanoic acid is active only at relatively high concentrations. The effects we report were obtained using 0.5 μg *n*-hexanoic acid. This amount is physiologically plausible, however, since Yarger et al. (1975) estimate that an adult female *T. glabrum* contains 0.2 μg *n*-hexanoic acid. As we report elsewhere (Cross et al., in press), an adult female *T. glabrum* emits *n*-hexanoic acid at the rate of only 10 ng/day. In the experiments we conducted on the activity of Porapak-collected pheromone, we used a maximum concentration of 1 beetle–day/μl (equivalent to 0.1 μg hexanoic acid). Thus, we would expect any effect of *n*-hexanoic acid in these experiments to be negligible.

The observed activity of *n*-hexanoic acid in mating provides a likely basis for homosexual behavior in *T. glabrum*. This phenomenon is observed among dermestid males (Burkholder and Dicke 1966), as well as among males of other beetle families (e.g., August 1971), and involves attempted copulation between conspecific males in the presence of airborne pheromone. The pheromone apparently acts as a releaser for arousal, but as a primer for copulatory behavior, under these conditions, the copulatory behavior presumably being released by a species-specific (but not gender-specific) chemical (or mixture) such as *n*-hexanoic acid. Yarger (1974) reported the presence of *n*-hexanoic acid at similar concentrations in extracts of both male and female *T. glabrum*.

The actual components of a natural pheromone and the roles they play in the behavior of the organism are frequently complex. Systems are known in which attractant sex pheromones are apparently single compounds (Silverstein et al. 1967) or mixtures that may interact nonlinearly (e.g., Tamaki and Yushima 1974). Also, the attraction may sometimes be concentration-

dependent (Kaae et al. 1973). Single blends may release an ascending series of behavior (Bartell and Shorey 1969), or different stages may require different chemical messengers (Cardé et al. 1975). These transitions may be mediated by the same sensory structures at a gross level (Cardé et al. 1975) or by different ones, as in *T. glabrum*. Certain components of the system may, in addition, have an effect on target organisms of another species (Sower et al. 1974). A complete understanding of the functions of the various components requires both detailed chemical analysis and comprehensive behavioral observation, leading to a synthetic theory of the behavior or behavioral sequence of interest. The real test of such a theory is our ability to use it in the manipulation of the behavior of the organism in the field.

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INFLUENCE OF CARBON SOURCES ON THE PRODUCTION OF THE TERMITE TRAIL-FOLLOWING SUBSTANCE BY *Gloeophyllum trabeum*

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Abstract—Efforts were made to study the effects of various carbon sources on the production of the trail-following substance (*Z*, *Z*, *E*)-3,6,8-dodecatrien-1-ol in the brown-rot fungus *Gloeophyllum trabeum* (= *Lenzites trabea*). The substance is found in a southern subterranean termite, *Reticulitermes virginicus*, and is known to function as the trail-following pheromone. The fungus could not produce it when grown on a low potato–dextrose medium. On addition of several simple carbon sources, however, the fungus actively produced the substance. Ethylene glycol, glyceraldehyde, and xylose yielded the highest production of the substance. Using 1,2-^{14}C-ethylene glycol as a tracer, we demonstrated that the given carbon source was incorporated into the trail-following substance by the fungus.

Key Words—biosynthesis of trail-following substance, *Gloeophyllum trabeum*, southern subterranean termite, influence of carbon sources.

INTRODUCTION

Esenther et al. (1961) discovered that woods decayed by the brown-rot fungus *Gloeophyllum trabeum* (= *Lenzites trabea* Pers. ex Fr.) produced a substance attractive to the eastern subterranean termite, *Reticulitermes flavipes* Kollar. It was later found to elicit trail-following behavior by the subterranean termites *R. flavipes* and *R. virginicus* (Smythe et al. 1967, Matsumura et al. 1969), and by *Coptotermes formosanus* and *Leucotermes speratus* (Matsumura et al. 1972) in open-field bioassays. A chemical corresponding to this substance was isolated both from a southern subterranean termite, *R. virginicus*, and from woods decayed by the fungus *G. trabeum*, and was characterized as a

highly unsaturated alcohol (*Z*, *Z*, *E*)-3,6,8-dodecatrien-1-ol (Matsumura et al. 1969). Several synthetic analogues of the trail-following pheromone from *R. virginicus* were prepared to study the structural basis of its biological activity in this species (Tai et al. 1971). It appeared, from comparative data, that the pheromone-mimicking compounds owed their activities to 2 strategically situated *Z* double bonds.

Two factors prompted us to initiate the current study: first, the necessity to know the production process of the trail-following substances in the wood by the fungus; second, to investigate the influence of the carbon sources on the rate of production in the fungus to maximize its yield from a practical point of view.

METHODS AND MATERIALS

The agar medium was steam-sterilized at 120°C for 15 min and cooled before the addition of the test compounds (see Tables 1 and 2). These compounds were directly dissolved in the medium to make their final concentration 2%. A 25-ml sample of such a preparation was dispensed aseptically into each 9 cm-diam, sterile-glass petri dish, which was then inoculated with minced mycelium obtained from potato–dextrose cultures. Control dishes contained only the potato–dextrose agar or plain agar solution (Difco). The petri dishes containing the fungi (originally supplied by G.R. Esenther) were incubated for 1–2 weeks at 25°C. After incubation, the mycelium was separated from the medium, oven-dried, and weighed. The oven-dried mycelium was extracted with 10–20 ml ether/petri dish at room temperature for 30 min and weighed. The amount of ether-extractable material was estimated by the difference of mycelium weight before and after ether extraction (see Table 2). Each ether extract was concentrated to 1 ml before the bioassay. The 3 media included either 39 g or 13 g potato–dextrose agar, or 15 g agar/liter distilled water.

To study the conversion of ethylene glycol to the trail-following substance, we used the low potato–dextrose agar medium. The surface of the medium (in 9 petri dishes) was flooded with 9 ml methanol containing 15.75 mmol unlabeled and 1.22 μmol 1,2-^{14}C-labeled ethylene glycol (specific activity 18.6 mCi/mmol, ICN Corp., Irvine, California). The methanol was allowed to evaporate over 3 h at room temperature, and the medium was then inoculated with minced mycelium. After 2 weeks' incubation at 32°C, the mycelium from 9 petri dishes was harvested and extracted with ether (200 ml). The ether extract was dried over sodium sulfate, and concentrated to 5 ml. Altogether, 64,278 dpm radioactivity (= 0.128% added ^{14}C-ethylene glycol) was found in the extract.

The extract was chromatographed on 3 different thin-layer (Silica gel HF-254) chromatographic (TLC) systems (System I: *n*-hexane:ether [1:1]; System II: benzene:ethyl acetate [7:1]; System III: benzene:acetone [4:1]) (Matsumura et al. 1969). A Beckman GC-4 gas chromatographic system (GLC with a hydrogen-flame ionization detector) with an SE52 glass column (1.2 m, ID 3 mm) was used at 126°C for both collection and detection of the trail-following substance.

The radioactivity of each fraction from TLC or GLC collection was measured by a liquid scintillation spectrometer with a 10-ml aliquot of counting solution.

The bioassay employed was essentially the same as that developed by Smythe et al. (1967). A 10-μl portion of the sample was taken from the extract and streaked along a 120° arc of a 10 cm–diam circle on the ground surface of a glass plate. Six workers of *C. formosanus* were placed within 1 cm of one end of the line, and the trail-following activity was recorded. The potency of the reference and *G. trabeum* samples was identical to that assayed by Matsumura et al. (1969).

TABLE 1. EFFECTS OF MEDIUM COMPOSITION AND CARBON SOURCES ON THRESHOLD VALUE[a] OF PRODUCTION OF TRAIL-FOLLOWING SUBSTANCE BY *G. trabeum*

Carbon source added	Medium used to grow: Pure agar	Standard[b] potato-dextrose agar	Low[c] potato–dextrose agar	Activity indices
D-Glucose	0.002	0.004	0.02	+
D-Mannose	0.003	0.002	0.009	+
D-Galactose	0.003	0.02	0.01	+
D-Xylose	0.02	0.001	0.001	++
L-Arabinose	0.2	0.02	1	—
α-Cellobiose	0.002	0.004	0.001	++
D-Dextrose	0.2	0.03[d]	0.5[d]	—

[a] The value 1 indicates that the original ether extract (10 μl of 1-ml extract from the total mycelium of each petri dish content) elicited questionable behavioral trail-following response in termites. If the response nearly disappeared on 100-fold dilution, a rating of 0.01 was given.

[b] Potato–dextrose agar (Difco), 39 g/liter distilled water.

[c] Potato–dextrose agar, 13 g/liter distilled water.

[d] In the case of potato–dextrose media, these values represent the media without any extra source of carbon.

RESULTS

The choice of medium is important in obtaining a clear-cut result (Table 1). The low potato–dextrose agar medium for culturing the fungus eliminated almost all the production activity of the substance by the fungus. The results (Tables 1 and 2) clearly indicate that production of the trail-following substance was influenced by the nature of the compounds added to the medium.

TABLE 2. EFFECTS OF ADDED CARBON SOURCES ON THRESHOLD VALUE OF PRODUCTION OF TRAIL-FOLLOWING SUBSTANCE BY *G. trabeum* GROWN IN LOW POTATO–DEXTROSE AGAR MEDIUM

Compound	Threshold value[a] as expressed per total mycelium produced	Threshold value[b] as expressed per gram extract of mycelium present in a streak
D-Glucose	4×10^{-3}	5×10^{-7}
D-Mannose	5×10^{-3}	5×10^{-7}
D-Galactose	1×10^{-2}	1×10^{-6}
Galacturonic acid[c]	3×10^{-2}	1×10^{-5}
2-Keto-D-gluconic acid	1×10^{-1}	1×10^{-6}
D-Xylose	1×10^{-3}	8×10^{-8}
L-Arabinose	4×10^{-1}	8×10^{-5}
D-Ribose	1×10^{-1}	4×10^{-5}
D-Levulose (fructose)	1×10^{-1}	1×10^{-5}
Glyceraldehyde	1×10^{-3}	7×10^{-8}
Glycerol	1×10^{-3}	2×10^{-7}
D-Cellobiose	2×10^{-3}	2×10^{-7}
D-Lactose	1×10^{-2}	2×10^{-6}
D-Sucrose	1×10^{-3}	2×10^{-7}
Ethylene glycol	4×10^{-4}	5×10^{-8}
α-Cellulose[c]	1×10^{-2}	1×10^{-6}
Wood powder[c]	1×10^{-1}	5×10^{-6}
Control[d]	5×10^{-1}	5×10^{-5}

[a] The entire mycelium grown in each petri dish, regardless of the amount grown, was extracted into 1 ml ether; a 10-μl portion was used per assay (as in Table 1).

[b] The weight of the extract of the mycelium was estimated by the difference in weights of the mycelium before and after extraction with ether (usually on the order of 0.05–0.2 g). To arrive at the figure, the corresponding threshold value (footnote *a*) was multiplied by the weight of the mycelium, and then divided by 100, since a 10-μl portion was used per streak for bioassay from a 1-ml stock solution.

[c] Tested on the standard potato–dextrose agar.

[d] Without any extra carbon source.

The compounds that allowed the fungus to actively produce the trail-following substance were: glucose, xylose, mannose, cellobiose, sucrose, glyceraldehyde, glycerol, and ethylene glycol. In addition to the compounds listed in Table 2, glycolic acid, acrolein, glycolaldehyde, glyceric acid, dihydroxy acetone, pyruvic acid, acetaldehyde, glyceraldehyde-3-phosphate, acetic acid, CoA, acetyl CoA, and ethyl pyruvate were added to the medium, but the fungus did not grow well. The lower threshold value shows that the added compound is metabolically utilized by the fungus to facilitate the production of trail-following substance.

The extracts from the incubates with ^{14}C-ethylene glycol that led to the strongest biological activity were purified by the method of Matsumura et al. (1969). The thin-layer chromatogram (Table 3) of the ether extract of the ^{14}C-ethylene glycol shows that the bioactive No. 7 fraction (R_f 0.3–0.4 in System I) corresponded to the dark spot on the radioautogram that represented high radioactivity, and to 0.009% (or 4,442 dpm) of the originally added ^{14}C-ethylene glycol. To ensure the purity of this radioactive fraction, the

TABLE 3. PERCENTAGE DISTRIBUTION OF RADIOACTIVITIES OF FRACTIONS ON THIN-LAYER CHROMATOGRAMS[a]

Fraction	Radioactivity[b] (%)			R_f value
	TLC I	TLC II	TLC III	
No. 1	2.40	1.72	1.84	0.9–1.0
2	0.41	0.58	1.18	0.8–0.9
3	0.33	1.17	0.66	0.7–0.8
4	0.61	0.76	*2.35*[c]	0.6–0.7
5	1.68	*1.02*	4.27	0.5–0.6
6	0.97	*3.58*	24.64	0.4–0.5
7	*6.90*	2.01	4.08	0.3–0.4
8	42.75	25.24	2.17	0.2–0.3
9	12.82	22.38	6.02	0.1–0.2
10	31.36	42.51	52.74	0.0–0.1

[a] Mobile phases are: System I: *n*-hexane:ether (1:1); System II: benzene:ethyl acetate (7:1); System III: benzene:acetone (4:1).

[b] The data are expressed in percentage of added total radioactivity found in each zone. In addition, radioautograms were developed for these TLC plates by using X-ray films to facilitate the visual recognition of the radioactive spots. In System I, the region with the biological activity was represented by a single symmetrical spot in 3 of 3 trials.

[c] The italicized areas are the ones in which the trail-following activities were observed.

silica gel band corresponding to the aforementioned TLC position (R_f 0.3–0.4) was scraped off, extracted with ether, and redeveloped on TLC with solvent System II. The fraction at R_f 0.4–0.6 with bioactivities and radioactivities was purified further on a gas-chromatographic system (Matsumura et al. 1969). Only the fraction that had the same retention time as that of the trail-following substance coincided with the region of bioactivity and radioactivity.

The extent of incorporation of ^{14}C radioactivity into the trail-following substance was at most 2.35% of the total radioactivities found in the ether extract, as judged by the result of TLC analysis on System III (see Table 3).

Although the approach of using a ^{14}C-labeled precursor was taken only for ethylene glycol, ether extracts of mycelia grown with glucose, D-xylose, and glyceraldehyde were also purified on TLC System I and then on the same GLC system. The resulting chromatogram from each test indicated the existence of 1 detectable peak with matching trail-following activity.

DISCUSSION

It is clear from our experiment that the fungus does not produce the trail-following substance on just any medium. The sources of carbon are very important in deciding the amount of the substance produced by the fungus. Our studies established that the fungus, which synthesized hardly any trail-following substance on low potato–dextrose agar, could be changed to produce it actively by the addition of a simple carbon source, such as ethylene glycol.

The key question is whether ethylene glycol is utilized to build the carbon skeleton of (*Z*, *Z*, *E*)-3,6,8-dodecatrien-1-ol, or whether it facilitates the synthesis of the latter in other ways, serving, for instance, as a precursor for an enzyme for synthesis. Our studies showed that when radioactive ethylene glycol is given to the fungus, the radioactivity is eventually incorporated into the trail-following substance, as judged by the results of successive TLC analyses and one GLC analysis. It is not certain just how many carbons of the final products are labeled, but so long as the radioactivity is found in the final product (i.e., so long as even 1 carbon of 12 has the radioactivity), one must conclude that ethylene glycol is partially or wholly utilized as a building block for (*Z*, *Z*, *E*)-3,6,8-dodecatrien-1-ol.

Thus, it is likely that the fungus is capable of synthesizing the trail-following substance from a compound with few carbons.

The finding that both the fungus and a southern subterranean termite, *Reticulitermes virginicus*, produce the same trail-following substance (Matsumura et al. 1969) was puzzling to many scientists. Some have suggested that

the fungus and the termites may rely on a readily available precursor in the wood, such as linoleic acid, as a common source for its synthesis. An example of the insect utilization of a specific precursor has recently been shown by Renwick et al. (1976), who demonstrated that verbenol, a pheromone of *Ips paraconfusus*, could be produced by the insect from α-pinene present in the host tree. Nevertheless, such a possibility for a specific requirement of a precursor appears to be remote for the fungus in the light of the present findings that it can produce the trail-following chemical from several simple carbon sources.

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DETERMINATION OF THE ENANTIOMERIC COMPOSITION OF SEVERAL INSECT PHEROMONE ALCOHOLS

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Abstract—Details are given for the determination of the enantiomeric composition of several insect pheromone alcohols. The two methods used in the determination were: formation of the derivative with (+)-α-methoxy-α-trifluoromethylphenylacetyl chloride and the use of chiral lanthanide shift reagents. The five alcohols studied and their enantiomeric compositions are: sulcatol from *Gnathotrichus sulcatus* 65:35 (+)/(−), *trans*-verbenol from *Dendroctonus frontalis* 60:40 (+)/(−), 4-methyl-3-heptanol from *Scolytus multistriatus* 100% (−), seudenol from *Dendroctonus pseudotsugae* 50:50 (+)/(−), and ipsdienol from *Ips pini* (Idaho) 100% (−). Determinations were done on 50–500 μg substrate.

Key Words—pheromone, *Gnathotrichus sulcatus*, *Scolytus multistriatus*, *Dendroctonus frontalis*, *Dendroctonus pseudotsugae*, *Ips pini* Idaho, enantiomeric composition, seudenol, sulcatol, *trans*-verbenol, chiral shift reagents, 4-methyl-3-heptonal.

INTRODUCTION

Since "odor" receptors in man and in insects can discriminate between enantiomers (Friedman and Miller 1971, Riley et al. 1974*a*, Riley et al. 1974*b*, Iwaki et al. 1974, Kafka et al. 1973, Lensky and Blum 1974, Staedler 1974), it is

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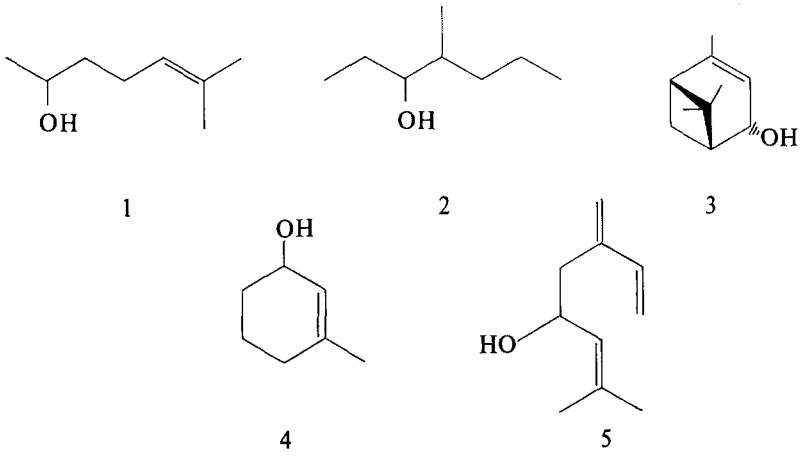
important to describe the enantiomeric composition of chiral molecules that impinge on these receptors. It has also been shown that one enantiomer can synergize the other (John Borden, private communication).

Usually, pheromone components are isolated in such small amounts that an accurate optical rotation cannot be obtained. Recourse may be had to two recently developed procedures for determining enantiomeric composition: the use of Mosher's reagent or of chiral shift reagents. We report here details of the application of these reagents to several chiral alcohols that are components of insect pheromones. The alcohols previously isolated and identified are: (1) sulcatol, 6-methyl-5-hepten-2-ol, from *Gnathotrichus sulcatus* (Byrne et al. 1974); (2) 4-methyl-3-heptanol, from *Scolytus multistriatus* (Pearce et al. 1975); (3) *trans*-verbenol, from *Dendroctonus frontalis* (Renwick 1967); (4) seudenol, 3-methyl-2-cyclohexen-1-ol, from *Dendroctonus pseudotsugae* (Vité et al. 1972*a*); and (5) ipsdienol, 2-methyl-6-methylene-2,7-octadien-4-ol from *Ips pini* Idaho (Vité et al. 1972*b*).



METHODS AND MATERIALS

Synthetic sulcatol (structure 1) was obtained by lithium aluminum hydride reduction of the commercially available ketone and was resolved as the brucine salt of the half-acid phthalate. The oily half-acid phthalate (Vogel 1962) was extracted with benzene–ether, and the extracts were taken to dryness in vacuo and used without further purification. A hot saturated acetone solution of this product (13.8 g) and 21.5 g brucine gave 13.3 g crystals on cooling to room temperature. Three recrystallizations gave 6.9 g brucine salt. The alcohol was recovered as described by Vogel (1962). The

gas-liquid chromatography (GLC) purified alcohol had a specific rotation of $[\alpha]_D^{20} = +14.8°$ (hexane). Natural sulcatol (structure 1) was obtained from the frass of the ambrosia beetle, *Gnathotrichus sulcatus* (Byrne et al. 1974).

Synthetic 4-methyl-3-heptanol (structure 2) was obtained by reduction of the commercially available ketone with lithium aluminum hydride; the diastereomers were separated by gas chromatography. The natural 4-methyl-3-heptanol (structure 2) isolated from the elm bark beetle, *Scolytus multistriatus* (Pearce et al. 1975), was one diastereomer.

One sample of synthetic *trans*-verbenol (structure 3) ($[\alpha]_D^{20} = +6.25°$, c = 9.9, $CHCl_3$) was obtained from Chemical Samples Co., Inc., and another ($[\alpha]_D^{20} = +165°$, c = 0.5, $CHCl_3$) was prepared from (+) α-pinene ($[\alpha]_D^{20} = 46.5°$, neat, by the method of Cooper et al. (1967). Natural *trans*-verbenol (structure 3) was obtained by collection for 96 h on Porapak Q (Byrne et al. 1975) of volatiles from 16 short leaf pine logs infested with 800 female *Dendroctonus frontalis*. The Porapak Q was extracted with pentane in a Soxhlet extractor and concentrated by distillation of the solvent through a glass bead packed column at atmospheric pressure. The concentrated sample was subjected to fractionation by gas chromatography as follows: Column A glass, 10% Carbowax 20M on Chromosorb W 60/80 mesh, 1·5 m × 10.3 mm (OD), 120 cm^3/min He flow rate, 130°C isothermal, collect fraction 24–30 min; Column B glass, 5% Apiezon L on Chromosorb G 60/80 mesh, 2.4 m × 6.3 mm (OD), 60 cm^3/min He flow rate, 110°C isothermal, collect fraction 14–18 min; Column C glass, 5% FFAP on Varaport 30, 1.5 m × 6.3 mm (OD), 50 cm^3/min He flow rate, 100°C isothermal, collect fraction 14–17 min.

Synthetic seudenol (structure 4) was obtained by lithium aluminum hydride reduction of 3-methyl-2-cyclohexen-1-one. Natural seudenol (structure 4) was obtained by homogenization in hexane in a Waring blender of about 400 g frass from female Douglas fir beetles, *Dendroctonus pseudotsugae*. The filtered extract was concentrated by distillation at atmospheric pressure of the solvent through a 250-mm column packed with glass helices. A crude concentrate, obtained by short-path vacuum distillation at 80°C at 0.005 mm, was fractionated by gas chromatography on the following glass columns: Column A, 10% Carbowax 20M on Chromosorb W 60/80 mesh, 1.5 m × 10.3 mm (OD), 100 cm^3/min He flow rate, 120°C isothermal, collect fraction 16–27 min; Column B, 5% SE-30 on Chromosorb G 60/80 mesh, 3 m × 6.3 mm (OD), 50 cm^3/min He flow rate, 75°C isothermal, collect fraction 13–18 min; Column C, 4% Carbowax 20M on Chromosorb G 60/80 mesh, 5.5 × 6.3 mm (OD), 60 cm^3/min He flow rate, 130°C isothermal, collect fraction 24–27 min.

Synthetic ipsdienol (structure 5) was prepared as reported by Riley et al. (1974*c*). Natural ipsdienol (structure 5) was obtained by Porapak Q aeration

of 1000 males of *Ips pini*, Idaho. The Porapak Q was Soxhlet extracted with pentane, and the pentane extracts were concentrated in the same manner as above. The ipsdienol (structure 5) was fractionated by gas chromatography as follows: Column A glass, 4% Carbowax 20M on Chromosorb G 60/80 mesh, 5.5 m × 6.3 mm (OD), 45 cm^3/min He flow rate, 135°C isothermal, collect fraction 38–47 minutes. Column A, temperature program starting at 100°C by 0.5°/min, 80 cm^3/min He flow rate, collect fraction 61–65 min. Column A, 155°C isothermal, 90 cm^3/min He flow rate, collect fraction 15–16 min.

A resolution of the ipsdienol alcohol was attempted by the method of Vogel (1962). The brucine salt of the ipsdienol–hydrogen phthalate was recrystallized to constant rotation $[\alpha]_D = 39.6°$. Decomposition of the brucine salt and subsequent reduction of the ipsdienol–hydrogen phthalate with lithium aluminum hydride gave a very poor yield of the desired allylic alcohol; however, a source of (+) ipsdienol was available from the species *Ips paraconfusus* Lanier ($[\alpha]_D^{20} = +10 \pm 0.9°$, Silverstein et al. 1966). The volatiles released by the males of *I. paraconfusus* were cold-trapped (Browne et al. 1974). The cold trap was rinsed with ether, and the condensate was continuously extracted with ether for 24 h. The combined ether solutions were dried over sodium sulfate and concentrated as described previously. The ipsdienol was fractionated from the concentrated ether solution in the same manner as was ipsdienol from *I. pini*, except that an additional GLC column was used: Column D, 5% Apiezon L on Chromosorb G 60/80 mesh, 2.0 m × 6.3 mm (OD), 60 cm^3/min He flow rate, 125°C isothermal, collect fraction 9–10 min.

(+)-α-Methoxy-α-trifluoromethylphenylacetyl chloride [(+)-MTPA-Cl] was prepared from (+)-α-methoxy-α-trifluoromethylphenylacetic acid (Aldrich lot 043017) by reaction with thionyl chloride (Dale et al. 1969). (+)-MTPA esters of synthetic and natural alcohols were prepared in the following way: GLC-purified samples were washed from collection tubes into 4 mm OD glass tubes with 50 μl dry carbon tetrachloride, and 5 μl (+)-MTPA-Cl was added along with 50 μl dry pyridine. The glass tube was purged with nitrogen, sealed, shaken, and left with occasional shaking for 24 h. The esters of the two secondary alcohols, 4-methyl-3-heptanol (structure 2) and sulcatol (structure 1), were isolated in pure form by GLC fractionation, but the thermally labile esters of the two allylic alcohols were purified by extraction–liquid chromatography: The reaction product was treated with 5 μl 3-dimethylamino-1-propylamine (Dale and Mosher 1973), 1 ml carbon tetrachloride was added, and the solution was sequentially washed with 1 ml 6 N hydrochloric acid, 1 ml water, 1 ml saturated sodium carbonate, and 1 ml water. The product was washed into a silica gel column (1.5 cm × 6.0 mm ID), washed with 3 portions of carbon tetrachloride, and eluted with benzene.

This solution was evaporated to dryness, and the residue was taken up in the appropriate solvent for NMR studies.

The following shift reagents were obtained and sublimed immediately before use: *tris*(3-heptafluorobutyryl-d-camphorato)europium III, $Eu(hfbc)_3$ (Norell Chemical Co., Inc., 170–180°/0.005 Torr; Aldrich Chemical Co., Inc., 170–180°/0.005 Torr); *tris*(3-trifluoromethylhydroxymethylene-d-camphorato)europium III [Alfa Inorganics, Inc., Eu-Opt, lot no. 111772, $Eu(facam)_3$, 200°/0.010 Torr]; and the achiral shift reagent *tris*(6,6,7,7,8,8,8, heptafluoro-2,2-dimethyl-3,5-octanedionato)europium III, $Eu(fod)_3$ (Alfa, 135–140°/0.010 Torr).

Coaxial tubes (inside sample capacity, 50 μl) in thin-wall (5 mm OD) NMR tubes (Wilmad Glass Co., Inc.) were used in all lanthanide shift reagent studies and most Mosher derivative studies. The remaining Mosher derivatives were studied in 100-μl capacity NMR tubes also obtained from Wilmad Glass Co.

Benzene-d_6 and chloroform-d, 100% deuterated, were obtained from Stoler Isotopes, Inc. Carbon tetrachloride was spectranalyzed grade from Fisher Scientific, predried for at least 1 week over 4A molecular sieves. In the coaxial tube experiments, tetramethylsilane and trifluorotrichloroethane in deuterated chloroform (100%) were placed in the outer tube for ^1H (PMR) and ^{19}F (FMR) studies, respectively.

Lanthanide shift reagent studies were performed in the following way: All operations that would involve exposure of the samples to the atmosphere were performed under an argon atmosphere in a dry box. A weighed quantity of freshly sublimed lanthanide shift reagent was dissolved in a known volume of dry carbon tetrachloride. This stock solution of lanthanide shift reagent was stored and used under argon, but was discarded after 72 h. A stock solution of GLC-purified synthetic substrate was prepared in carbon tetrachloride, and its concentration was determined by GLC integration both before and after shift studies. The substrate and shift reagent stock solutions were mixed, then filtered through a glass-wool plug into the NMR tube. Total sample volume was 40–50 μl and was measured exactly. Except where noted in the text, substrate concentration was held constant and was approximately 0.05–0.08 M (500 μg in 50 μl solvent). Separate samples were prepared for each lanthanide-to-substrate ratio tested.

Gas-liquid chromatography was performed on a Varian Model 2740 gas chromatograph fitted with flame ionization detectors and a splitter with a split ratio of 100:1. A thermal gradient collector (Brownlee and Silverstein 1968) was used to collect fractions of column effluent in 30.5-cm glass capillary tubes. Optical rotations were obtained on either a Durrum Jasco Model ORD/UV/CD-5 recording spectropolarimeter or a Perkin–Elmer Model 531 polarimeter. NMR spectra were obtained on a Varian XL-100 Fourier Transform spectrometer with an internal deuterium lock.

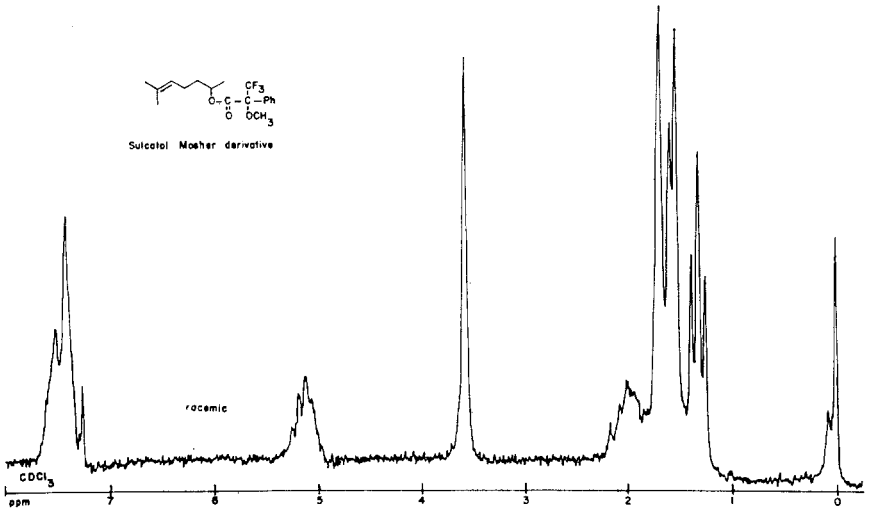
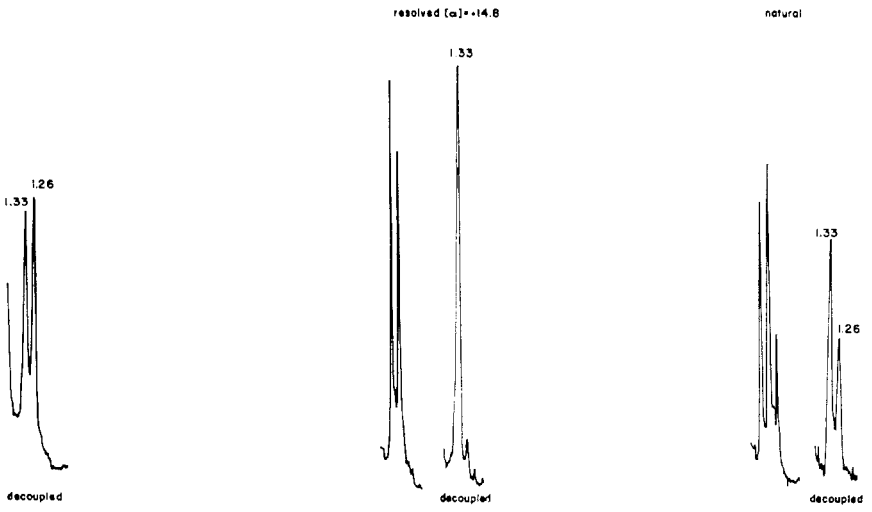


FIG. 1a. NMR spectrum of the Mosher derivative of racemic sulcatol.



(b) (c) (d)

FIG. 1b–d. The C-1 methyl signal from the decoupled spectra of (b) racemic, (c) resolved, (d) and natural sulcatol.

RESULTS AND DISCUSSION

Mosher Derivative Studies

The PMR spectrum of the (+)-MTPA ester of synthetic racemic sulcatol is shown in Fig. 1a. The only signal that reflected the enantiomeric composition was that of the C-1 methyl protons. Decoupling by irradiation of the C-2 methine proton (Fig. 1b) collapsed the C-1 methyl triplet to a pair of singlets, at 1.33 and 1.26 ppm, indicating that the original triplet was actually two overlapping doublets, each representing an enantiomer. In the PMR spectrum of the (+)-MTPA ester of (+)-sulcatol, the C-1 methyl signal was a doublet (Fig. 1c), which collapsed to a singlet at 1.33 ppm when the C-2 methine proton was irradiated. The (+)-MTPA ester of sulcatol, isolated from the insect (120 μg), gave a PMR spectrum in which the C-1 methyl proton signal appeared as an unsymmetrical triplet (Fig. 1d). This signal collapsed to two singlets of unequal intensity, the dominant singlet at 1.33 ppm and the smaller singlet at 1.26 ppm. The natural sample thus consists of a 65(+)/35(−) mixture of enantiomers. The FMR spectrum of each of the sulcatol derivatives showed only a singlet for the trifluoromethyl fluorine resonance.

Of the two diastereomeric pairs present in synthetic 4-methyl-3-heptanol (structure 2), only one was observed in the natural product, as indicated by GLC analysis. The (+)-MTPA ester of the synthetic diastereomer corresponding to that isolated gave the PMR and FMR spectra reproduced in Fig. 2. The carbinyl proton signal (5.0 ppm) appeared as two overlapping quartets, reflecting the racemic composition of the alcohol. The same composition gave rise to two apparent singlets for the trifluoromethyl fluorine resonance in the FMR spectrum. The (+)-MTPA ester of the natural alcohol gave the PMR and FMR spectra shown in Fig. 3. Clearly, only one enantiomer was present in the isolated alcohol, which is responsible for the upfield apparent singlet in the FMR spectrum of the racemic ester. The natural product has an optical rotation of $[\alpha]_D^{20} = -15°$, and it can be concluded from these data that it is enantiomerically pure. The OCH_3 absorption ($\sim$ 3.56 ppm) reflects small long-range coupling to the CF_3 group.

The (+)-MTPA esters of *trans*-verbenol (structure 3), seudenol (structure 4), and ipsdienol (structure 5) gave PMR and FMR spectra that showed incomplete separation of the enantiomeric nuclei. The separation of enantiomeric nuclei observed in (+)-MTPA ester of 4-methyl-3-heptanol may be the result of the steric hindrance by the 4-methyl group. In addition to the small enantiomeric shift differences noted for the esters of these alcohols, it was found that the allylic esters were totally decomposed on attempts at GLC purification. This difficulty was avoided by use of the extraction–liquid chromatography procedure, but large losses were incurred in handling

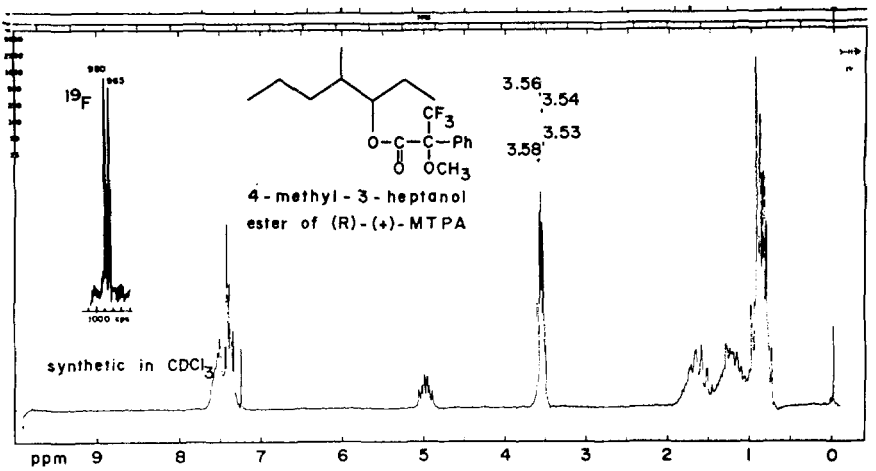


FIG. 2. PMR and FMR spectra of the (+)-MTPA ester of one of the diastereomers (racemic) of 4-methyl-3-heptanol.

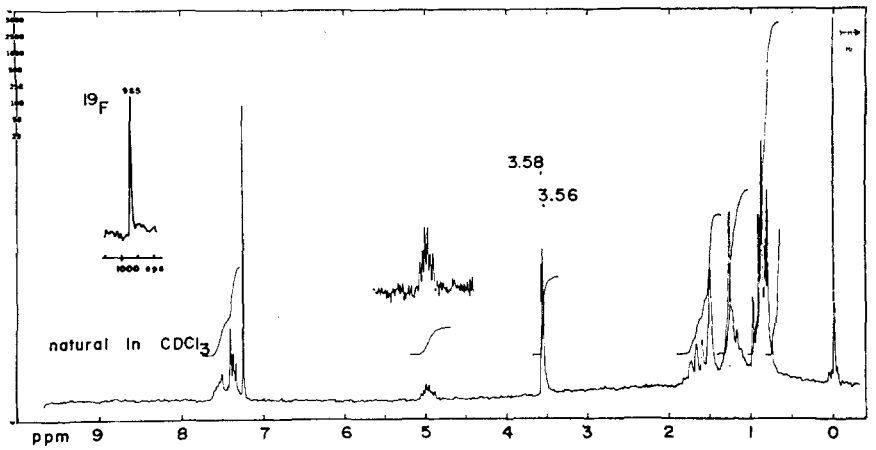


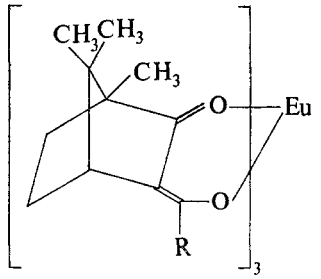
FIG. 3. PMR and FMR spectra of the (+)-MTPA ester of the natural (isolated) 4-methyl-3-heptanol.

small samples. Larger enantiomeric shift differences and thus applicability to a larger spectrum of alcohols were found with the use of chiral lanthanide shift reagents.

Lanthanide Shift Reagent Studies

Two chiral europium (III) shift reagents are commercially available and

have been extensively evaluated (Goering et al. 1974 and references cited therein). These are *tris*(3-trifluoroacetyl-d-camphorato)europium (III), $Eu(facam)_3$, and *tris*(3-heptafluorobutyryl-d-camphorato)europium (III) $Eu(hfbc)_3$, structures 6 and 7, respectively.



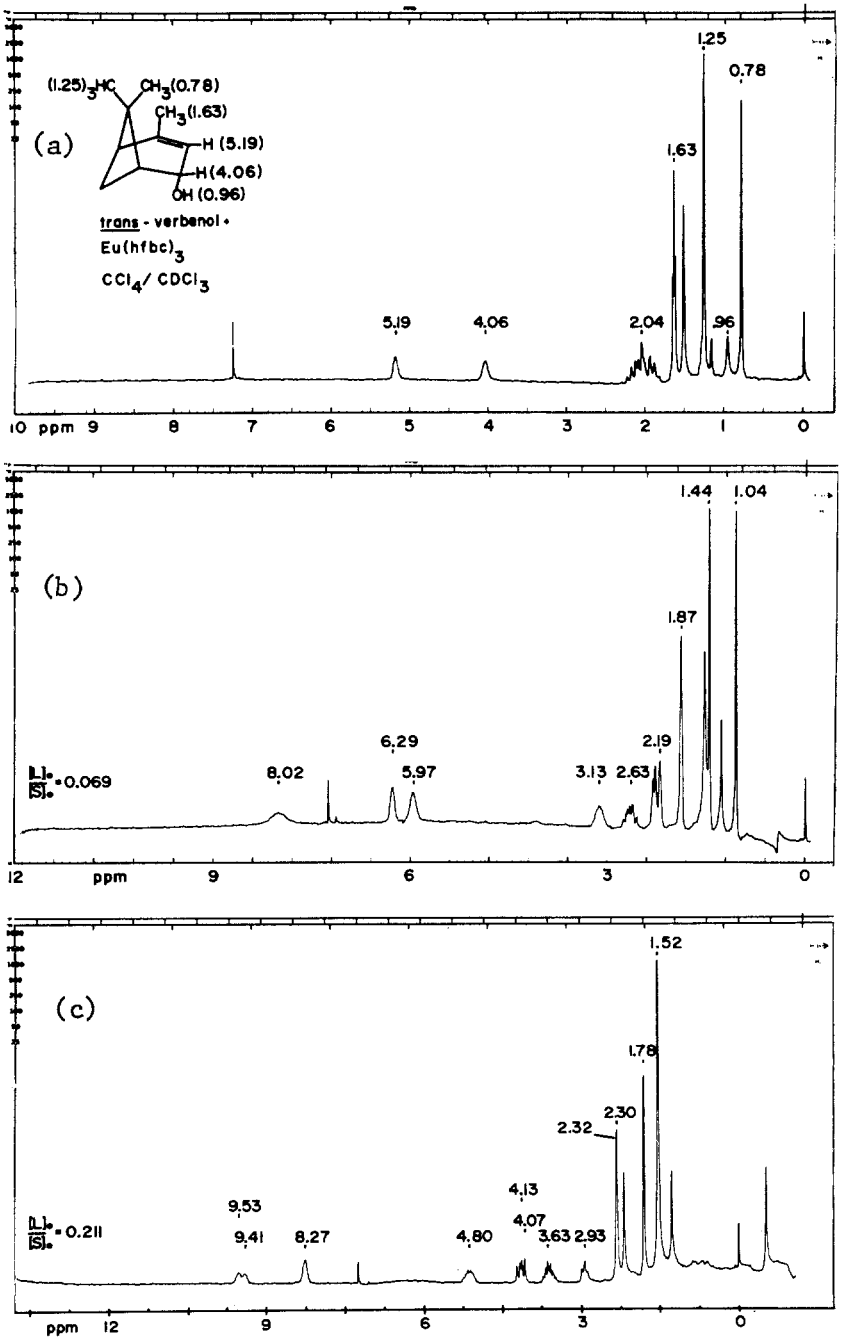
6. R = CF_3
7. R = C_3F_7

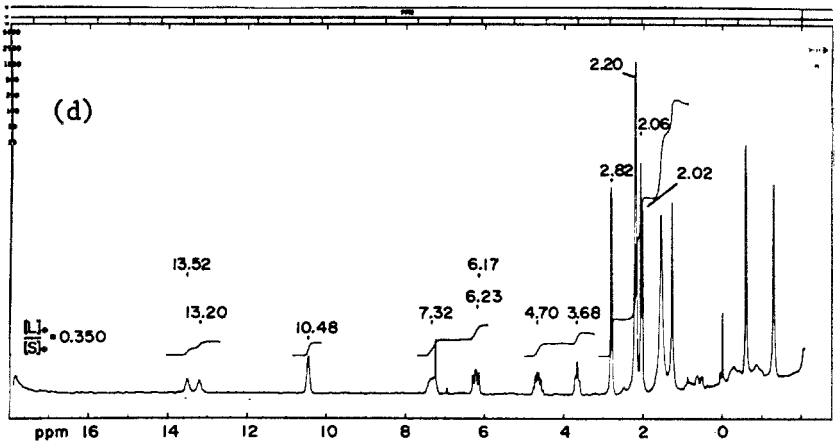
Goering et al. (1974) have noted that the magnitude of induced shifts ($\Delta\delta$) and of enantiomeric shift differences ($\Delta\Delta\delta$) for substrates in the presence of $Eu(facam)_3$ and $Eu(hfbc)_3$ are greater in carbon tetrachloride than in deuterobenzene or deuterochloroform. Our experience confirmed this finding. It is also easier to remove trace quantities of water from carbon tetrachloride than from either benzene or chloroform; it is the least hygroscopic of the three solvents and, with reasonable care in handling, can be used without serious interference from water.

The 50-μl capacity coaxial NMR tubes are suitable for 100–500-μg samples, since both induced shifts and enantiomeric shift differences dropped off severely at lower molar concentrations of substrate even when the lanthanide-to-substrate ratio was maintained. It was found that solutions of 50–100-μg samples could be used with these tubes if the solvent volume was reduced to less than 50 μl to maintain the concentration of the substrate in the 0.02–0.08 M region.

Commercial shift reagent samples were sublimed before use to eliminate impurities that caused serious broadening of spectra. Even with this step and the minimization of sample exposure to water, it was found necessary, especially at very high shift reagent–to–substrate ratios, to filter prepared solutions through a glass-wool plug in a constricted glass tube (McCreary et al. 1974).

Goering et al. (1974) have observed that, in general, both induced shifts ($\Delta\delta$) and enantiomeric shift differences are greater in the presence of $Eu(hfbc)_3$ than in the presence of $Eu(facam)_3$. In preliminary studies on ipsdienol (see below), the same results were obtained. In general, it was found that with substrate concentrations of from 0.02 to 0.08 M, signal broadening was experienced when shift reagent–to–substrate ratios exceeded about


(a)
(1.25)3HC
CH3(0.78)
CH3(1.63)
H (5.19)
H(4.06)
OH (0.96)
trans - verbenol -
Eu(hfbc)3
CCl4/ CDCl3
1.25
0.78
1.63
5.19
4.06
2.04
.96
10 ppm 9 8 7 6 5 4 3 2 1 0
(b)
1.44
1.04
1.87
6.29
2.19
[L]o/[S]o = 0.069
8.02
5.97
3.13
2.63
12 ppm 9 6 3 0
(c)
1.52
1.78
2.32
2.30
4.13
4.07
9.53
9.41
8.27
4.80
3.63
2.93
[L]o/[S]o = 0.211
ppm 12 9 6 3 0



FIGS. 4a–d. NMR spectrum of *trans*-verbenol and the chemical shifts caused by increasing L_o/S_o.

0.5:1. In two cases tested, this broadening was reversed when substrate was added to decrease the shift reagent–to–substrate ratio.

One important note should be stressed in the interpretation of spectra involving either chiral or achiral shift reagents. The two protons of a CH_2 group in an enantiomer may be diastereotopic, but they may appear to be a sharp singlet (fortuitous chemical shift equivalence). In the presence of a chiral or achiral shift reagent, they may pull apart into two signals. This separation can lead to confusion in the determination of enantiomeric composition. The determination should be based on the signals arising from CH or CH_3 groups.

Figure 4 shows NMR spectra obtained on a synthetic sample of *trans*-verbenol (structure 3) ($[\alpha]_D = +6°$) in the presence of Eu(hfbc)$_3$. The carbinyl proton, observed at 4.06 ppm in the unshifted spectrum, moves past the olefinic proton, and at a lanthanide–to–substrate molar ratio of 0.211 ($[L]_o/[S]_o$) separates into two signals. At a lanthanide–to–substrate ratio of 0.350 ppm, the separation of the enantiomeric proton signals reaches baseline (13.20, 13.52 ppm). None of the other peaks showed useful separation.

It has been reported (McCreary et al. 1974) that when the probe temperature is lowered, both $\Delta\delta$ and $\Delta\Delta\delta$ increase. Figure 5 shows that, for the carbinyl proton of *trans*-verbenol (structure 3) ($[\alpha]_D = +6°$), $\Delta\Delta\delta$ is 32 Hz at 38°C (13.20, 13.52 ppm), 57 Hz at 10°C (14.41, 14.98 ppm), and 60 Hz at 0°C (14.72, 15.32 ppm). In addition, at 0°C, the enantiomeric olefinic protons (11.71, 11.99 ppm) reached baseline separation ($\Delta\Delta\delta = 28$ Hz), and the methyl signals showed significant enantiomeric shift differences.

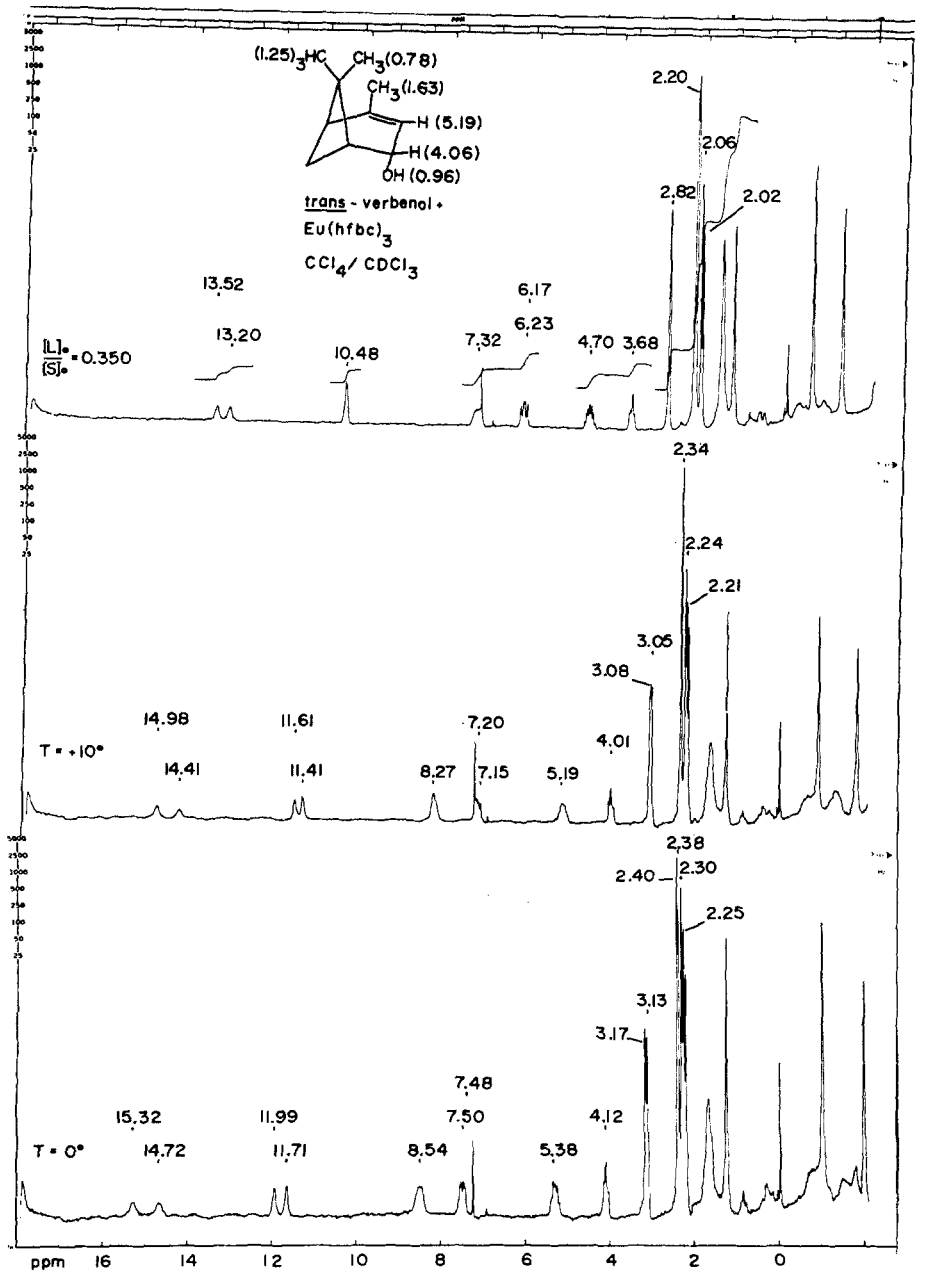


FIG. 5. Temperature dependence of enantiomeric shift differences of *trans*-verbenol induced by $Eu(hfbc)_3$.

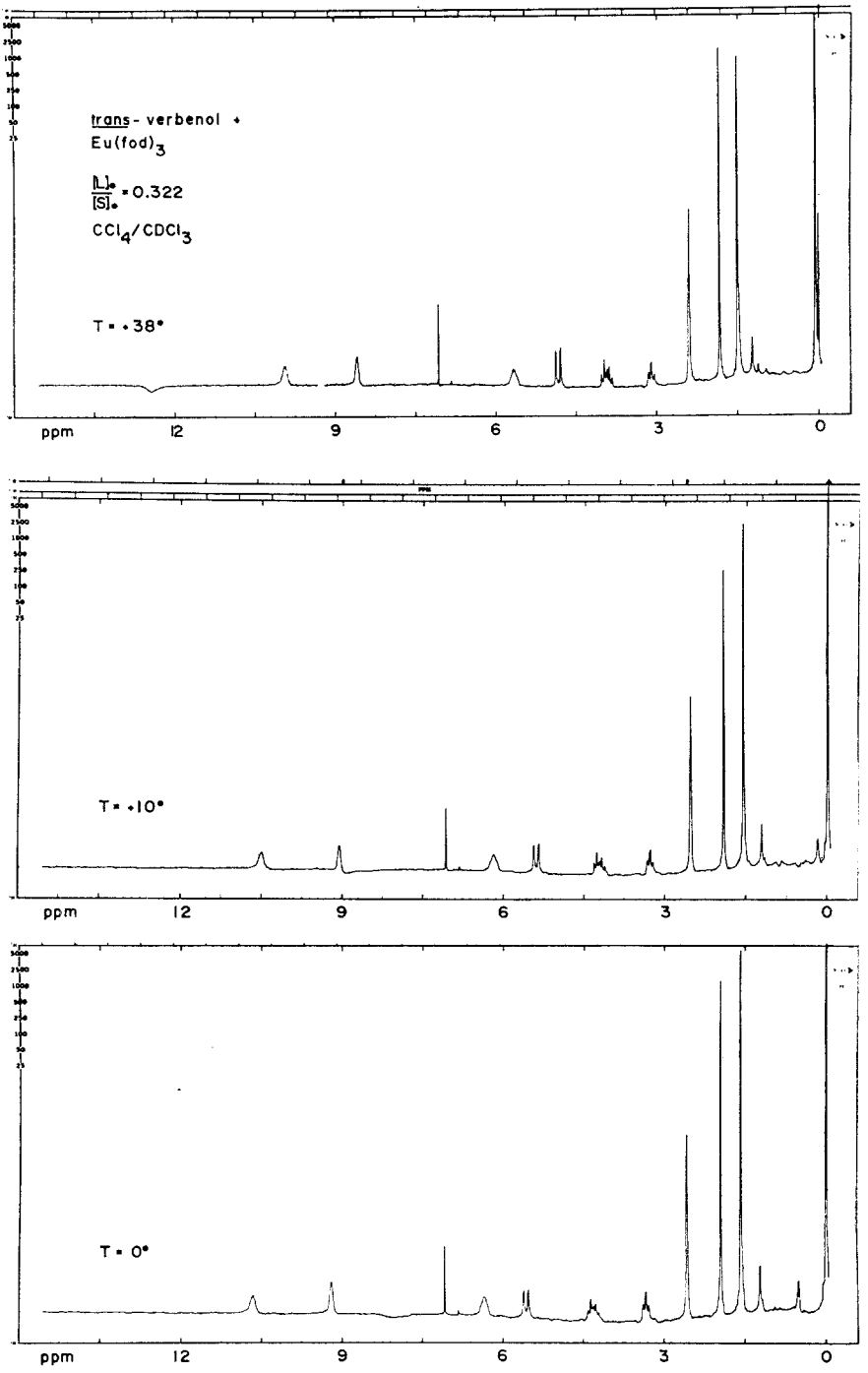


FIG. 6. Temperature dependence of enantiomeric shift differences of *trans*-verbenol induced by $Eu(fod)_3$.

To confirm that the observed shift differences reflected enantiomeric composition, a 500-μg sample of *trans*-verbenol (structure 3) ($[\alpha]_D = +6°$) was observed in the presence of the achiral shift reagent Eu(fod)$_3$. Comparison of Figs. 5 and 6 shows that at the same lanthanide–to–substrate ratio, the carbinyl proton is a broad singlet in the presence of Eu(fod)$_3$ (10.00 ppm) (Fig. 6), whereas in the presence of Eu(hfbc)$_3$, two signals are observed (Fig. 5). On lowering the temperature to +10°C and 0°C, no separation of the signals was observed with the achiral shift reagent.

Spectra of a sample of about 200 μg *trans*-verbenol (structure 3) (isolated from female southern pine beetles) in the presence of Eu(hfbc)$_3$ at an approximate $[L]_o/[S]_o = 0.349$ are reproduced in Fig. 7. Clearly, this sample was a mixture of enantiomers, and this was confirmed when the probe temperature was reduced to −15°C; there is a 102-Hz shift difference for the enantiomeric carbinyl proton signals (17.95, 18.97 ppm), a 33-Hz shift difference for the enantiomeric olefinic protons (14.18, 13.85 ppm), and a 25-Hz shift difference for the bridghead proton adjacent to the hydroxy bearing carbon (10.51, 10.76 ppm). It should be noted that the relative shifts of the enantiomeric peaks may change for each of the enantiomeric protons observed. The isolated *trans*-verbenol was a 60:40 mixture of enantiomers.

To assign peaks to the enantiomers, we spiked the synthetic (+) *trans*-verbenol (structure 3) ($[\alpha]_D = +165°$) with synthetic *trans*-verbenol (structure 3) ($[\alpha]_D = +6°$) and determined the NMR of this sample in the presence of Eu(hfbc)$_3$ (Fig. 8). It can be seen that the (+) enantiomer is represented by the downfield signal of the two signals arising from the enantiomeric carbinyl protons (average position ~ 12.0 ppm) and by the upfield signal of the two signals from the olefinic proton (average position ~ 10.3 ppm). If only one enantiomer were present, a single signal would appear, and the shift would depend on the lanthanide–to–substrate ratio, the temperature, and other factors. Spiking eliminates errors due to changes in the various factors controlling the downfield position of the singlet by showing both enantiomers with one enhanced.

Figure 9 shows spectra of 500 μg synthetic seudenol (structure 4) in the presence of varying amounts of Eu(hfbc)$_3$. The olefinic proton absorption that appears at 5.33 ppm in the unshifted spectrum pulls apart at $[L]_o/[S]_o = 0.102$, reaches baseline separation at $[L]_o/[S]_o = 0.205$, and gives $\Delta\Delta\delta$ of 18 Hz at $[L]_o/[S]_o = 0.255$ (8.93, 9.11 ppm). Although the 3-methyl singlet also separates, it does not reach baseline separation, and the olefinic proton is the only signal that can be used to determine quantitatively enantiomeric composition. In Fig. 10, a low-temperature study of the $[L]_o/[S]_o = 0.205$ sample shows enhancement of $\Delta\Delta\delta$ for the olefinic proton (8.82, 9.14 ppm), but an enantiomeric shift difference large enough to determine enantiomeric composition quantitatively is not observed for any other signal.

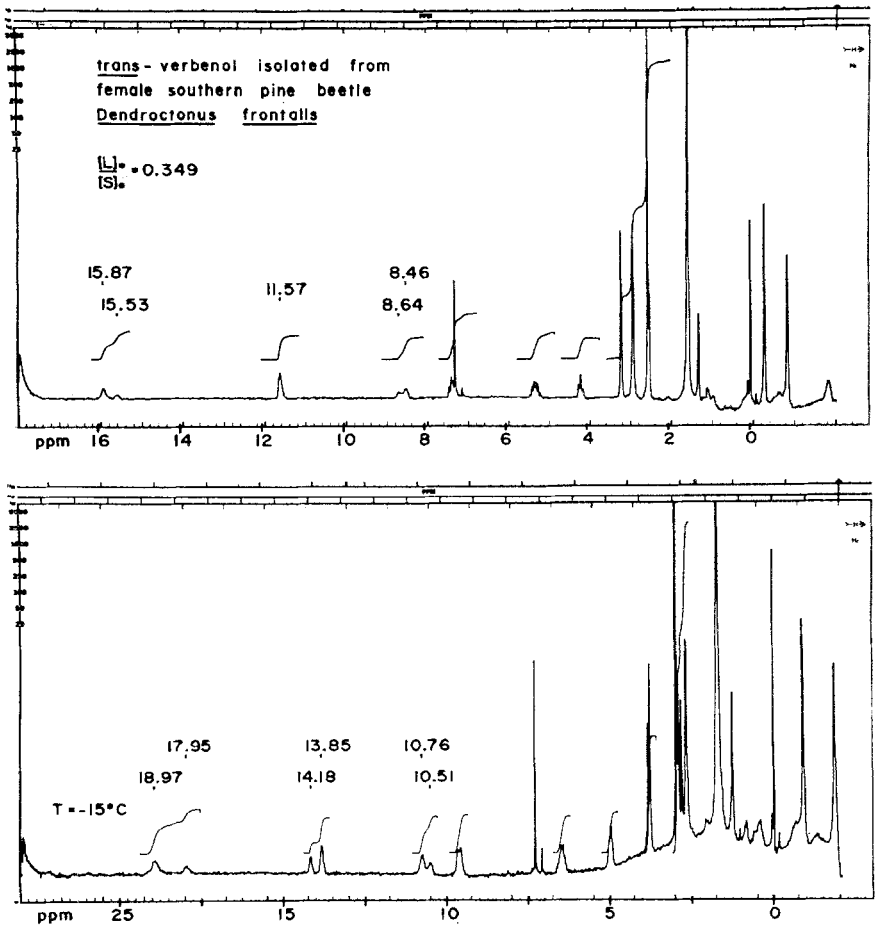


FIG. 7. NMR spectrum of natural *trans*-verbenol at +38°C and −15°C.

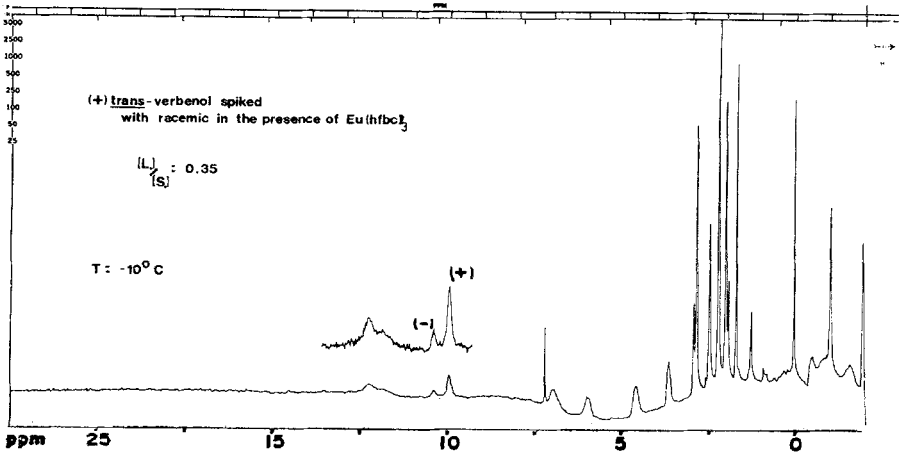
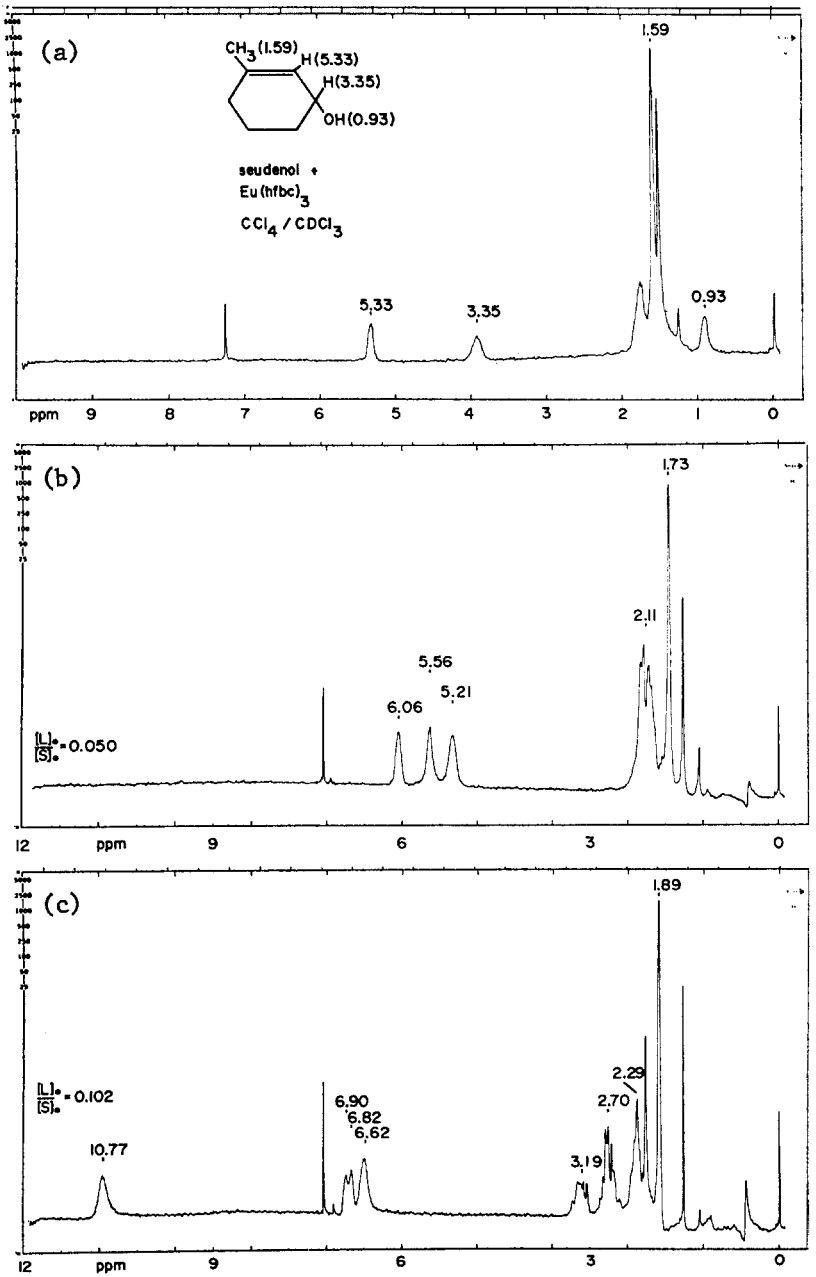
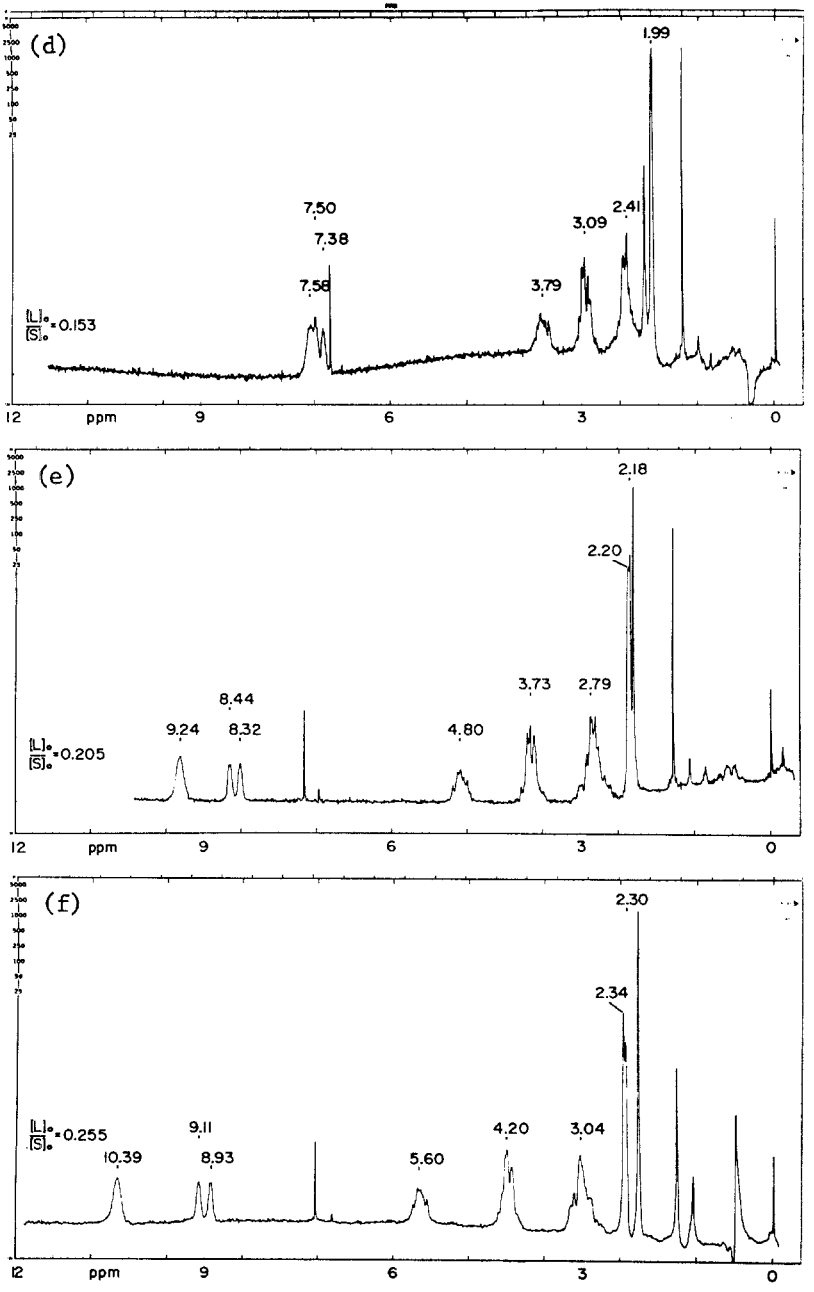
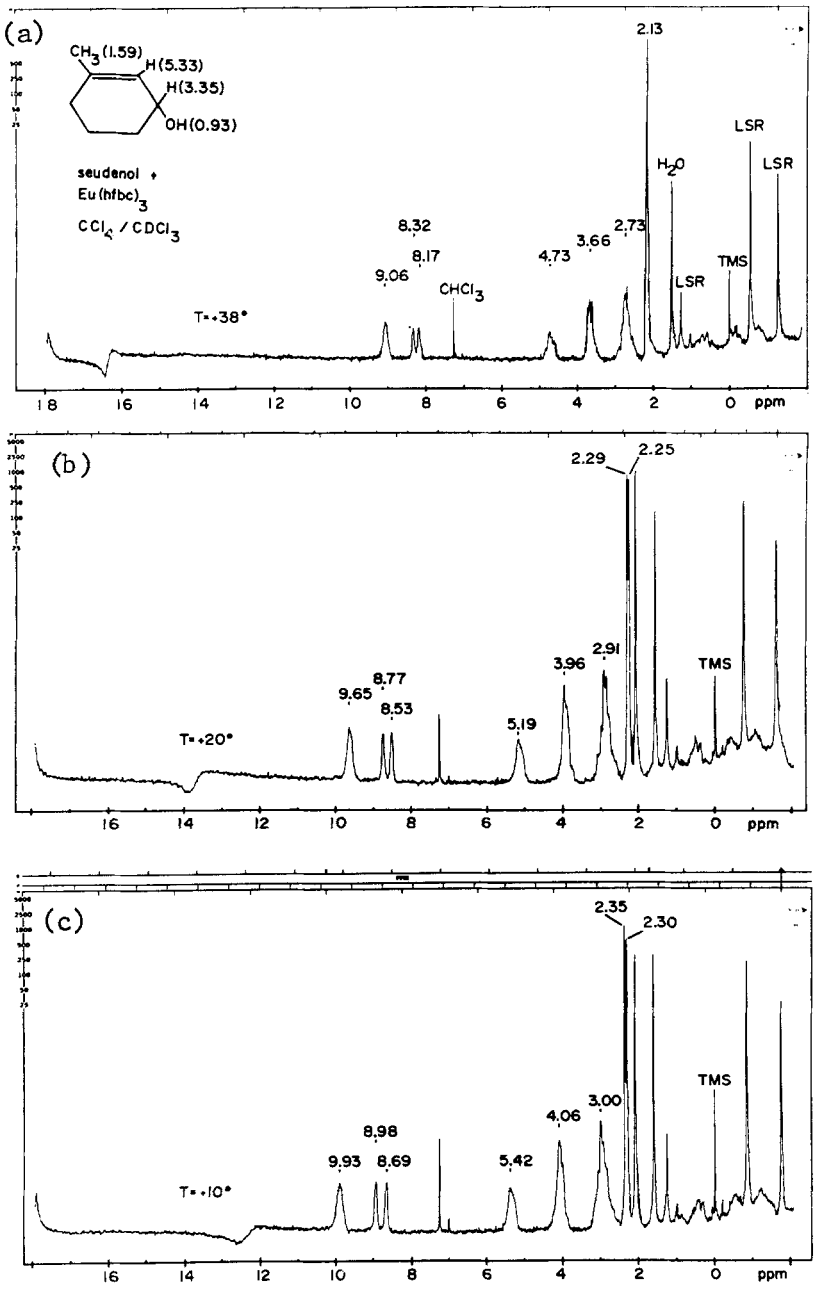


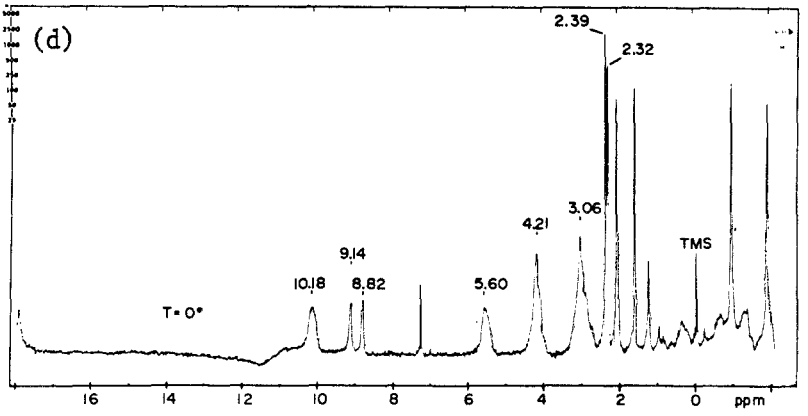
FIG. 8. Synthetic (+) *trans*-verbenol spiked with racemic in the presence of Eu(hfbc)$_3$ at −10°C.



FIGS. 9. (a) NMR spectrum of seudenol; (b–f). Chemical shifts of enantiomeric signals as a result of increasing $[L]_o/[S]_o$.


(d)
1.99
7.50
7.38
7.58
3.79
3.09
2.41
[L]o/[S]o=0.153
12
ppm
9
6
3
0
(e)
2.18
2.20
8.44
9.24
8.32
4.80
3.73
2.79
[L]o/[S]o=0.205
12
ppm
9
6
3
0
(f)
2.30
2.34
[L]o/[S]o=0.255
9.11
10.39
8.93
5.60
4.20
3.04
12
ppm
9
6
3
0


(a)
CH3 (1.59)
H(5.33)
H(3.35)
OH(0.93)
seudenol +
Eu(hfbc)3
CCl4 / CDCl3
T=+38°
9.06
8.32
8.17
CHCl3
4.73
3.66
2.73
2.13
H2O
LSR
TMS
LSR
LSR
18 16 14 12 10 8 6 4 2 0 ppm
(b)
T=+20°
9.65
8.77
8.53
5.19
3.96
2.91
2.29
2.25
TMS
16 14 12 10 8 6 4 2 0 ppm
(c)
T=+10°
9.93
8.98
8.69
5.42
4.06
3.00
2.35
2.30
TMS
16 14 12 10 8 6 4 2 0 ppm



FIGS. 10a–d. Temperature dependence of enantiomeric shift differences of seudenol in the presence of Eu(hfbc)$_3$.

Figure 11 shows spectra of 128 μg seudenol (structure 4), isolated from the frass of female *D. pseudotsugae*, in the presence of Eu(hfbc)$_3$ at $[L]_o/[S]_o$ = 0.195. The olefinic proton shows $\Delta\Delta\delta$ of 11 Hz at ambient probe temperature (7.85, 7.96 ppm), and 36 Hz at -15°C (8.61, 8.97 ppm). The material isolated is racemic.

Spectra of synthetic ipsdienol (structure 5) are shown in the presence of Eu(facam)$_3$ (Fig. 12) and of Eu(hfbc)$_3$ (Fig. 13). The spectra of ipsdienol in the presence of Eu(facam)$_3$ were obtained by incremental addition of the shift reagent to a 100-μl solution of 1 mg ipsdienol in carbon tetrachloride. The spectra in the presence of Eu(hfbc)$_3$ were obtained at constant lanthanide concentration by using separate samples of substrate varying from 152 μg/100 μl to 1.19 mg/100 μl. In practice, these techniques were inferior to that of using separate samples of constant substrate concentration, since extensive instrument retuning was not required for the latter technique.

At an Eu(hfbc)$_3$–to–substrate ratio of 0.400, enantiomeric composition is reflected by the separation of the methyl signals at 3.45 and 3.00 ppm. The double quartets for the C-7 conjugated olefinic proton also reflect the enantiomeric composition. These enantiomeric shift differences were enhanced at 0°C, but the separation was not baseline for both types of protons. It should be noted that the enantiomeric olefinic protons at C-3 were found to give baseline separation at $[L]_o/[S]_o$ = 0.40 (not shown in Fig. 9) with an enantiomeric shift difference of 40 Hz.

A sample of approximately 250 μg ipsdienol (structure 5) isolated from *Ips pini* Idaho was observed in the presence of Eu(hfbc)$_3$. The spectra obtained on this sample, both at ambient probe temperature and at 0°C, are compared

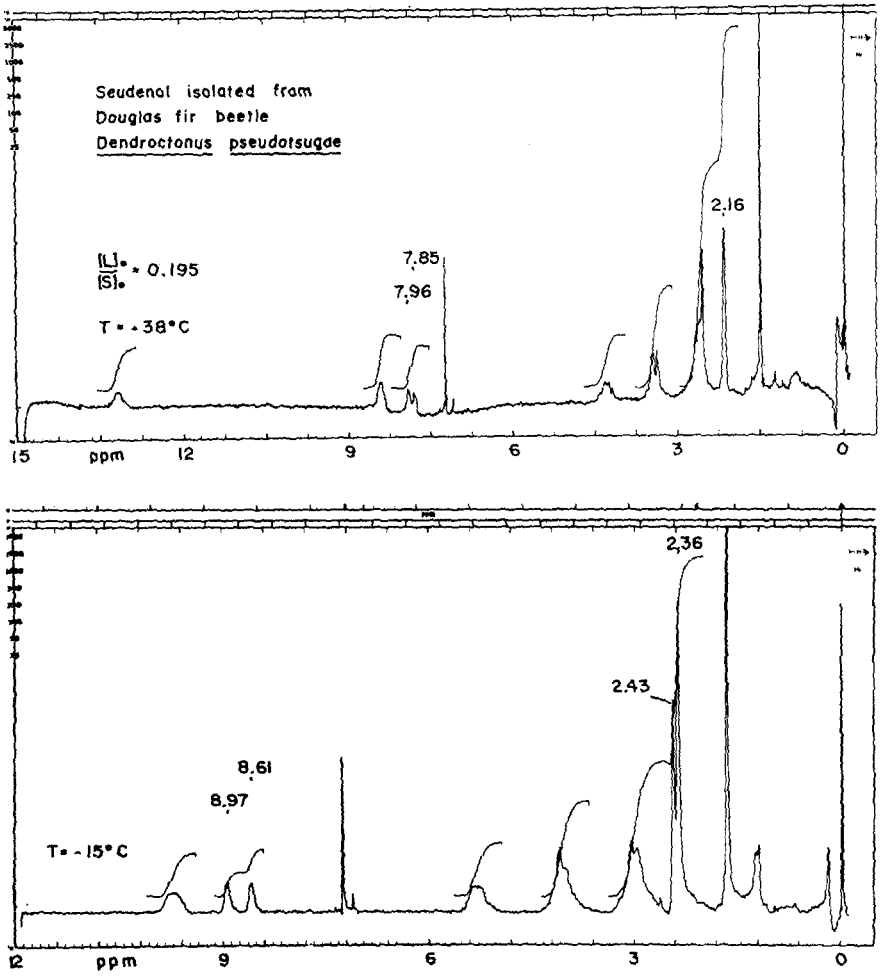


FIG. 11. NMR spectra of natural seudenol at +38°C and −15°C.

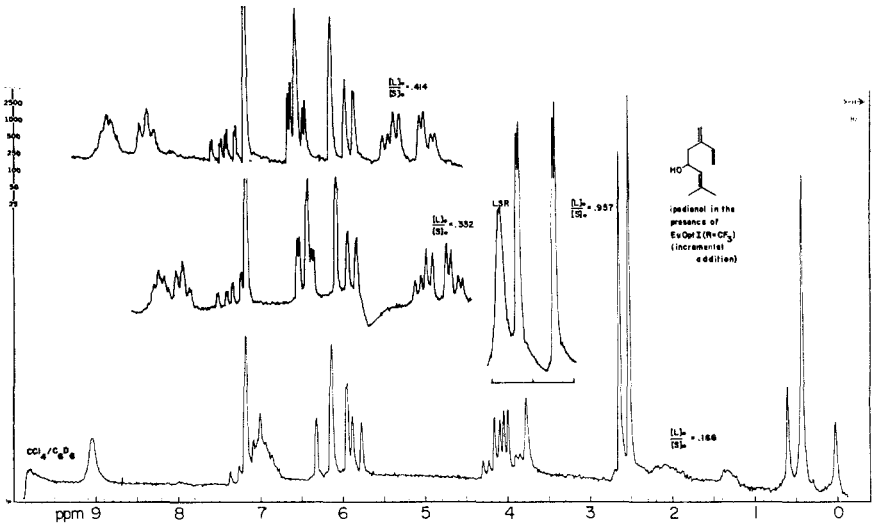


FIG. 12. NMR spectra of ipsdienol in the presence of Eu-Opt I (R = CF_3).

with the spectrum of synthetic racemic ipsdienol (structure 5) in Fig. 14. The calculated $[L]_o/[S]_o$ is the same for all spectra. It is apparent that the natural sample is a pure enantiomer—but which? Since the resolution of ipsdienol (structure 5) was unsuccessful, we turned to ipsdienol (+10°, Silverstein et al. 1966) isolated from the species *I. paraconfusus* Lanier to answer this question.

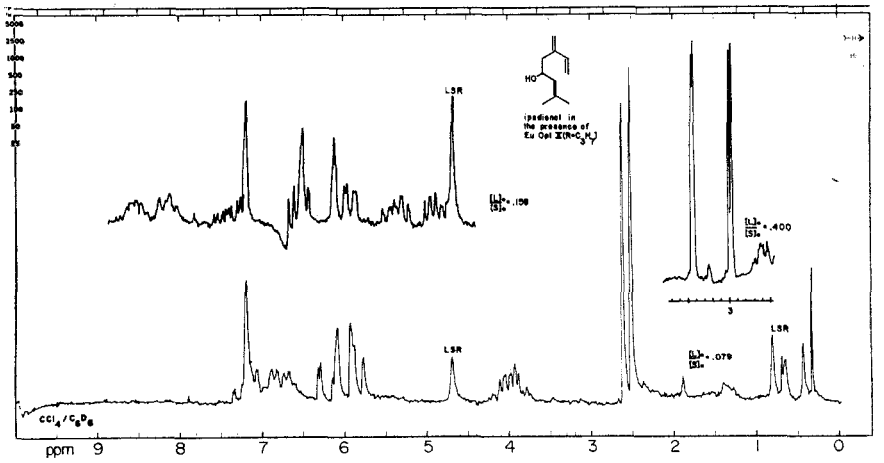


FIG. 13. NMR spectra of ipsdienol in the presence of $Eu(hfbc)_3$.

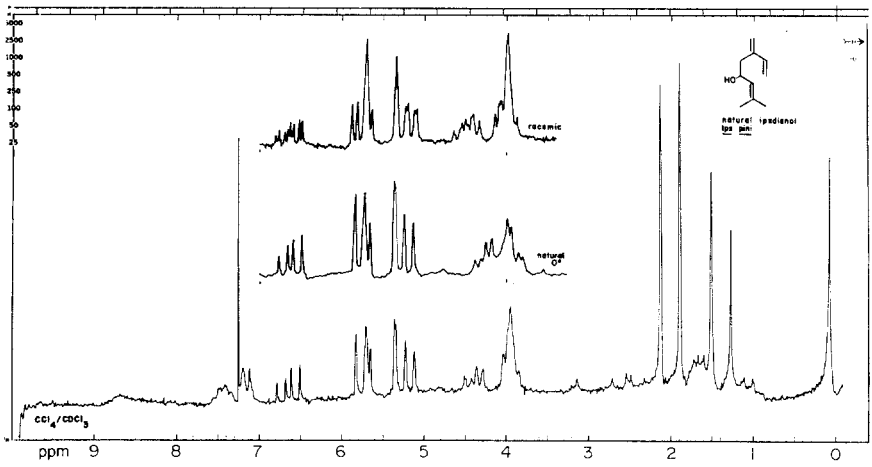


FIG. 14. NMR spectra of racemic and natural ipsdienol in the presence of Eu(hfbc)$_3$.

Figure 15 shows a sample of ipsdienol (structure 5) from *I. pini* spiked with racemic material. Note the shift and separation of signals from the methyl groups of both enantiomers (2.20, 3.00 ppm). The spectrum of 50 μg ipsdienol (structure 5) isolated from *I. paraconfusus* in the presence of Eu(hfbc)$_3$ is shown in Fig. 16. The enantiomeric peaks are reversed from those of Fig. 15 (2.20, 3.40 ppm). Since the ipsdienol (structure 5) from *I. paraconfusus* is (almost entirely) the (+) enantiomer, then ipsdienol (structure 5) from

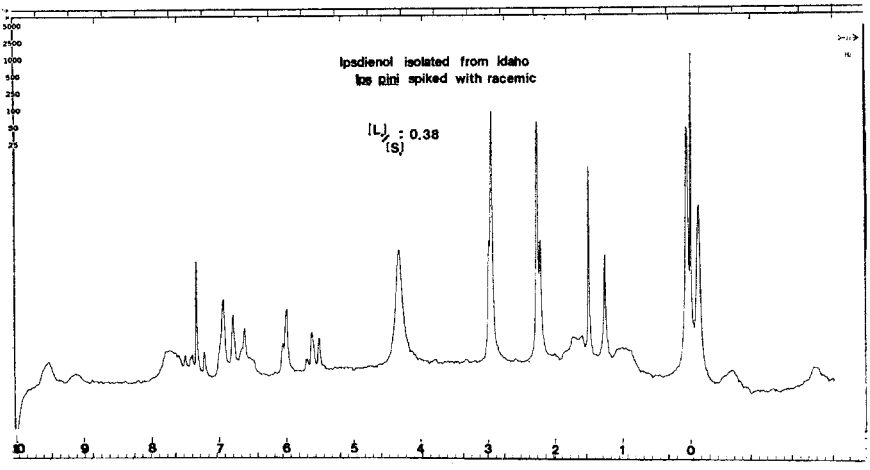


FIG. 15. NMR spectrum of natural ipsdeniol isolated from *I. pini* Idaho, spiked with racemic in the presence of Eu(hfbc)$_3$.

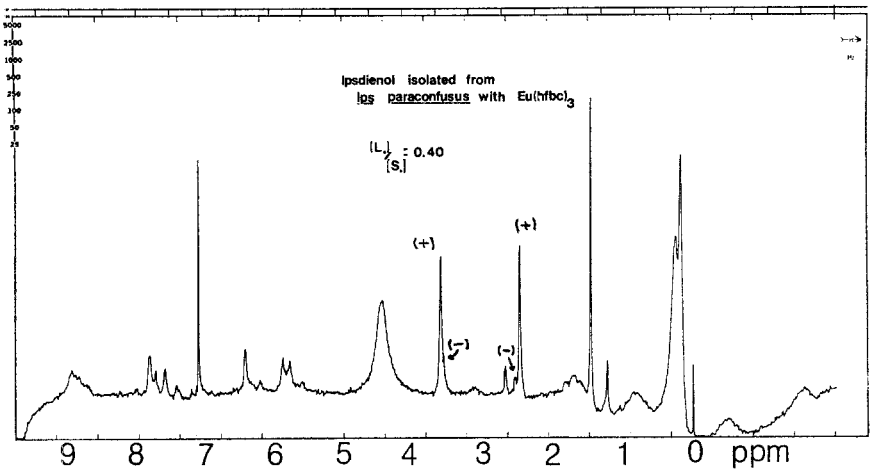


FIG. 16. NMR spectrum of natural ipsdienol isolated from *I. paraconfusus* in the presence of $Eu(hfbc)_3$.

I. pini Idaho is (−). Note that the sample of ipsdienol (structure 5) from *I. paraconfusus* was *not* spiked with a racemic mixture; it contained a small percentage of the (−) enantiomer.

The methylene protons on the C-5 carbon underwent a transformation from a doublet to a complex pattern when shift reagent, either $Eu(facam)_3$ or $Eu(hfbc)_3$, was added to the sample. This transformation merely reflects that these protons are at a prochiral center and are diastereotopic, but are fortuitously chemical shift equivalent at ambient probe temperatures.

SUMMARY

Both methods described in this paper can be successfully applied to the determination of enantiomeric composition in the submilligram range. The Mosher procedure requires more chemical manipulation of the sample and has been used to date only with alcohols; however, correlations have been made with absolute configurations (Dale and Mosher 1973). Chiral shift reagents have the following advantages: relative ease of experimental procedure and interpretation of the spectra, larger enantiomeric shift differences, smaller amounts of sample, and quantitative recovery of the samples by GLC. Disadvantages of this procedure are that the substrate must be a strong enough Lewis base to coordinate with the europium chelate, and that the experimentation to determine the best $[L]_o/[S]_o$ ratio is tedious.

Acknowledgments—We wish to thank Dr. William Gore for the samples of natural and synthetic 4-methyl-3-heptanol, Dr. Lew Edson for the aerated material from *D. frontalis*, Dr. John Borden for the frass from *D. pseudotsugae*, Dr. G.W. Lanier for the aerated material from *I. pini* Idaho, and Mr. Larry McCandless for NMR spectra. We gratefully acknowledge the support of the National Science Foundation, the Rockefeller Foundation, and the U.S. Forest Service.

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BEHAVIORAL RESPONSES OF MALE *Argyrotaenia velutinana* (LEPIDOPTERA: TORTRICIDAE) TO COMPONENTS OF ITS SEX PHEROMONE

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Abstract—Males of the redbanded leafroller, *Argyrotaenia velutinana* (Walker) (Lepidoptera: Tortricidae), were studied for their behavioral responses in laboratory olfactometers and in the field to the 3 components of the female-produced sex pheromone: *cis*-11-tetradecenyl acetate (c11-14:Ac), *trans*-11-tetradecenyl acetate (t11-14:Ac), and dodecyl acetate (12:Ac). Dodecyl acetate, when evaporated with c11-14:Ac (8% *trans*) in the field, modified the behavior of feral males nearby the chemical source, causing an increase in the frequency of landing and close approach to the pheromone dispenser. Apparently, an in-flight behavioral modification concerning landing or not landing occurs within 60 cm of the source and is mediated by 12:Ac. In laboratory olfactometers, c11-14:Ac (8% *trans*) demonstrated a lower threshold for male activation than pure c11- and t11-14:Ac and blends of the two isomers. Additionally, over a wide range of dosages, males responded with optimum wing-fanning response to c11-14:Ac (8% *trans*) compared to pure c11-14:Ac, c11-14:Ac (30% *trans*), and pure t11-14:Ac, suggesting that the *cis*:*trans* ratio rather than absolute amounts of either isomer, is a crucial factor in eliciting male response. When presented with c11-14:Ac (8% *trans*) (1:1), dodecyl acetate caused a significant prolongation of wing-fanning over c11-14:Ac (8% *trans*) alone and resulted in a greater percentage of males moving upwind to the source. Since the increase in wing-fanning and orientation occurred at higher concentrations of the 3-component mixture, the effect of 12:Ac in the laboratory may reflect the close-range role of 12:Ac in the field.

Key Words—*Argyrotaenia velutinana*, redbanded leafroller, sex pheromone, attractant, *cis*-11-tetradecenyl acetate, *trans*-11-tetradecenyl acetate, dodecyl acetate, male behavior, synergist, inhibitor.

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INTRODUCTION

The redbanded leafroller moth, *Argyrotaenia velutinana* (Walker) (Lepidoptera: Tortricidae), uses a female-produced sex pheromone comprised of 3 active components (Roelofs et al. 1975). The primary sex pheromone component of *A. velutinana* was identified as *cis*-11-tetradecenyl acetate (c11-14:Ac) by Roelofs and Arn (1968). Two other pheromone components, *trans*-11-tetradecenyl acetate (t11-14:Ac) and dodecyl acetate (12:Ac), were shown to be instrumental in increasing the trap catch of male *A. velutinana* in the field. The former was shown to be necessary for attractancy when present in low ratios to the *cis* isomer, and gave optimum attractancy when mixed in a *trans*:*cis* ratio of approximately 7:93 (Klun et al. 1973, Roelofs et al. 1975). At ratios higher than 7:93, trap catch of males was reduced. In field screening tests, dodecyl acetate was found to increase trap catch of *A. velutinana* males when evaporated with the other two components (Roelofs and Comeau 1968, 1971). Further tests showed that optimum attractancy was obtained with the addition of 12:Ac at ratios greater than 3:2 to the *trans*:*cis* (8:92) blend (Roelofs et al. 1975).

Recently, both t11-14:Ac and 12:Ac were isolated and identified from female *A. velutinana* abdominal tip extract and calling female effluvia (Roelofs et al. 1975). The former was found to be present in a ratio of about 9:91 to the *cis* isomer, whereas in airborne collection of calling female effluvia, 12:Ac was found to be emitted at a 5:4 ratio of 12:Ac to △11-14:Ac.

The chemical requirements for optimum attractancy of males in the field had thus been defined, and the chemicals identified from female tip extract and effluvia, but the role of the 3 pheromone components in eliciting the appropriate behavioral responses culminating in trap catch—or, in the case of live females, copulation—remained undefined. In this paper, we describe some of the differences in male behavioral responses that can account for the dramatic increase in trap catch when all 3 components are present in their correct ratios.

METHODS AND MATERIALS

General

Adult redbanded leafrollers were reared on a pinto bean diet modified from Shorey and Hale (1965). Wax-coated drinking cups (5.2 cm diameter bottom, 12.5 cm long), containing pinto bean diet and covered with plastic snap-on lids, served as containers for developing larvae. Approximately 75 larvae were reared in each cup until pupation, when pupae were removed and sexed. Male pupae were placed in a screened cage (38 × 38 × 47 cm)

containing a 5% sucrose solution on cotton in a petri dish and held in a walk-in growth chamber. All stages of larval growth and the holding of adults occurred at 24°C on a 16 h:8 h light:dark photoperiod regime, with photophase at 1,400 lx.

Eggs for each generation of diet-reared larvae were produced by adults from a greenhouse colony maintained on fava bean plants (Glass and Hervey 1962). Rearing on diet in growth chambers allowed strict control over photoperiod and temperature and the development of large numbers of adults in a small space.

Solutions of synthetic pheromone components (various ratios of c11- and t11-14:Ac) were prepared by making dilutions in 2 ml Skellysolve B in 10-fold increments, starting with neat material. Ratios of c11- and t11-14:Ac (Farchan Corp.) were analyzed by gas-liquid chromatography (GLC). The following mixtures of the 2 isomers were used: thin-layer chromatography (TLC)–pure c11-14:Ac, 3% t11-14:Ac, 8% t11-14:Ac, 15% t11-14:Ac, 30% t11-14:Ac, 50% t11-14:Ac, 80% t11-14:Ac, and TLC-pure t11-14:Ac. Dodecyl acetate (Eastman Kodak) was purified by preparative GLC. All solutions were stored at −10°C in 1-dr screw-cap vials with Teflon-lined lids and were used within 18 m. Laboratory bioassay treatments were prepared by dispensing 20 μl solution onto a filter-paper tab for presentation to males. For field experiments, the neat pheromone components were loaded directly into polyethylene caps (Glass et al. 1970).

Laboratory Observations of Behavior

Laboratory Courtship Behavior. Cardé et al. (1975*c*) demonstrated in *A. velutinana* that at 24°C, male responsiveness to female abdominal tip extract occurs only during scotophase. However, responsiveness can be advanced as much as 6 h prior to onset of darkness by dropping the ambient temperature from 24° to 16°C 15 min before assay. This feature of the *A. velutinana* chemical communication system was exploited in all bioassays so that the viewing of male behavior could be accomplished during photophase at 1,400 lx.

About 2 h before light-off, 1 virgin female and 1 unmated male were placed in each of a number of clear plastic boxes (12.4 × 9.0 × 7.0 cm). The box to be observed was transferred to a 16°C room on an identical photoperiod regime. Once the female commenced "calling", usually within a matter of minutes (Cardé et al. 1975*c*), the male's behavior was observed and recorded on a portable casette recorder and later transcribed. A second male was sometimes added to a box with a calling female and nonresponding male. Twenty courtship sequences ending with copulation were observed.

Box Olfactometers. Approximately 1 h before bioassay, males were

drawn from their screened holding cage in the 24°C chamber and placed in clear plastic olfactometer boxes (12.4 × 9.0 × 7.0 cm) (Roelofs and Feng 1967), 10 males/box. Care was taken to sample all areas of the cage equally, so that a uniform distribution of floor-dwelling and ceiling-active males was obtained for each box. One hour later, during the last 2 h of photophase, boxes were transferred to an adjoining chamber of equal light intensity, but with a temperature of 16°C. The moths were allowed to chill for 15–45 min, and during this time, chemical treatments were prepared outside the chamber.

Preparation of chemical treatments involved cutting 2-cm^2 filter-paper tabs with crescent-shaped handles (the tabs resembled mushrooms) from Whatman No. 1 filter paper (9.0 cm diam) and impregnating them with 20 μl test solution. The solvent was allowed to evaporate before the tabs with their nonimpregnated handles were placed in numbered glass shell vials stoppered with corks. A randomized complete block design was employed. Treatments were tested double-blind.

Wing-fanning was the "key" response recorded, except in the two earliest series, in which activation (either rapid walking, flight, or wing-fanning) was the response scored. The males in each box were assessed for background activity for 60 sec immediately before presentation of the treatment, and males exhibiting a prestimulus key response were eliminated from further consideration. Each tab was inserted through a slit in the side 2 cm long and 1 cm from the floor of each box, and the number of males exhibiting wing-fanning response during the next 60 sec was recorded. The nontreated filter-paper "handle" attached to each tab remained outside the box. Percentage response was calculated by the formula:

$$\frac{\text{stimulus response} - \text{background response}}{10 - \text{background response}} \times 100 = \text{percentage response}$$

At the end of each block, males were returned to the 24°C room and released into the emergence cage before lights-off. Boxes were soaked in strong detergent solution overnight, rinsed thoroughly, and allowed to air-dry after each assay.

Orientation Tube Olfactometers. Orientation tube olfactometers, glass tubes 2 cm in diameter and 99 cm long including a ground-glass joint on one end, were used to monitor orientation toward the chemical source (Sower et al. 1973) as well as percentage response of *A. velutinana* males. Each tube was fitted internally with a screen barrier 8 cm from the upwind end. The downwind end was plugged with a cheese cloth–covered plastic ring that permitted air to flow out. Laboratory air from an outside source was filtered through charcoal. Twelve plastic tubes linked to a spherical glass manifold were connected to glass connecting tubes (105°) that delivered air at 0.36 m/sec to the orientation tubes. The third arm of each connecting tube was

used for the introduction of the chemical treatments and was closed with a ground-glass stopper. The apparatus for delivery of chemically impregnated filter paper into the airstream was as follows: A piece of copper wire 7.5 cm long with a loop clip at one end was inserted into the end of a cork stopper, and a filter paper disc (Whatman, 2.4 cm diameter), folded in half, was lodged in the loop. After being impregnated with chemical, the filter paper was stored in a glass shell vial with the cork end forming a stopper until time of assay.

One hour or more before assay time, males were drawn from their emergence cage and distributed (10 males/tube) in glass orientation tubes, with care taken to sample uniformly among all areas of the emergence cage. Males were held in these tubes at 24°C until approximately 2 h before lights-off, and then transferred by block in staggered fashion to the 16°C room, where 15 min elapsed before commencement of the assay. Males were allowed to acclimate to the air flow for at least 5 min. A randomized complete block design was employed, and treatments were tested double-blind. Background activity of the key response, wing-fanning, was monitored for 60 sec; then, after introduction of a treatment, wing-fanning was recorded at 10, 30, and 60 sec. The most active treatments also commonly evoked higher-level responses, such as clasper extension and copulatory attempts with other males. Upwind orientation was scored by assessment of the number of males occupying the upwind 10 cm of the tube before assay, and then after 30 and 60 sec of treatment exposure. In calculating percentage orientation, pre-stimulus upwind males were not scored.

After assays, males were returned to the emergence cage, and all tubes were washed with a strong detergent solution and rinsed with hot water followed by redistilled acetone.

Field Observations of Behavior

Sticky Tables and Pherocon® 1C Traps. Sheet-metal tables of 60 cm *r* were coated with Stikem Special®. In the center of each table was placed a polyethylene dispenser (Glass et al. 1970) containing either 10 mg c11-14:Ac (8% *trans*) or 10 mg c11-14:Ac (8% *trans*) plus 15 mg 12:Ac. The tables were stationed 1 m off the ground in an abandoned orchard in Lakemont, New York, from July 23 to July 26, 1974. Captured males' distances from the lure were measured, the males removed, and the tables rerandomized daily.

Pherocon® 1C (Zoecon Corp., Palo Alto, California) insect traps using the same 2 treatments were deployed at Lakemont, New York, July 5–15, 1974. Males were removed and the traps rerandomized every 2 days.

Nonsticky Tables and Nonsticky Pherocon Traps. Circular sheet-metal tables of 60 cm *r* with concentric circles engraved every 10 cm from the center

were employed to observe behavior of feral males close to 2 pheromone sources in the field: (1) 10 mg c11-14:Ac (8% *trans*), and (2) 10 mg c11-14:Ac (8% *trans*) plus 15 mg 12:Ac. Both treatments were loaded in polyethylene caps. The tables were located 1 m off the ground in the grassy aisles between rows of apple trees in an experimental orchard in Geneva, New York, during May 5–15, 1975. This period coincided with the spring flight of *A. velutinana*, and males responded to the pheromone during the afternoon (Comeau et al. 1976), thus making detailed observations of these small moths possible. Observers, one for each treatment, were stationed about 3 m downwind and slightly crosswind of the pheromone source. Portable cassette recorders were used to record observations, which later were transcribed.

Pherocon 1C insect traps were assembled using a nonsticky top as the floor instead of the normal sticky floor. The 2 pheromone treatments used above were again employed. Observations took place from May 5 to May 15, 1975, in an orchard in Sodus, New York, abandoned for 10 yr. Traps were hung on the outer branches of apple trees at a height of about 1.5 m.

Trap locations were changed about every 10 min for tables and every 5 min for Pherocon traps to minimize the possibility of multiple observations of a single individual.

RESULTS

Laboratory Courtship Behavior

For a quiescent male (antennae pointing posteriorly and parallel to the substrate), a sequence of behavior in response to a calling female involved the following steps:

(1) Antennae were elevated, perpendicular to the substrate,
(2) Male preened antennae, drawing first one foretarsus, then the other, along the full length, sometimes bringing it into contact with the mouthparts and face in between wiping an antenna.
(3) Male walked rapidly or flew a short distance toward the female.
(4) Male fanned its wings while walking rapidly toward the female, approaching either posteriorly or laterally.
(5) Male touched the costal tip of the female's slightly raised forewing with its antennae and head, while facing at a 45° angle from behind and continuing to fan its wings (sometimes a male's head would become concealed beneath the wing).
(6) Male's abdomen, claspers extended, curled toward the female's abdomen and probed until successful in grasping the tip.

(7) Male's wings stopped fanning and were folded back once more to a resting position, but beneath the female's wings—the two moths now faced in opposite directions.

Steps 5–7 usually occurred within 1 or 2 sec. The durations of the earlier steps were quite variable.

Some steps in this sequence of behavior either were omitted sometimes or perhaps occurred so quickly that they could not always be observed. For example, some males commenced wing-fanning without first either flying or walking rapidly. Some responses, however, occurred in all 20 successful courtships, these being steps 1, 4, and 5–7. No contact between any part of the male's body and the female's antennae was observed; the male's abdomen and genitalia always curled toward the tip of the female's abdomen during copulatory attempts.

Box Olfactometer Experiments

TLC-pure c11-14:Ac alone at 2×10^{-3} μg elicited 27% activation, but the 3% *trans* treatment at this dosage raised the response significantly, to 49% (Fig. 1). Furthermore, 8% *trans* elevated the response to a significantly higher level of 76%. Raising the percentage of *trans* to 15% caused a significant decrease in the response to 37%, while treatments with higher ratios of *trans*:*cis* continued to elicit even lower responses. The addition of 2×10^{-3} μg 12:Ac to each of the *cis*:*trans* ratios resulted in a significant increase of percentage response for the TLC-pure c11-14:Ac and 15% *trans* treatments only (χ^2 2×2 test of independence, $P < 0.05$). It can be seen, however, that

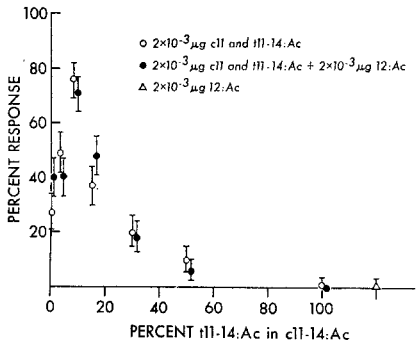

FIG. 1. Percentage activation response of *A. velutinana* males to mixtures of c11- and t11-14:Ac, both with and without the addition of 12:Ac. Brackets above and below the means denote the 95% binomial confidence limits (n = 160).

8% *trans* both with and without addition of 12:Ac gave a percentage activation response significantly higher than any of the other 13 treatments.

Since the decline in response with increasing percentages of *trans* could have been due to the presence of either more *trans* or less *cis*, a second series of treatments was assayed. The amount of *cis* presented remained absolute at 2×10^{-3} μg, and *trans* was added to *cis* to make 30% *trans* and 50% *trans*, 2 treatments in which the large amounts of *trans* would have had the greatest effects. There was no statistical difference between responses to 30% and 50% *trans* treatments prepared either way using the χ^2 2×2 test of independence ($P > 0.05$).

In order to observe responses to various ratios of *cis* and *trans* over a large range of concentrations, a dosage series of TLC-pure *cis*, pure *trans*, 8% *trans*, and 30% *trans* was tested in box olfactometers. The key response recorded was wing-fanning.

One unexpected result was the occurrence of wing-fanning response when males were exposed to 10^1 μg and 10^2 μg pure t11-14:Ac (Fig. 2). At lower concentrations, pure *trans* had elicited almost no response, but at these higher concentrations, the male response appeared to be similar to that elicited by the pure *cis* or 8% *trans* treatments in terms of wing-fanning persistence or movement toward the filter paper. Additionally, over 4 orders of magnitude of dosage (10^{-2}–10^1 μg), pure *cis*, pure *trans*, and 8% *trans* elicited levels of wing-fanning response significantly different from each other, using the binomial confidence interval (C.I.) at 95%, and the χ^2 2×2 test of independence for 10^{-2} μg (Fig. 2).

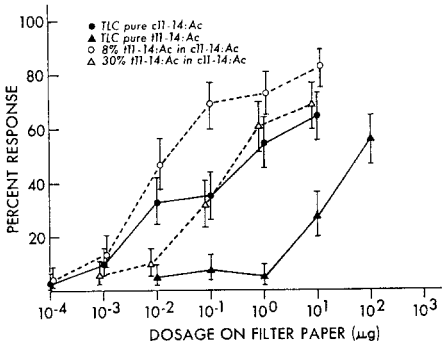

FIG. 2. Percentage wing-fanning response by *A. velutinana* males in box olfactometers to various concentrations of c11- and t11-14: Ac alone and in mixtures. Brackets above and below the means denote the 95% binomial confidence limits (n = 130).

The data in Fig. 2 also reveal that in box olfactometers, about 100 times more pure *cis* and 10,000 times more pure *trans* than 8% *trans* was required to elicit wing-fanning response in 50% of the males. Thus, the threshold for 50% wing-fanning appears to be about 100 times lower for 8% *trans* than for pure *cis*, and about 10,000 times lower than pure *trans*.

Orientation Tube Olfactometer Experiments

Differences in the amount of orientation to the pheromone source could not be easily elucidated in box olfactometers, so experiments in long glass tubes with a directional airflow were conducted (Sower et al. 1973).

Testing was initiated with a dosage level for each treatment that would elicit about 50% wing-fanning response. The dosage-response box olfactometer series showed that 10^2 μg pure t11-14:Ac, 1 μg TLC-pure c11-14:Ac, and 10^{-2} μg c11-14:Ac (8% *trans*) each elicited approximately 50% wing-fanning response, and these 3 treatments were used both with and without an equivalent amount of dodecyl acetate (Fig. 3).

It is apparent that the addition of 10^2 μg 12:Ac to 10^2 μg pure *trans* did not alter significantly the percentage of either wing-fanning or orientation

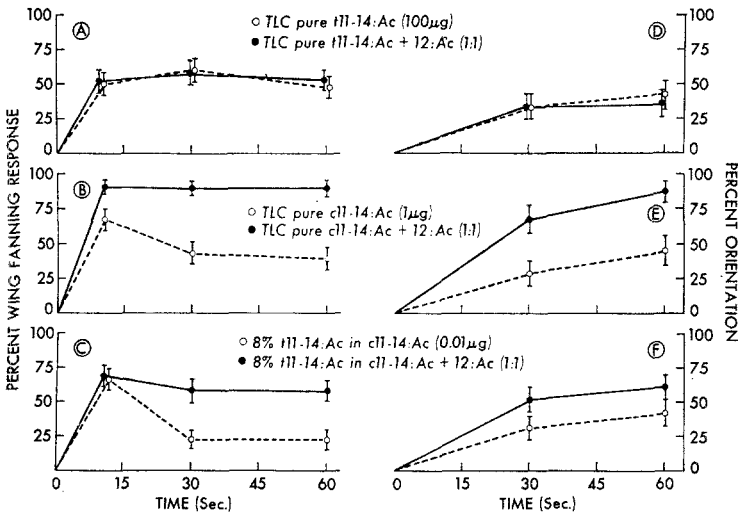

FIG. 3. Percentage wing-fanning response and orientation by *A. velutinana* males in glass tube olfactometers to mixtures of c11- and t11-14:Ac and 12:Ac at dose levels eliciting 50% response in box olfactometers. Brackets above and below the means denote the 95% binomial confidence limits (n = 160).

during the 60-sec period (Figs. 3A and D). However, 1 μg 12:Ac evaporated with 1 μg pure *cis* increased the percentage wing-fanning response during the initial 10 sec and caused it to persist at a significantly higher level throughout the entire 60 sec (Fig. 3B). Dodecyl acetate also increased the duration of wing-fanning in response to 10^{-2} μg 8% *trans*, but did not elicit a higher response during the first 10 sec (Fig. 3C).

For all 3 treatments not containing 12:Ac, the percentage of males orienting to the upwind 10 cm of the tube was approximately 45% after 60 sec. The addition of 12:Ac to pure *trans* produced no increase in percentage orientation (Fig. 3D). However, adding 1 μg 12:Ac to 1 μg pure *cis* (Fig. 3E) resulted in a significant increase in orientation to nearly 90%, after 60 sec. The effect of 12:Ac on the 8% *trans* treatment was not as dramatic, yet 12:Ac elicited a significant increase in orientation after both 30 and 60 sec (95% C.I.).

In order to test the effect of 12:Ac at lower dosages, treatments of 10^{-1} μg pure *trans*, 10^{-3} μg pure *cis*, and 10^{-3} μg 8% *trans* were chosen from the box bioassay dosage-response experiment to approximate a 10% wing-fanning response level. An equivalent amount of 12:Ac was added to each treatment. No significant increase in wing-fanning response was noted with the addition

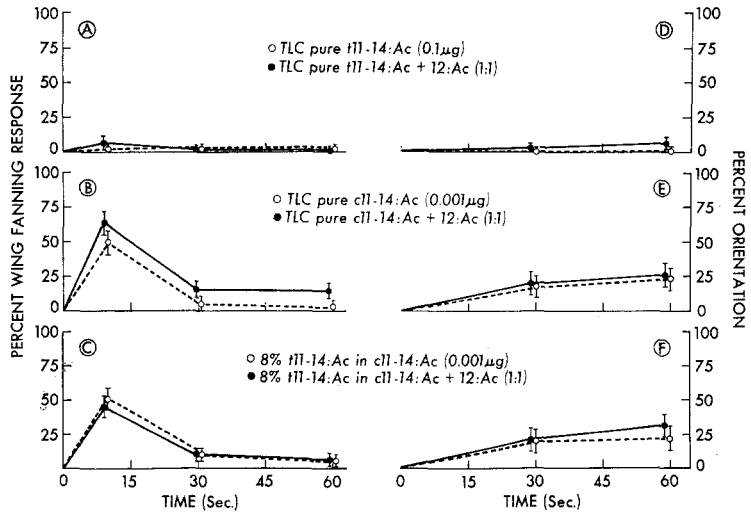

FIG. 4. Percentage wing-fanning response and orientation by *A. velutinana* males in glass tube olfactometers to mixtures of c11- and t11-14:Ac and 12:Ac at dose levels eliciting 10% response in box olfactometers. Brackets above and below the means denote the 95% binomial confidence limits (n = 160).

TABLE 1. PERCENTAGE WING-FANNING RESPONSE AND ORIENTATION BY *A. velutinana* MALES TO 12:Ac

	Percentage wing-fanning response[a]			Percentage orientation[b]		
Dosage 12:Ac	10 sec	30 sec	60 sec	0 sec	30 sec	60 sec
10^{-3} μg	1.7*	0.8*	0.8*	0	4.9*	4.9*
10^{0} μg	1.7*	2.5*	0*	0	3.4*	4.5*
10^{3} μg	19.2†	20.8†	12.5†	0	9.4*	16.5†
Control[c]	85.0‡	10.0‡	11.3†	0	13.4*	30.3†

[a] Percentages in same column with different superscript symbols are significantly different using 95% binomial confidence limits ($n = 120$).
[b] Percentages in same column with different superscript symbols are significantly different using a χ^2 2×2 test of independence with Yates' correction ($P < 0.01$).
[c] c11-14:Ac (8% *trans*), 10^{-2} μg.

of 12:Ac (Fig. 4), except in the case of pure *cis* (Fig. 4B), where 10^{-3} μg pure *cis* plus 10^{-3} μg 12:Ac elicited significantly higher levels of wing-fanning at 30 and 60 sec than 10^{-3} μg pure *cis* alone. No differences in percentage orientation occurred at this dosage level.

Dodecyl acetate was then tested by itself at 3 different dosages—10^{-3} μg, 1 μg and 10^{3} μg—to examine its possible effects, since it seemed to enhance the response to the treatments mentioned above. Surprisingly, at 10^{3} μg, the highest level tested, 12:Ac elicited 19%, 21%, and 13% wing-fanning response at 10, 30 and 60 sec (Table 1). However, percentage response to the 2 lower concentrations was near 0. Hence, at the levels used in previous bioassays, 12:Ac alone was at subthreshold levels for wing-fanning response. Its enhancement of response to pure *cis* and 8% *trans* could not have resulted from any separate elicitation of wing-fanning, but instead was due to some other effect that occurred in combination with the other sex pheromone components.

Field Observations of Behavior

Sticky Tables and Pherocon Traps. Only 2.0 times as many males were caught on table traps with the treatment containing 12:Ac as on traps lacking this component, whereas in the smaller Pherocon traps, the 12:Ac-containing treatment caught 5.4 times more males (Table 2). Males were caught at a mean distance of 49.0 cm on the tables lacking 12:Ac, and at 46.7 cm on tables containing 12:Ac (no significant difference).

Nonsticky Tables. Feral males were observed orienting upwind toward

TABLE 2. EFFECT OF TRAP SIZE ON RATIOS OF MALES CAUGHT IN TRAPS LACKING AND CONTAINING 12:AC IN DISPENSERS WITH C11-14:AC (8% *trans*)

Type of trap	Mean males captured per trap		Ratio of males caught in traps lacking and containing 12:Ac
	10 mg c11-14:Ac (8% *trans*)	10 mg c11-14:Ac (8% *trans*) +15 mg 12:Ac	
Sheet-metal tables, 60 cm *r*, Summer 1974[a]	9.7	19.7	1:2.0
Pherocon 1C traps, 14 cm *r*, Summer 1974[b]	2.7	14.7	1:5.4
Sectar traps, 7.6 cm *r*, Spring 1973[c]	2.0	24.6	1:12.3

[a] 3 replicates. [b] 18 replicates. [c] 15 replicates (from Roelofs et al. 1975).

the tables from as far away as 15 m downwind. Many males flew very low to the ground and landed in the grass periodically before proceeding onward. This seemed to occur more often when the breeze was strong or increased suddenly in velocity. Males that landed in the grass sometimes exhibited wing-fanning behavior as they walked up to the top of a blade of grass, and then proceeded upwind toward the table. Antennal preening, consisting of dragging each foretarsus over the entire length of an antenna, occurred both in the grass and on the table surface while males were stationary. Many times, the foretarsi would be brought into contact with the mouthparts before being dragged over the antennae. Antennal preening was almost always followed immediately by renewed wing-fanning, walking, or flight. When a male finally reached the vicinity of the table, he would usually spend many seconds casting (oscillating vertical or horizontal flight with little forward progress) at the edge of the metal surface before landing. The mean landing distance from the dispenser was 50.6 cm for both treatments.

Many males made more than one approach toward the table. After spending some time on the surface, males would often fly downwind 0.5 to 10 m and begin approaching the trap again. Of the males landing on the table in response to the treatment lacking 12:Ac, 43% made 2 or more upwind approaches, compared with 51% (not significantly different, χ^2 2×2 test of independence) of those responding to the treatment containing 12:Ac. As many as 6 approaches were observed for single males in response to both treatments. All males approaching closer than 10 cm from the dispenser on

their initial approach exhibited wing-fanning behavior either near the dispenser or while walking on the dispenser surface. No copulatory attempts with the dispenser were observed. Departure for the final time usually occurred after an extended stationary period, and involved vertical flight up to 5 m in height immediately on leaving the surface, followed by rapid downwind flight to more than 20 m away.

The addition of 15 mg 12:Ac to 10 mg c11-14:Ac (8% *trans*) resulted in a significant increase in the number of males landing on the table surface, and in the mean closest approach to the attractant dispenser among all males scored (Table 3). When considering only landers, there was no significant difference between the 2 treatments in mean closest approach, although there was a trend toward a closer approach to the 12:Ac-containing treatment.

TABLE 3. BEHAVIOR OF *A. velutinana* MALES NEAR DISPENSERS BAITED WITH c11-14.AC (8% *trans*) LACKING OR CONTAINING 12:AC AND LOCATED AT THE CENTER OF CIRCULAR SHEET-METAL TABLES

Male behavior	10 mg c11-14:Ac (8% *trans*)	10 mg c11-14:Ac (8% *trans*) +15 mg 12:Ac
No. of males observed	41	67
Percentage approaching to <0.5 m from table[a,b]	70.7	82.1‡
Percentage landing on table[a,b]	39.0	71.6*
Percentage fanning while walking on table:[a,b]		
all males	26.8	56.7*
landers only	78.6	81.0‡
Percentage approaching <10 cm from dispenser:[a,b]		
all males	17.1	52.2†
landers only	50.0	74.5‡
Mean closest approach to dispenser ±SD:[b,c]		
all males[d]	40.9±25.18 cm	18.2±24.77 cm†
landers only	20.4±22.14 cm	11.5±19.19 cm‡
Mean time spent fanning while walking by landers ±SD[b,c]	20.0±23.72 sec	18.6±21.95 sec‡

[a] Percentages in the same row tested for significance by a χ^2 2×2 test of independence with Yates' correction.

[b] *$P<0.01$; †$P<0.001$; ‡$P>0.05$.

[c] Means in the same row tested for significance using the *t*-test.

[d] Males flying within 0.5 m of the table edge were scored as approaching to 60 cm; males not approaching to within 0.5 m were not scored. The remaining approaches were by males walking on the table surface.

The percentage of all males approaching to within 10 cm of the dispenser was significantly higher for the treatment containing 12:Ac than for the treatment lacking 12:Ac, but among landers, there was no significant difference between the 2 treatments (Table 3). (The approximate radius of the sticky surface of a Pherocon 1C trap is 10 cm.) The percentage of males approaching to within 0.5 m of the table was higher for the treatment containing 12:Ac, but not significantly different from the treatment lacking 12:Ac. The percentage of males exhibiting wing-fanning behavior was directly proportional to the percentage landing on the table surface. While the percentage of all males exhibiting wing-fanning behavior was significantly different for the treatments containing and lacking 12:Ac, the percentage of landers fanning their wings was similar.

Nonsticky Pherocon Traps. Males were observed orienting toward the Pherocon 1C traps from as far away as 10 m downwind. Flight sometimes appeared to be erratic and of high velocity at first, but as males neared the trap edge, their forward progress slowed and the flight pattern was refined into small (10–20 cm) vertical or horizontal casting motions, which lasted as

TABLE 4. BEHAVIOR OF *A. velutinana* MALES NEAR DISPENSERS BAITED WITH c11-14:Ac (8% *trans*) LACKING OR CONTAINING 12:Ac AND LOCATED IN NONSTICKY PHEROCON 1C TRAPS

Male behavior	10 mg c11-14:Ac (8% *trans*)	10 mg c11-14:Ac (8% *trans*) +15 mg 12:Ac
No. of males observed	49	40
Percentage approaching to 0.5 m from trap[a,b]	85.7	87.5†
Percentage landing on trap[a–c]	26.5	87.5*
Percentage fanning while walking on trap[a,b]	26.5	87.5*
Percentage "caught"[a,b,d]	20.4	87.5*
Percentage touching dispenser:[a,b]		
all males	14.3	62.5*
landers only	54.0	71.4†
Mean time fanning while walking by landers ±SD[b,e]	42.0±39.68 sec	51.5±38.41 sec†

[a] Percentages in the same row tested for significance by a χ^2 2×2 test of independence with Yates' correction.

[b] * $P<0.001$; † $P>0.05$.

[c] Males alighting anywhere on the trap surface for more than 1 sec were scored as "landing."

[d] Males walking on the area on the trap floor normally coated with Stickem were judged to have been "caught."

[e] Means in the same row tested for significance using the *t*-test.

long as 17 sec before landing was attempted. Some males approached at grass level and were well below trap altitude, but proceeded to rise to trap level and land. Many males fanned their wings while walking either on both sides of the roof of the trap, on the underside of the floor, or on the connecting wires before walking onto what would have been the sticky floor of a normal Pherocon trap. Only rarely would a male fly directly into the trap onto the floor without first fanning while walking on the edge of the trap. Males always approached the attractant dispenser by walking while fanning their wings on the initial approach.

The simultaneous evaporation of 12:Ac from the dispenser resulted in a significantly greater percentage of males landing on the trap surface compared with the treatment lacking 12:Ac, although both treatments lured an equivalent percentage of males to within 0.5 m of the trap (Table 4). All males that approached to within 0.5 m of the trap emitting 12:Ac landed, whereas only 31% of the males approaching to within 0.5 m of the trap lacking 12:Ac landed. Of the males observed orienting toward the trap containing 12:Ac, 87.5% would have been ensnared had there been a sticky floor, whereas only 20.4% of observed males would have been caught in the trap lacking 12:Ac. The percentage of males observed fanning their wings while walking was the same as the percentage landing on the trap in both treatments: All males that landed were observed to fan. Of males that landed in response to both treatments, similar percentages oriented to and touched the dispenser, although the percentages were very different if the total number of males observed orienting in both groups is considered.

DISCUSSION

Laboratory Observations of Behavior

In a response sequence to calling virgin females, *A. velutinana* males exhibited certain behavioral characteristics that were useful for assaying synthetic chemicals. The most evident response during courtship was wing-fanning while walking, and not one male approached and touched a calling female without fanning his wings simultaneously. This behavior was easily discernible from steps occurring earlier in the sequence that in some contexts may have no relationship to sexual behavior. Except for the first two series of tests, then, wing-fanning behavior was selected as the "key response" to be observed and reflected a naturally occurring behavior requisite to successful courtship.

In laboratory activation assays, males responded optimally to c11-14:Ac when it contained 8% of the *trans* isomer (see Fig. 1). Interestingly, Bartell and Roelofs (1973) postulated a missing component or components in addi-

tion to *cis*, as detected by laboratory bioassays of their female tip extract. Extrapolating from their data, 2×10^{-3} μg pure *cis* elicited about 25% male activation response, while 2×10^{-3} μg female tip extract elicited about 65% response. Figure 1 shows that TLC-pure c11-14:Ac elicited 27% activation response in our series, while 8% *trans* in c11-14:Ac gave 75% response. The difference in behavior in response to crude extract and pure *cis* observed by Bartell and Roelofs (1973) can be explained by the addition of 8% *trans* to *cis*. Although the addition of 12:Ac to pure *cis* significantly increased the percentage response to 40%, this level was not sufficient to account for their observed difference.

The 8% *trans* mixture proved to be more potent in eliciting wing-fanning response than pure *cis*, pure *trans*, and 30% *trans* over a wide range of dosages covering 4 orders of magnitude (see Fig. 2). It can be concluded that in responding preferentially to treatments containing 8% *trans*, male *A. velutinana* were not determining the absolute quantity present of either isomer. At any one dosage level from 10^{-2} μg to 10^{1} μg, the reduced response to 30% *trans* compared to 8% *trans* can be attributed to either too much *trans* present or too little *cis*. However, when one considers the reduced response to pure *cis* compared to 8% *trans* at each dosage, the argument for too little *cis* is eliminated. Additionally, the increase in response elicited by progressively higher dosages of 30% and 8% *trans* (with the amount of *trans* increasing 10-fold each step) nullifies the argument that reduced response is a result of too much *trans*. The insect must be detecting the *cis*:*trans* ratio and responding accordingly. In these dosage-response series, then, it can be argued that the 2 isomers are at various times "excitants," "synergists," and "inhibitors," depending on their dosage and proportion. These terms thus become inappropriate for describing the behavior elicited by these pheromone components and may be misleading.

The wing-fanning response elicited by high concentrations of *trans* was unexpected. Roelofs and Comeau (1971) reported a "short-lived buzzing response" when *trans* was tested at 1 μg, which was 1,000 times the observed threshold for *cis*. Our data also show very little wing-fanning response at 1 μg, but at 10^{1} μg and 10^{2} μg, the wing-fanning response to *trans* was qualitatively indistinguishable from that to *cis* or 8% *trans*.

Likewise, the wing-fanning response to 1 mg (detectable by the human nose) 12:Ac in orientation tube olfactometers was unexpected, and was qualitatively indistinguishable from that to 8% *trans*. The higher dosages required for response to these 2 components may represent the relative degree of affinity and intrinsic activity that these compounds have for the *cis* antennal receptor sites (O'Connell 1972, 1975) to produce the intensity and quality of sensory neuron impulse generation required for wing-fanning response.

The 2 assays in orientation tube olfactometers (see Figs. 3 and 4) can be viewed as support for the role of 12:Ac as a modifier of behavior close to the source. At low dosages in the laboratory (i.e., long-range in the field), 12:Ac had no effect on wing-fanning response to 8% *trans*, the most active *cis*:*trans* mixture (Figs. 4C and F). However, at a higher dosage of 8% *trans* (close-range in the field), 12:Ac became effective in prolonging the wing-fanning response and increasing percentage orientation toward the source (Figs. 3C and F). Since wing-fanning behavior always occurs as a male approaches a female before attempting to copulate, these assays are important in explaining a close-range role for 12:Ac. The enhancement of response by 12:Ac (Fig. 3) to pure *cis* and 8% *trans*, but not pure *trans*, indicates that 12:Ac might be acting in conjunction with *cis* rather than *trans* receptor sites in modulating wing-fanning response.

Field Observations of Behavior

Observations of male *A. velutinana* in the field indicate that 12:Ac modified the behavior of males close to the c11-14:Ac (8% *trans*) source. This modification is evidenced by the greater percentage of landing and closer mean approach to the dispenser on large nonsticky tables (see Table 3) and the greater percentage of landing in nonsticky Pherocon 1C traps by males that approached to within 0.5 m of 12:Ac-containing traps (see Table 4). A large percentage (69%) of the males that approached to within 0.5 m of the Pherocon traps in response to the lure lacking 12:Ac turned away and flew rapidly downwind, whereas all males that approached within 0.5 m of the trap containing 12:Ac landed.

Interestingly, for nonsticky tables and traps, no significant differences in frequency or duration of wing-fanning or mean closest approach to the dispenser could be discerned between the 2 treatments once males had landed on their surfaces. For both surface sizes, however, the significant differences in the percentage of males landing after flight close to the surface edge suggest that an in-flight behavioral step involving landing (or not landing) is mediated by 12:Ac.

Further evidence to support the role of 12:Ac as a close-range modifier of behavior resulted from the relative numbers of males caught in traps having progressively smaller sticky surfaces. Large sticky table traps with a radius of 60 cm gave a 2-fold increase in catch with the addition of 12:Ac to the 8% *trans* in *cis* mixture, whereas the 12:Ac-added treatment in Pherocon 1C traps with a sticky radius of about 14 cm resulted in a 5.4-fold increase of males trapped compared to the treatment lacking 12:Ac. In addition, Roelofs et al. (1975) reported a 12-fold increase in males caught using still smaller Sectar traps with a radius of 7.6 cm. All these findings suggest that

the presence of 12:Ac becomes increasingly critical for trap catch as trap size is reduced.

The near-maximal mean distance from the dispenser (49 and 47 cm) of males caught on the surface of 60 cm *r* sticky tables and observed landing (50.6 cm) on the surface of nonsticky tables for both treatments indicate that male *A. velutinana* will take the first opportunity to land and complete the approach when a suitable surface is presented. This, along with their casting flight at the table's edge before landing, suggests that visual cues play an important role in their approach to a pheromone source. Likewise, males were observed to slow their forward progress and cast sideways and vertically a few centimeters from the edge of Pherocon traps before either landing or departing. A visual response to the trap surface is again suggested, because a purely chemical response to odor concentration should have caused a cessation of forward progress and casting to occur at the same distance from the dispenser for the Pherocon traps as for the tables. The phenomenon of multiple approaches to tables by a large percentage of the males observed indicates that male *A. velutinana* may become dishabituated to the pheromone by flying downwind and again beginning the behavioral sequence with upwind anemotaxis. This behavioral pattern occurred even after coming in direct contact with the dispenser for uninterrupted periods as long as 1 or 2 min. Thus, males do not appear to become habituated easily to a single source of pheromone at optimum concentration for attractancy. Another explanation could involve the more transient phenomenon of sensory adaptation, allowing the insect to recover its odor-perceiving ability relatively quickly after returning to an area of lower odor concentration.

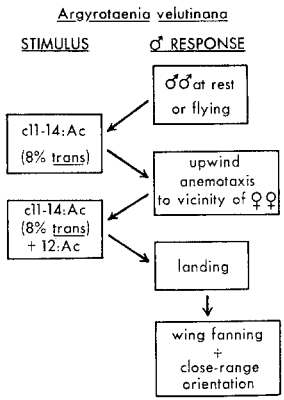

FIG. 5. Stimulus–response reaction chain diagramming possible steps involved in the location of an *A. velutinana* female by conspecific males.

The laboratory and field results from this study can be used to construct a stimulus–response action chain diagramming the possible steps involved in the location process (Fig. 5). In the field, a greater percentage of males at rest or flying would become activated and fly upwind to a blend containing 8% t11-14:Ac in c11-14:Ac than to other mixtures or to c11-14:Ac alone (see Fig. 1). After the males had flown to the vicinity of the female, the higher concentration of pheromone and the presence of 12:Ac would cause a greater frequency of landing, wing-fanning while walking, and close-range orientation by males. The smaller the available landing surface, the more pronounced becomes the effect of 12:Ac, so a female calling from a small twig or leaf might receive few male visitors unless she were emitting 12:Ac along with c11-14:Ac (8% *trans*).

Although both laboratory bioassays and field observations were employed to try to ascertain the behavioral function of the *A. velutinana* pheromone components, the latter were the key to elucidating the role of 12:Ac. Laboratory assays demonstrated the enhancement of activity by this component only at certain discrete concentrations, but a behavioral function mediated by 12:Ac could be described only after observing males during response in the field.

The finding of a close-range chemical modifier of behavior in *A. velutinana* is similar to the communication system of the Oriental fruit moth, *Grapholitha molesta* (Cardé et al. 1975*a,b*). Dodecyl alcohol (12:OH), when presented with *cis*-8-dodecenyl acetate (c8-12:Ac) with 7% of the *trans* isomer present, elicited increases in the percentage of males landing near the source, fanning while walking, and exhibiting hairpencil display behavior near the dispenser. In addition, the mean approach to the dispenser was significantly closer with treatments containing 12:OH. However, 12:OH has not been identified from Oriental fruit moth female tip extract. For *A. velutinana*, however, all 3 compounds investigated in this study are known to be either emitted by female *A. velutinana* or present in the pheromone gland. Thus, the close-range mediation of behavior by 12:Ac appears to represent a naturally occurring phenomenon in the location of a female by a male redbanded leafroller moth.

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AUTOINTOXICATION MECHANISM OF *Oryza sativa* I. PHYTOTOXIC EFFECTS OF DECOMPOSING RICE RESIDUES IN SOIL[1]

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Abstract—The aqueous extracts of decomposing rice residues in soil exhibited inhibition on the radicle growth of lettuce and rice seeds and the growth of rice seedlings. The phytotoxicity was found in extracts obtained from the early stage of decomposition (first month), and gradually declined thereafter. The inhibition was also found in extracts obtained from rice fields, and was persistent for 4 months. The root initiation of hypocotyl cuttings of mungbeans was suppressed by extracts of decaying rice residues and extracts obtained from paddy soil. Five phytotoxins, *p*-hydroxybenzoic, *p*-coumaric, vanillic, ferulic, and *o*-hydroxyphenylacetic acids, and several unknowns were found in the decomposing rice residues under waterlogged conditions. At 25 ppm, *o*-hydroxyphenylacetic acid revealed significant inhibition on the radicle growth of rice and lettuce seeds and suppressed root initiation of mungbean seedlings. It was concluded that the growth of rice seedlings was retarded by decaying rice residues in soil; thus, this appeared to be an autointoxication phenomenon.

Key Words—autointoxication, phytotoxin, allelopathy, decomposing rice residues, *p*-hydroxybenzoic acid, *p*-coumaric acid, vanillic acid, *o*-hydroxyphenylacetic acid, ferulic acid.

INTRODUCTION

It is well known that a certain quantity of the unharvestable portion of a plant is left in the soil. The debris of crops in soil has always been thought

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to be beneficial to succeeding crops. However, evidence indicates that the crop residues left in soil are sometimes harmful to plant growth (Börner 1960, McCalla 1971, Patrick 1965). Patrick and his associates demonstrated that residues of rye, timothy, tobacco, and barley that remained in the soil released great amounts of phytotoxic substances after 6–8 weeks of decomposition (Toussoun et al. 1968). The phytotoxins arising during the decomposition of these plant residues in soil inhibited the respiration, germination, and growth of several different kinds of plants (Patrick and Koch 1958). Many "sick" soils contain phytotoxins that produce a detrimental effect on some other plants or suppress the growth of the same plants from which the phytotoxins were derived (Börner 1971; Chou and Chung 1974; Muller 1966, 1974; Rice 1974; Wang et al. 1967*b*). Wang and his associates studied the decline of sugarcane yield in Taiwan, and found that 5 growth inhibitors were present in the sugarcane soil. They indicated that these inhibitors were plant-originated and came from the debris of sugarcane decomposed in soil. Similarly, the productivity of rice in the second crop is generally lower than that in the first crop. Although there are many factors involved in determining the reduction of rice yield in the second crop, phytotoxic inhibition could be one of the factors involved. In Taiwan, the farmers have always left a large amount of rice straw in the soil after harvesting. It is possible that the rice residues submerged in soil could release phytotoxic substances during the decomposition period, especially under waterlogged conditions. Thus, it was the aim of this study to investigate the hypothesis of an autointoxication mechanism of *Oryza sativa*, particularly emphasizing the phytotoxic effect of decomposing rice residues in soil.

METHODS AND MATERIALS

Materials

After rice was harvested in Nankang, Taiwan, in June 1973, many straw–root–soil samples were taken from the upper 15-cm layer of ground from various rice fields and brought into the greenhouse of Academia Sinica. Part of the fresh sample was immediately used for extraction, and another part was allowed to air dry in the greenhouse. The dry soil was separated from the straw and root and screened with a 2-mm sieve, thus removing all visible root fragments. Some of this dry soil was placed in pots in which chopped rice residues were mixed, and enough water added to moisten the soil.

For bioassay, seeds of rice (*Oryza sativa* var. Taichung 65) and lettuce (*Lactuca sativa* var. Great Lakes 366) were used as test materials. The rice seeds were presoaked with water for about 72 h until the radicle was just visible, and then transferred into test solutions for 6 h, while the lettuce seeds

were soaked with test solutions for 2 h. In other experiments, 3-week-old rice seedlings were used as test plants. In a third bioassay, the hypocotyl cuttings of mungbeans (1 week old) were used to determined if their root initiation was affected by decomposing rice residues.

Field Experimental Design

To determine the effect of rice residue after decomposition on the growth of rice, a field experiment was designed in Nankang by using a 4-fold split-plot design with 2 replications. In this design, the fallowing periods between the first crop and the second crop were set at 7, 14, and 21 days. In addition, there were two plots for complete fallowing during the first crop period; one plot was planted with sweet potatoes, another was left unplanted. At the beginning of the fallowing period, rice residues were removed from some plots, while in other plots the residues were not removed and served as controls. Each plot was then divided into two subplots, and planted with two varieties of rice (*Oryza sativa* var. Taichung 65 and Ai-chueh-wu-chien).

Extraction of Soils from Field and Greenhouse Settings

Once a month, about 5 kg soil was collected from the field, placed on a 10-liter container, and water added; after stirring, the mixture was then centrifuged at 5,000 rpm, and the supernatant was stored in a freezer until use.

Rice residues chopped into small pieces about 2.5 cm long were mixed with 3 kg soil and 2 liters distilled water and left to decompose for 1, 2, 4, or 8 weeks in a greenhouse. At the end of each time period, an aqueous extract was obtained by squeezing the residue–soil through cheesecloth and centrifuging at 5,000 rpm. The extract was used for chemical analysis and bioassay. The residual soil was returned to the 10-liter container, to which 1,500 ml water was added. This mixture was then allowed to decompose further for another week, after which this same extraction process was repeated.

Bioassay Techniques

Three bioassay techniques were employed to determine the phytotoxicity of the extracts. The sponge bioassay technique as described by Muller (1966) was conducted, using lettuce seeds as test material. The second bioassay technique, using the presoaked rice seeds as the test material, was a modification of Muller (1966). In this bioassay, 10 rice seeds were planted on a 5×5 cm sheet of chromatographic paper moistened with test solutions, and placed in a petri dish. The ends of the seeds were fastened by the sponge

to prevent movement during growth, and the sponge was also moistened with the test solution. The petri dish was then sealed with a sheet of cellophane and allowed to incubate at 25°C for 72 h. After incubation, the lengths of radicle and coleoptile were measured in millimeters.

The third bioassay was designed to determine the inhibitory effects of extracts on root initiation of mungbean seedlings. Five hypocotyl cuttings of mungbean seedlings were placed in a 50-ml test tube filled with 30 ml test solution. The tube was then sealed with a sponge, and covered with a piece of black paper. The experiment was set up in triplicate and was incubated at 25°C for 7 days in the light. After incubation, the number of roots initiated from the hypocotyl cuttings was counted.

Isolation and Identification of Phytotoxins in Extracts

The aqueous extracts of the decomposing rice residues were concentrated to a small volume in vacuo, and then reextracted with ethyl ether. The isolation procedures of the phytotoxins from the extracts and from the soil were described by Chou and Young (1975). The final clean extracts were placed in a 5-ml vial and sealed with a cover.

To identify the responsible phytotoxins present in the extracts, paper chromatography was used. About 50 μl (1 μl solution containing the product of about 0.1 g rice residue) of the ethanolic solution was chromatographed on a paper strip. The synthetic chemicals were simultaneously developed with a solvent of 2% acetic acid and of BAW (butanol:acetic acid:water, 4:1:5, vol/vol/vol). After being developed the paper was sprayed with the following three detecting reagents: (1) diazotized *p*-nitroaniline (DPNA), followed by 10% sodium carbonate (Hais and Mecek 1963); (2) 2,6-dichloroquinone chlorimide (DQC), followed by saturated sodium borate (Vázquez et al. 1968); (3) 0.3% ethanolic ninhydrin solution. The chromatograms were also examined under a short UV light, and some revealed absorption or fluorescence.

RESULTS

Inhibition of Rice Growth As Affected by Decomposing Rice Residues in Soil

To understand the effect of decomposing rice residues in soil on rice growth, the following experiments were designed: First, a soil–straw mixture (3 kg:200 g) was saturated with water and allowed to decompose for the time intervals of 1, 2, and 4 weeks under greenhouse conditions. The soil alone was treated in the same manner and served as control. At the end of each decomposition time, 5 rice seedlings (3 weeks old) were planted in a pot containing rice residues. After transplantation for 1 month, the rice plants

TABLE 1. INHIBITION OF GROWTH OF RICE SEEDLINGS AFFECTED BY ADDITION OF RICE RESIDUES DECOMPOSED FOR DIFFERENT PERIODS IN SOIL[a]

Decomposition period (week)	Treatment	Length of seedling (mm)	Inhibition (%)	Dry weight	
				Gram	Decrease (%)
One	Soil (control)	66	0	0.29	0
	Soil–residues	29	56[b]	0.11	62[b]
Two	Soil (control)	79	0	0.41	0
	Soil–residues	36	54[b]	0.14	66[b]
Four	Soil (control)	96	0	0.51	0
	Soil–residues	24	75[b]	0.19	63[b]

[a] At the end of each decomposition period, 5 rice seedlings (3 weeks old) were transplanted in a pot containing rice residues. After transplantation for 1 month, the length of rice seedlings and dry weight were obtained from the means of 5 seedlings in triplicate.
[b] Statistical significance at 1% level by using analysis of variance.

were taken for examination and measurement. It was found that the rice seedlings grown under control conditions were normal and usually over 66 cm tall. However, the rice seedlings grown in the decomposing material were less than 36 cm tall, and the roots were shorter and dark brown. The degree of inhibition of rice growth did not vary with length of decomposition time (Table 1).

The second experiment was set up to determine the amount of straw required to cause inhibition, but it consisted of a series with 0, 25, 50, 75, and 100 g straw mixed with 3 kg soil and saturated with water. The treatments were left to decompose for 2 weeks, after which 3-week-old rice seedlings were transplanted into the pots. The growth of rice was significantly retarded at a concentration as low as 50 g straw, and the dry weight decreased significantly with increasing amount of straw mixed (Table 2).

Effects of Extracts of Decomposing Rice Residues on Germination of Rice Seeds

The aqueous extracts obtained from a 2-week decomposition of soil–straw mixture, in which 3 kg soil was mixed with 0, 25, 50, 75, and 100 g straw, were bioassayed, using presoaked rice seeds as the test material. Distilled water was also bioassayed to serve as a control. Results expressed as the percentage of growth of the radicle and coleoptile of the control are shown in Table 3. The data reveal that the radicle was significantly suppressed by the amount of straw applied; however, the coleoptile growth was not inhibited.

TABLE 2. INHIBITION OF GROWTH OF RICE SEEDLINGS AFFECTED BY ADDITION OF DIFFERENT AMOUNTS OF RICE STRAW DECOMPOSED IN 3 KG SOIL FOR 2 WEEKS[a]

Amount of straw mixed (g)	Length of seedling (mm)	Inhibition (%)	Dry weight	
			Grams	Decrease (%)
0	59	0	0.51	0
25	52	12	0.30	41[c]
50	44	25[b]	0.23	55[c]
75	39	34[c]	0.20	61[c]
100	37	37[c]	0.12	76[c]

[a] After decomposition, 3-week-old rice seedlings were transplanted into pots; 1 month later, the length and dry weight of seedlings were obtained from the means of 5 seedlings in triplicate.

[b,c] Statistical significance at 5% (*b*) and 1% level (*c*) by using the analysis of variance.

TABLE 3. EFFECT OF EXTRACTS ON GROWTH OF RICE SEEDLINGS[a]

Condition	Amount of straw mixed (g)	Radicle growth		Coleoptile growth	
		Length (mm)	% of Control	Length (mm)	% of Control
Aerobic	0	30.3	79.1	13.3	101.8
	25	24.3	63.4[c]	14.6	111.7
	50	18.0	47.0[c]	14.8	113.4
	75	22.3	58.1[c]	13.6	104.2
	100	14.8	38.7[c]	11.6	89.1
Anaerobic	0	26.0	68.0[b]	13.4	102.0
	25	24.4	63.7[c]	15.8	121.0
	50	15.6	40.7[c]	12.0	92 0
	75	11 5	30.1[c]	13.2	101.0
	100	10.4	27.2[c]	14.2	109.0

[a] The extracts were obtained from the soil (3 kg) mixed with different amounts of straw under aerobic and anaerobic decomposition conditions for 2 weeks. Distilled water was used as control.

[b,c] Statistical significance at 5% (*b*) and 1% level (*c*) by using the analysis of variance.

In another experiment, the aqueous extracts obtained from a soil–straw mixture (3 kg:200 g) after different decomposition periods were bioassayed by using the same techniques. The results show that the radicle growth was significantly inhibited by the extracts of decomposing rice residues in the soil. The phytotoxicity decreased with the length of decomposition; thus, more than 70% inhibition was found in the extract of a 1-week decomposition, and 40% inhibition in that of a 4-week decomposition.

Phytotoxicity of Soil Extracts from Field and Greenhouse Settings

The soil extracts obtained from the rice field were bioassayed against lettuce seed, using the sponge bioassay. Results expressed as percentage of distilled water control are shown in Fig. 1. The phytotoxicity was found in extracts of field soils, where the field experiments were designed. The inhibition differed significantly among the different sampling times and the durations of fallowing, but did not differ between the treatments of residues

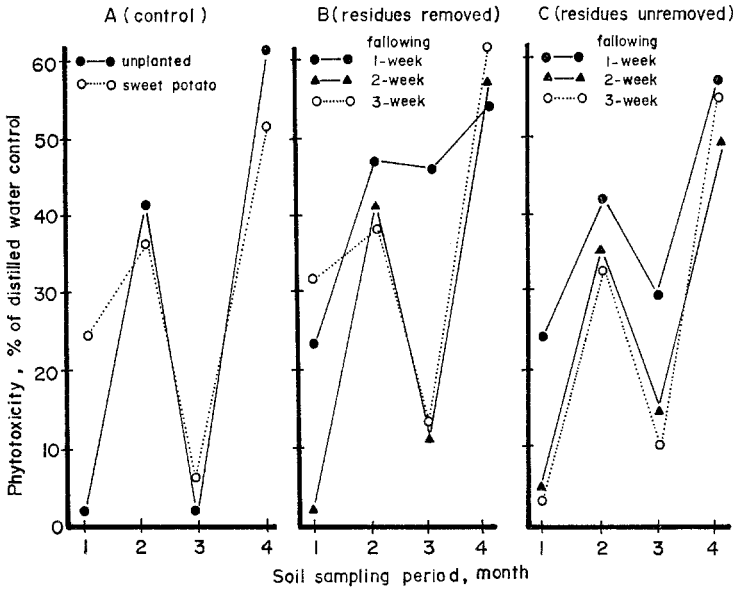

FIG. 1. Phytotoxic effect of water extract of paddy soil on growth of lettuce. The extracts were obtained from the paddy once a month during the second crop. (A) Extracts from the pots planted with sweet potatoes compared with the ones unplanted; (B) extracts from the pots with rice residues removed before the start of fallowing periods; (C) essentially the same as in (B) except that residues were not removed.

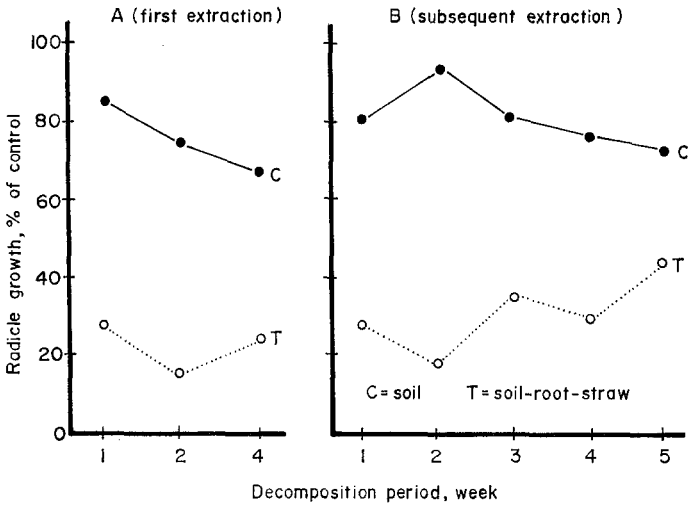

FIG. 2. Phytotoxic effect of extracts from decomposition of rice residues in soil on radicle growth of lettuce. (A) Extracts were obtained from the soil alone (C) and from the soil–root–straw after the indication of decomposition; (B) subsequent extractions were made each week on the soil and soil–root–straw samples, the residues being returned to the same pot each time for a period of further decomposition.

removed and unremoved from the plots. The phytotoxicity was persistent until the maturation of the second crop.

Two additional bioassays were also performed to determine the phytotoxicity of extracts obtained from the decomposition of rice residues and of soil alone against lettuce growth. In the first experiment, extracts were prepared from the mixture of dry straw–root–soil (100:100:3000, g/g/g), which was left to decompose for 1, 2, and 4 weeks. It was found that the extract from soil alone showed no phytotoxicity; however, the extracts from the decaying rice residues revealed more than 70% inhibition (Fig. 2A). Extracts made from the same pot 1 week later also showed significant inhibition, and subsequent extracts in the following 4 weeks continued to show inhibition (Fig. 2B).

In a second experiment, extracts were obtained from a decaying mixture of straw–soil (200 g:3,000 g) by using the same preparation process. The phytotoxicity of the extracts from the decaying rice straw was significantly higher than that from the soil alone (Fig. 3A). The toxicity was obviously high in the straw–soil extract, but gradually decreased with increased decomposition time and subsequent extractions. Nevertheless, the phytotoxicity

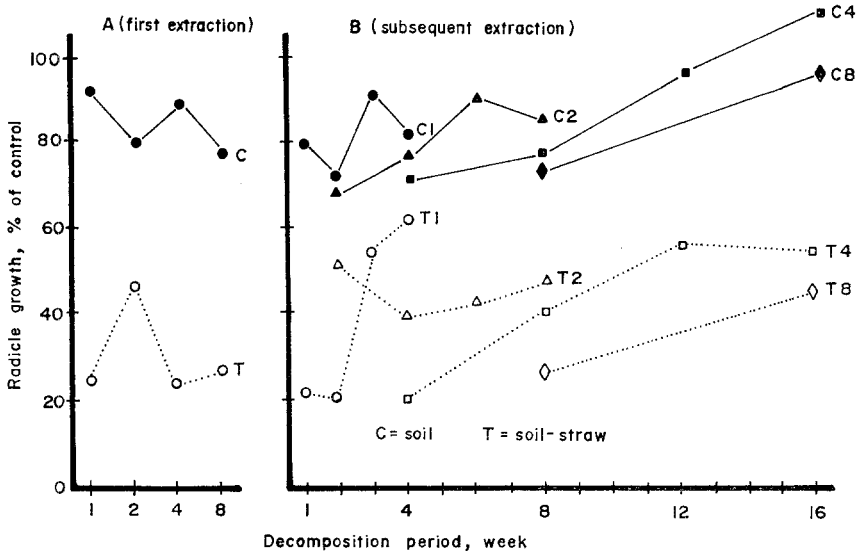

FIG. 3. Phytotoxic effect of extracts from decomposition of rice residues in soil on radicle growth of lettuce. (A) Extracts were obtained from the soil alone (C) and from soil–straw (T) after the indicated periods of decomposition; (B) subsequent extractions were made from the soil and soil–straw samples. On the C_1,T_1 sets of pots, extractions began at the end of the first week; on the C_2,T_2 at the end of the second week; on the C_4,T_4, at the end of the fourth week; on the C_8,T_8, at the end of the eighth week. After each extraction, the residues were returned to the same pot for a period of further decomposition.

(above 40%) was still persistent in extracts after 16 weeks of decomposition (Fig. 3B).

Effects of Aqueous Extracts of Decomposing Rice Residues on Root Initiation of Mungbean Hypocotyl Cutting

The earlier results have shown (see Tables 1 and 2) that the rice roots growing in the decomposing rice residues were much poorer than those in the control. An investigation, therefore, was attempted to understand the inhibition of adventitious root initiation from the hypocotyl cuttings of mungbeans to see if they were affected by rice–straw–soil extracts. The results showed that root initiation of mungbeans was greatly retarded by extracts from decomposing residues, but proceeded normally in control soil extracts. The degree of inhibition decreased with increased decomposition time (Fig 4), and the retarded hypocotyl cuttings became dark brown and quite fragile.

Using the same bioassay method, extracts obtained from paddy soil were also performed. The bioassay results agreed with those of the above

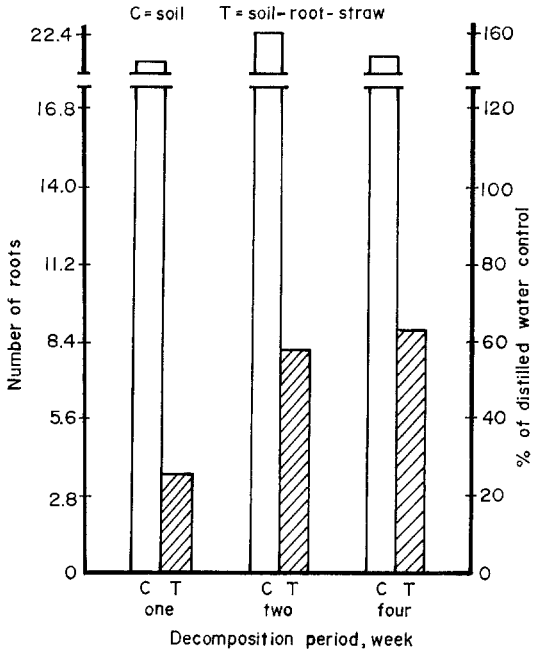

FIG. 4. Inhibition of root initiation of hypocotyl cuttings of mungbeans as affected by extracts of soil alone (C) and soil–residue (T) at different decomposition periods.

findings (Chou et al. 1975, unpublished data). From the above experimental results, it is clear that phytotoxicity is caused by decaying residues in soil. This finding suggests that some phytotoxic substances must be present in the extracts.

Identification of Phytotoxins in Decomposing Rice Residues in Soil

To identify the responsible phytotoxins in the extracts of decomposing rice residues in soil, paper chromatography was employed. The ether fraction of aqueous extracts, ethanol extracts, and alkaline ethanol extracts (Chou and Young 1975) were chromatographed separately with solvents of 2% acetic acid, and with BAW. Chromatograms were then examined under short UV light and sprayed with 3 detecting reagents, as mentioned previously. When the chromatograms of the extracts were compared with those of synthetic chemicals, some phytotoxins present in the extracts were identified. These are ferulic, *p*-coumaric, vanillic, *p*-hydroxybenzoic, and *o*-hydroxyphenylacetic acids, and several unknown compounds (Table 4). It was found

TABLE 4. DYNAMICS AND DISTRIBUTION OF PHYTOTOXINS PRESENT IN EXTRACTS OF DECOMPOSING RICE RESIDUES IN SOIL BY USING DIFFERENT EXTRACTION TECHNIQUES AND DECOMPOSED DURING DIFFERENT TIME INTERVALS.
(Quantitative comparison was made by the order ++++ > +++ > ++ > +.)

Compound	Ether fraction of aqueous extract			Ethanol extract			Alkaline ethanolic extract		
	1-Week	2-Week	4-Week	1-Week	2-Week	4-Week	1-Week	2-Week	4-Week
o-Hydroxyphenylacetic acid	++++	+	+	++	+	+			
p-Hydroxybenzoic acid	+	+	+	+	+		++	++	+
cis-p-Coumaric acid				++	++	++	+	+	+
trans-p-Coumaric acid							++++	++++	++++
Ferulic acid				+	+		+++	+++	++
Vanillic acid							++	++	+
Unknown 1[a,b]							++	++	++
Unknown 2[a,b]							+	+	+
Unknown 3[a,b]							+	+	+

Detection	Unknown 1	Unknown 2	Unknown 3
[a] R_f with 2% acetic acid	0.28	0.33	0.88
[b] Short UV light	fluorescence	absorption	absorption

that in the ether fraction of the aqueous extract of decomposing rice residue, *p*-hydroxyphenylacetic acid was present in large quantites, *p*-hydroxybenzoic acid was present only in small amounts, and there was one unidentified toxic spot on the chromatogram. The amount of *o*-hydroxyphenylacetic acid was very high (about 1×10^{-2} M) in the first week of the decomposition period, and was about 1×10^{-4} M in the second and the fourth week of decomposition. In the ethanol extract, *o*-hydroxyphenylacetic, *p*-hydroxybenzoic, and ferulic acids and one unknown were found in the extract after the first week of decomposition, but ferulic acid disappeared in the fourth week (Table 4). Furthermore, in the alkaline ethanol extraction, more phenolic compounds were found. The quantities of these compounds did not differ significantly in the various decomposition periods. Among them, *p*-coumaric acid was found in the largest amount, and three unknowns were found. These unknowns appeared to be nonphenolic in nature, and could be nitrogen-containing compounds, according to their positive ninhydrin reactions.

Inhibitory Effect of o-*Hydroxyphenylacetic Acid on Plant Growth*

Inasmuch as *o*-hydroxyphenylacetic acid was found in the aqueous extracts of decomposing rice residues in soil, the pure isolate of this compound from the chromatogram was bioassayed by using the chromatographic bioassay technique (McPherson et al. 1971). The results showed that this isolate at the concentration of 1×10^{-4} M suppressed the radicle growth of lettuce and rice seeds. In addition, the aqueous solution of this synthetic chemical was also bioassayed in a series of concentrations, such as 0, 25, 50, 75, 100, 200, and 300 ppm, using presoaked rice seed as the test material. The results of these bioassays showed that the radicle growth of rice was significantly suppressed at 25 ppm, and was completely inhibited at concentrations above 50 ppm (Fig. 5A). However, the coleoptile growth was not inhibited.

Furthermore, aqueous solutions of *o*-hydroxyphenylacetic acid in these concentrations were also tested to determine their effect on root initiation of hypocotyl cuttings of mungbeans. It was found that the root initiation of hypocotyl cuttings was inhibited at a concentration as low as 10 ppm (Fig. 5B). Above this concentration, growth was greatly retarded, and the roots began to decay.

DISCUSSION

The experimental results clearly demonstrate that rice residues submerged in soil release phytotoxic substances and that the amount depends

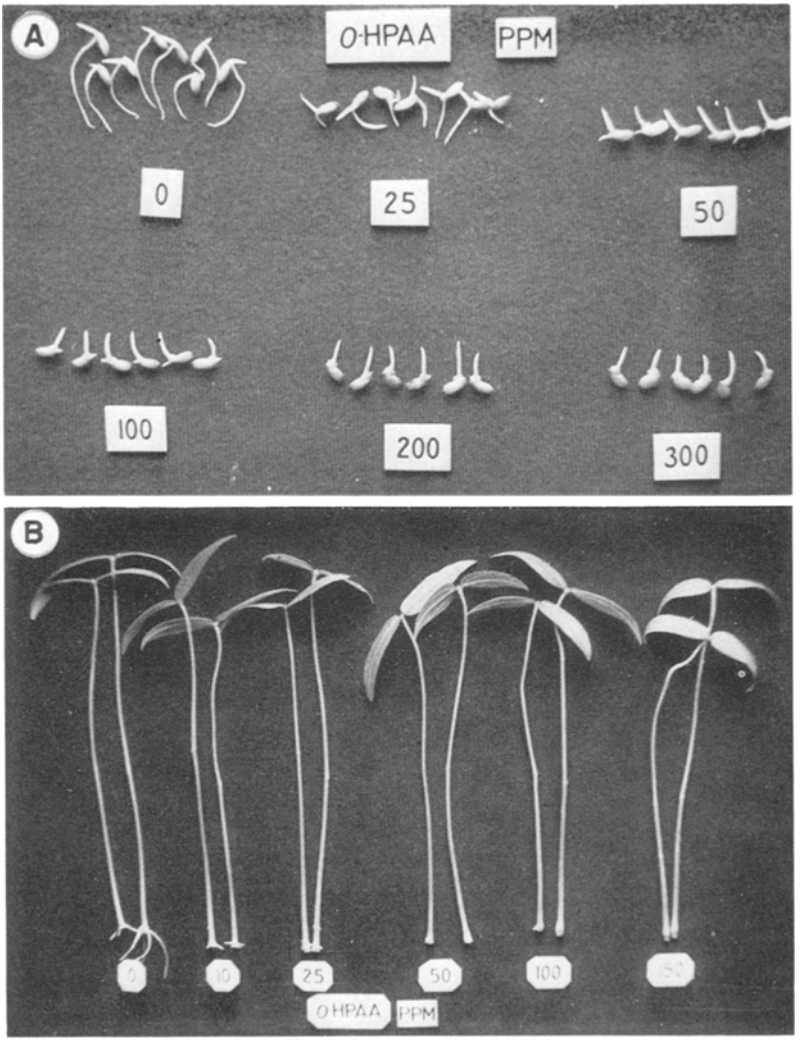

FIG. 5. Inhibitory effects of aqueous solution of *o*-hydroxyphenylacetic acid of different concentrations on the radicle growth of rice seeds (A), and on root initiation of hypocotyl cuttings of mungbeans (B).

(*Facing p.* 364)

on the amount of residues left in soil. The extract obtained from the decomposition of the rice residues in soil exhibited significant inhibition on the radicle growth of rice seedlings. This same toxicity was also revealed by soil samples from paddy soil. In addition, the root initiation of hypocotyl cuttings of mungbeans was suppressed by the extracts from the paddy soil and the decaying rice residues. Under greenhouse conditions, phytotoxicity reached its maximum at the decomposition period of 4 weeks and started to decline thereafter. However, some toxicity was persistent for 4 months. In fact, it is easy to find phytotoxicity from rice residues in paddy soil even at the end of the second crop. The undecomposed residue continues to be decomposed, resulting in release of toxic substances. Without doubt, the amount of residue accumulated in the paddy soil is greater during the second crop period than the first. Yet we still lack sufficient evidence to elucidate the real mechanism for the reduction of rice productivity in the second crop, even though the phytotoxic effect is clear. Reports from rice research in the IRRI (International Rice Research Institute, Philippines) showed that the height, tiller number, and plant weight of rice growing in the early stages in the wet season appeared to be retarded where rice straw had been applied (IRRI 1965). Our results agree with their findings.

Under waterlogged conditions, and such is the case with all rice grown in Taiwan, soil is poorly drained and oxygen is deficient, resulting in a condition favorable to the production of phytotoxic substances. Although many responsible phytotoxins, including aliphatic organic acids, phenolic acids, and other kinds of compounds, have been identified by the present authors and other researchers (Takijima 1960; Stevenson 1967; Wang et al. 1967*a*, 1967*b*; Chandrasekaran and Yoshida 1973; Kuwatsuka and Shindo 1973), several phytotoxic substances still remain unidentified. Volatile toxic compounds are thought to be present in the decomposing rice residues in soil. The unknown toxic compounds, including the volatile compounds, need to be identified. Some phytotoxins can be fixed by the humic acid in soil, making it difficult to extract them (Wang et al. 1971). Thus, other reagents such as EDTA may be helpful in extracting toxins (C.H. Muller, personal communication).

During the decomposition process, soil microorganisms play an important role. Their metabolic by-products are present in the extracts of decaying rice residues. It is difficult to determine which phytotoxins come from the residues and which are produced directly by the microorganisms. In addition, nitrogen-fixing bacteria may also be involved in the situation. The Japanese workers in the IRRI have studied the relationship between nitrogen fixation and rice straw applied and the transformation of organic debris in the soil (IRRI 1971, 1972, 1973). Chou and his associates found that the concentration of available soil nitrogen, such as NO_3^- and HN_4^+, was much

lower in the second crop of rice fields than in the first crop (Chou et al. 1976, unpublished data). Rice and his associates studied vegetation of abandoned fields in Oklahoma and found that plants in the first two successional stages produced substances that were very inhibitory to nitrogen-fixing bacteria, whereas the climax plants produced substances inhibitory to nitrification (Rice 1965, 1974; Rice and Pancholy 1972, 1973, 1974; Blum and Rice 1969). Thus, the relationship between available soil nitrogen as mentioned and phytotoxic substances is very important and is being studied.

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IDENTIFICATION AND PHYTOTOXIC ACTIVITY OF COMPOUNDS PRODUCED DURING DECOMPOSITION OF CORN AND RYE RESIDUES IN SOIL[1]

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Abstract—Residues from corn and rye plants were allowed to decompose in soil for periods up to 30 days at 22–23°C, and the identity of some of the compounds produced as well as their relative phytotoxicity to lettuce seed and seedlings were determined. Paper, thin-layer, and gas chromatography were the principal methods used to identify the various compounds formed. The identities were confirmed by comparison with known synthetic compounds. Eighteen compounds were identified in the decomposing corn residues. Of these, salicylaldehyde, and butyric, phenylacetic, and 4-phenylbutyric acids were "volatile", and benzoic, *p*-hydroxybenzoic, vanillic, ferulic, *o*-coumaric, *o*-hydroxyphenylacetic, salicylic, syringic, *p*-coumaric, *trans*-cinnamic, and caffeic acids were "not volatile". Resorcinol, *p*-hydroxybenzaldehyde, and phloroglucinol were also found. In the decomposing rye residues, nine compounds were identified, including vanillic, ferulic, phenylacetic, 4-phenylbutyric, *p*-coumaric, *p*-hydroxybenzoic, salicylic, and *o*-coumaric acids, and salicylaldehyde. In the lettuce seed bioassay, most of the above compounds from corn and rye decomposition products exhibited some phytotoxicity. Phenylacetic, 4-phenylbutyric, salicylic, benzoic, and *o*-hydroxyphenylacetic acids were highly inhibitory to the growth of lettuce at concentrations between 25 and 50 ppm. The others reduced growth significantly at 100 ppm. Most of the phototoxic spots were located in the R_f 0.37–0.97 zone when developed in 2% acetic acid solvent.

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Key Words—decomposing residues, phytotoxic activity, phytotoxin, allelopathy, rye, corn, phenolic acids.

INTRODUCTION

Organic matter from plants, in its various forms, constitutes an important component of the soil. During microbial decomposition of this plant material, a wide variety of chemical compounds are formed and released into the soil solution. In the soil, many of these compounds are involved in and affect many important biological activities. Because of the broad range of chemical compounds formed and their specific functions, extensive studies have been carried out on this subject. Some of these studies have recently been reviewed (Patrick et al. 1964; Patrick 1971; Rice 1967, 1974).

In the case of root diseases, plant residues and their decomposition products affect disease severity directly and indirectly through their effects on plant growth, pathogen survival, and plant–pathogen interactions (Patrick and Koch 1963, Patrick et al. 1963). They also have important roles in other aspects of soil microbiology (Millar 1955, Patrick and Toussoun 1965, Patrick 1971). Although the importance of these compounds in root-disease problems is well recognized, their precise role is obscure and often controversial because of the complex nature of the interactions (Guenzi and McCalla 1966, McCalla 1971, Patrick 1971, Muller 1970).

The main problems associated with plant residues and their decomposition involve the identity of some of the chemical compounds formed, their concentration in soil, and their biological activities. It was shown by various investigators (Millar 1955, Patrick et al. 1964, Patrick 1971) that some of the substances formed during the decomposition of certain plant residues have phytotoxic properties (Patrick and Koch 1958). Some of these substances have also been identified and were shown to be various types of phenolic acids (Toussoun et al. 1968, Patrick 1971). However, many other compounds with phytotoxic properties have not been identified.

The main purpose of the present study, therefore, was to identify additional compounds formed during the decomposition in soil of two of the more frequently used cover crops in Ontario, namely, rye and corn. Because of the important role of phytotoxins, the isolation and identification was confined mainly to substances with phytotoxic properties.

METHODS AND MATERIALS

Source of Crop Residues and Soils

Plant materials from corn and rye were used in the decomposition

studies. The corn was harvested from field plots when nearly mature and about 1 m high, while the rye was young, green in color, and about 15 cm high. Entire plants, including the roots, were harvested at the stages of growth mentioned, cleansed of soil, chopped into pieces about 2 cm long, and then either used immediately or air-dried and stored for future use.

Soils were sampled in the fall from fields at the Horticultural Experiment Station, Vineland Station, Ontario, Canada. The soil was placed in plastic bags, brought back to the laboratory, and stored in the freezer before use. The soil moisture of all samples was close to field capacity, and the pH of the soil ranged between pH 6 and 7.

Experimental Design and Extraction of Decomposition Products

To study the decomposition of corn residues in soil, three series of experiments were carried out using different ratios of corn residues to soil. The ratios of corn to soil by weight were 1:1, 1:2, and corn alone. In each series of experiments, 6 decomposition periods were used, namely, 5, 10, 15, 20, 25, and 30 days.

Based on the ratio of plant to soil, 400 g soil sample was mixed with either 400 g or 200 g chopped corn material. The mixture was placed in a 5-liter glass jar with a cover, and enough distilled water was added to saturate the mixture. Corn plant material without soil was treated in the same manner.

In the rye decomposition series, only 200 g soil and 200 g rye were used.

The samples were incubated at 22–23°C and allowed to decompose for the time period mentioned. After each decomposition period, aqueous extracts were obtained from each treatment, using the following procedures: The aqueous soil–plant mixture was filtered through two layers of cheesecloth, and the filtrate was centrifuged at 7,000 rpm for 15 min. The supernatant was decanted and stored in plastic bottles at 1°C. The soil precipitate obtained by centrifugation was combined with the decomposing residue from the filtering process. This combined mixture was returned to the glass jars, and enough distilled water was added to resaturate the mixture. The jars were again incubated for the time sequence mentioned earlier and reextracted. Four consecutive extractions were made from each series of experiments.

Each of the centrifuged extracts was divided into two samples. One was used for pH determination and phytotoxicity studies. The other was concentrated to less than 10% volume in a flash evaporator at a temperature below 50°C. The concentrated extract was extracted with anhydrous ether in a separatory funnel for 10 min. The upper fraction was collected, and the aqueous fraction was reextracted three times. The ether fractions were combined, and the final aqueous fraction was discarded. The ether fraction was evaporated to dryness at room temperature, then dissolved in 2 ml

100% ethanol. The alcoholic solution was used for chromatographic analysis or stored in a freezer.

Bioassay Techniques

The phytotoxicity of the crude aqueous extracts and of the partially purified phytotoxins obtained from paper chromatograms was determined by two bioassay methods. To determine volatile toxins present in the crude aqueous extract, a bioassay method that is designated as the "volatile bioassay" was used. The other bioassay method, called "chromatographic bioassay," was used to determine the toxins localized on paper chromatograms.

The volatile bioassay method was a modification of Muller (1966) and his associates. It was carried out in 100×15 mm plastic petri dishes. Four half-pieces of test tube plug sponge, $1/2 \times 3.14 \times 8 \times 40$ mm, were placed around the inside periphery of the petri dish. The sponges were saturated with the test extracts, or with distilled water as the control, before they were placed in the petri dish. A piece of 2×2-cm Whatman 3MM chromatographic paper, prewashed with distilled water and dried, was placed in the center of the petri dish. The paper was kept moist with distilled water during the bioassay period. Lettuce seeds, *Lactuca sativa* var. Great Lakes, previously washed for 2 h under running water, were used for the bioassays. Ten seeds were placed on the test paper, and the plate was sealed with a 10×10-cm sheet of parafilm. Three replicate dishes were used for each treatment and for the control. The dishes were incubated at 21–22°C for 72 h. The germination of the control seeds was uniformly above 93%. Results were expressed as percent of controls, using the radicle lengths measured to the nearest millimeter.

To determine what group of chemicals was present in the extracts, a specific bioassay method called "chromatographic bioassay" (McPherson et al. 1971, Chou and Muller 1972) was used. For the test, 30-μl samples of the ether fraction of the aqueous extracts were spotted on the chromatographic paper strips. The same amount of soil extract without crop residue was spotted on separate paper strips as the control. Paper strips were developed with 2% acetic acid. The paper strips were dried carefully after developing to avoid a solvent contamination of the paper. The chromatograms were examined under short-wavelength UV light, and sprayed with the reagents described later. Based on the R_f values of known compounds, the corresponding spots and the control chromatograms were cut out for bioassay. The bioassay techniques used to determine activities of these spots were similar to those described for the volatile bioassay, except that the sponges were soaked with distilled water instead of the test extracts.

Identification of Phytotoxins

Chromatography was used as the principal method to identify the phytotoxins. Paper, thin-layer, and gas chromatography were employed. The combined use of these three methods provided more accurate data for the quantitative and qualitative analyses of identifiable compounds. As indicated earlier, most of the phytotoxin identification was directed to phenolics.

For paper chromatography, the final alcoholic solution of the ether fraction of aqueous extracts was spotted on a 2 × 57-cm Whatman 3 MM paper strip (Wang et al. 1967*a*). The papers were developed by one-dimensional descending chromatography with 3 solvent systems: (1) 2% acetic acid (Smith 1960); (2) BuAW, 1-butanol:acetic acid:water (4:1:5, vol/vol/vol, using the organic layer) (Seikel 1964); and (3) BzAW, benzene:acetic acid:water (125:72:3, vol/vol/vol) (Steck and Wender 1965). After being developed, the papers were examined under short-wavelength UV light and sprayed with two spray reagents: (1) DPNA, diazotized *p*-nitroaniline (Hais and Macek 1963) followed by 10% sodium carbonate (Wang and Chang 1966); and (2) DQC, 0.1% ethanolic 2,6-dichloroquinone chlorimide fol-

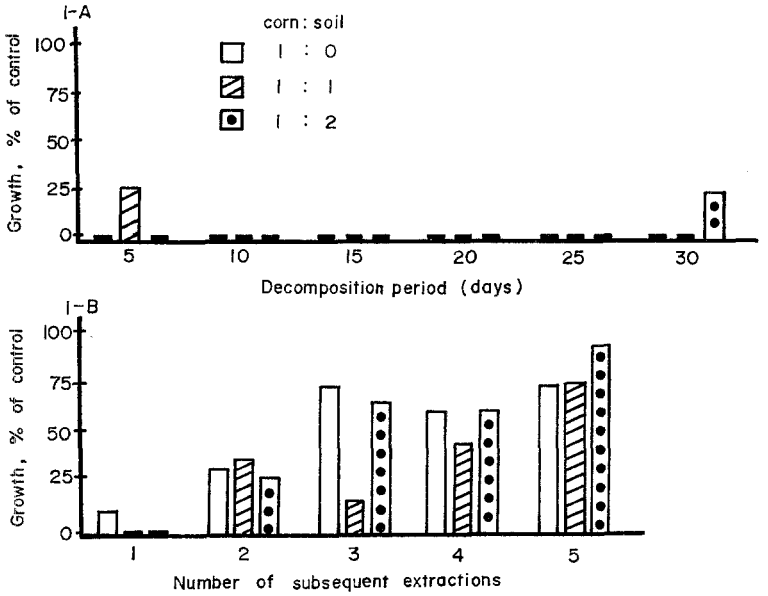


FIG. 1. Relative phytotoxicity to lettuce of compounds formed during decomposition in soil of corn residues for different periods (1-A); relative phytotoxicity of subsequent extracts made at every 25 days of decomposition (1-B).

lowed by saturated sodium borate solution (Vázquez et al. 1968). Phenolics appeared as absorbing or variously fluorescing spots under short-wavelength UV light, and as distinguishable colors after the spray reagents.

For thin-layer chromatography, precoated TLC plates, silica gel F-254 (2-mm layer), were used and developed with the solvent, methanol:chloroform (1:19, vol/vol) (Méndez and Brown 1971). The extracts and known chemicals were run simultaneously in order to make a precise identification of each spot. Each R_f value was based on the mean of three replications.

For gas chromatography, a Pye Unicam series 105 FID gas chromatograph was used. The analytical column was 152 cm long, 4 mm ID, packed with 10% cyclohexane dimethanol succinate on 100–120 mesh celite. The programmed temperature was set from 100°C to 235°C at an increasing rate of 3°C/min. The pressures of the gases were: N_2 at 14 kg/cm^2, H_2 at 11 kg/cm^2, and compressed air at 27 kg/cm^2. The recorder speed was 38 cm. The attenuation was set depending on the concentration of the sample, usually at 5×10^2. Samples of 3 μl of the ether fraction of the aqueous extracts were injected into the gas chromatograph. The highly purified compounds eluted from the paper chromatograms and the standard synthetic known compounds were run under the same conditions. The peak area and its retention distance in millimeters for each sample following the solvent peak were obtained, and the significant phytotoxins present in the crop residue–amended soil were identified.

RESULTS AND DISCUSSION

Bioassay of Aqueous Extracts from Decomposed Crop Residues in Soils

Phytotoxins from Corn Residue Decomposition Products. The phytotoxicity of aqueous extracts obtained from the decomposing corn residues was determined by using the volatile bioassay method. In this bioassay, the test seeds received the volatiles from the surrounding extracts held by sponges without direct contact with the extract. The bioassays of these extracts showed that they were extremely toxic to the growth of lettuce (Fig. 1-A). Two extracts exhibited 75% inhibition of growth of lettuce; the rest exhibited 100% inhibition. The seeds exposed to the toxic atmosphere from the extracts were damaged, and some turned black or dark brown. Some of the seeds were also swollen. In addition, the radicle was abnormal and lacked root hairs, and there was necrosis of the root tips. The growth of lettuce in the control was normal. The abnormal growth effects were similar to the earlier findings of Patrick and Koch (1958), Patrick (1971), and Chou and Muller (1972).

It is believed that the radicle damage is localized to the meristematic tissue, resulting in suppression of root elongation (Muller 1971). The volatile

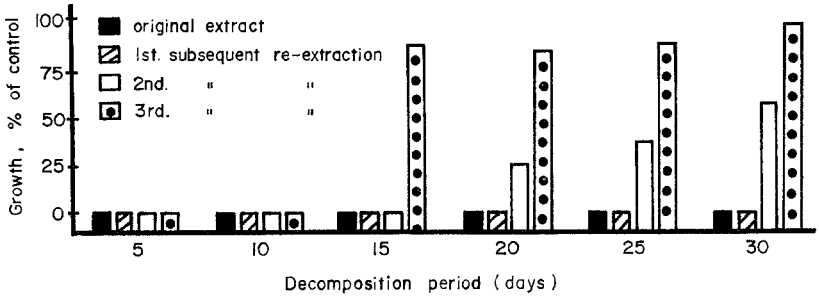


FIG. 2. Relative phototoxicity of compounds formed during decomposition of rye residues as affected by decomposition and number of subsequent reextractions.

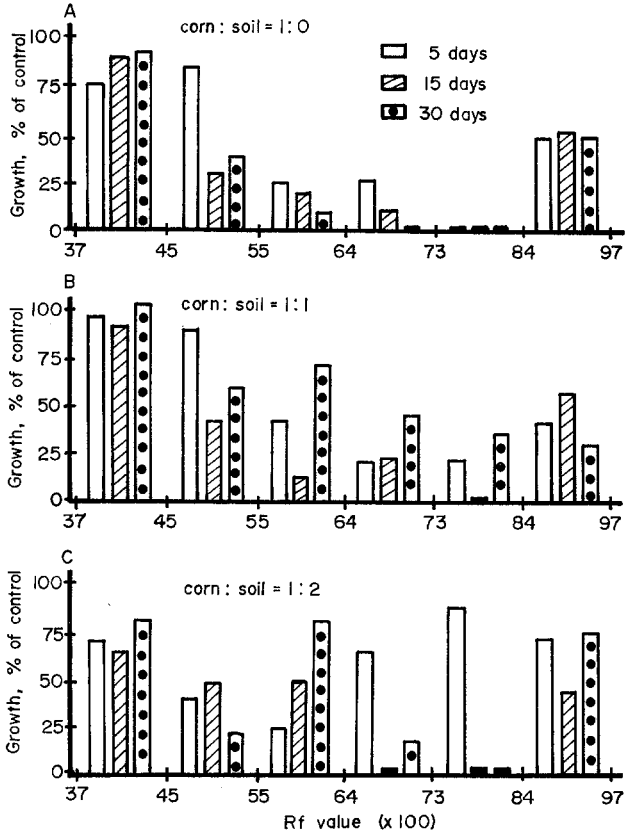
components of the extracts were investigated, and were shown to be mainly simple aliphatic compounds and simple phenolic compounds.

As shown in Fig. 1-B, the toxicities tended to decrease with the second extraction, and were almost lost after the fifth extraction. It appears that the phytotoxicity of the extracts is dependent mainly on the amount of organic substrate decomposing in the soil. The toxicity decreased as the amounts of soil incorporated into the corn residue was increased or as the decomposition period was increased. These results support those described earlier (Patrick 1971).

Phytotoxins from Rye Residues. The aqueous extracts of rye residue decomposing in soil were obtained by using the same techniques described above. Aqueous extracts of each treatment were obtained at three different periods, and their phytotoxicity determined using the volatile bioassay. The bioassay results are shown in Fig. 2. The extracts exhibited 100% inhibition of growth of lettuce. The toxicity decreased after the second extraction. As the decomposition period was increased from 15 to 30 days, the toxicities of the third set of extracts were almost negligible, and were similar to the controls (Fig. 2). Severe root damage, growth inhibition, absence of root hairs, and necrosis of apical meristem were seen in the lettuce seeds exposed to extracts of the rye–soil mixture. The pH of these extracts was 5.5–7.5, indicating that pH does not seem to be the significant factor determining the toxic effect. As in the corn decomposition studies, it appears that the volatiles present in the decomposed rye residue in soil are the phytotoxic agents.

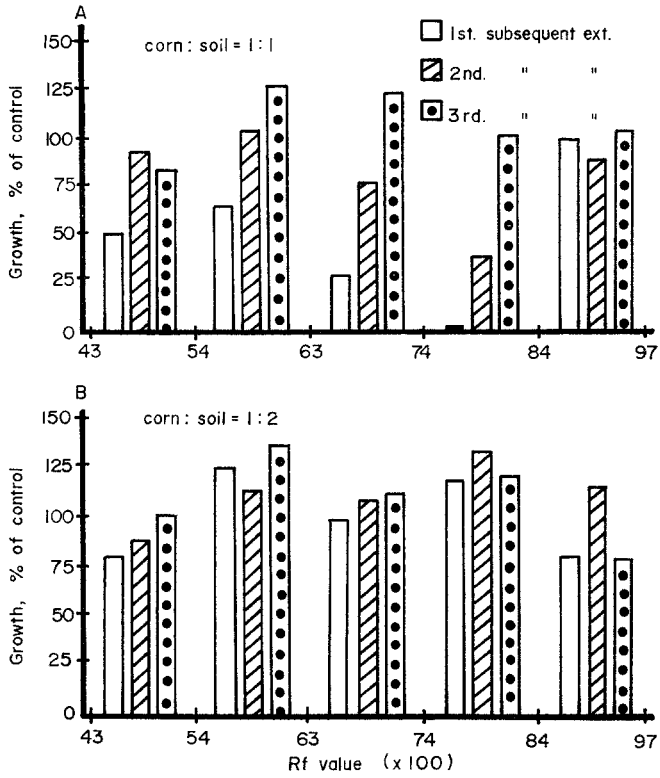
Chromatographic Bioassay of Phytotoxic Extracts

Phytotoxins from Decomposing Corn Residues. By using the chromato-



FIGS. 3A–C. Relative phytotoxicity of decomposing corn residues as determined by chromatographic bioassay against lettuce growth.

graphic bioassay described earlier, the phytotoxicity was determined on 30 μl (about 1.5% concentration of extracts) of the ether fraction of each extract. The control series consisted of soil without added crop residue. The results are shown in Figs. 3A–C. Statistical analysis of the bioassay results were made by using the Student's t-test. Most of the toxic spots were located in the zone between 0.37 and 0.97. Two distinguishable toxic spots were located on the chromatogram at R_f 0.63–0.73 and at R_f 0.73–0.84. These two toxic spots not only inhibited lettuce seed germination, but also suppressed radicle growth. The rest of the toxic spots showed suppression of radicle growth, rather than inhibition of seed germination. Toxicity was maximum after 25–30 days' decomposition. The results in Fig. 3 agree with the volatile bioassay. As more soil was incorporated with the corn residue, the

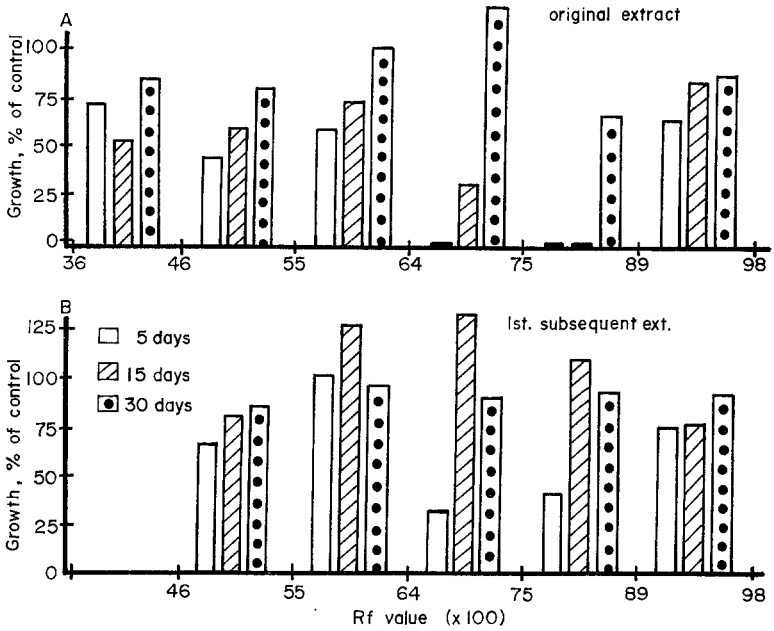


FIGS. 4A,B. Relative phytotoxicity of decomposing corn residues as affected by serial extractions of the extracts made at every 25 days of decomposition period.

toxicity was decreased. Chromatographic bioassay of three subsequent extracts was also performed. The results shown in Fig. 4, at 25 days' decomposition, indicated that the toxic spots remain in the R_f 0.63–0.84 zone. However, the toxicity was almost lost in the third subsequent extracts, in both the 1:1 and 1:2 corn-to-soil ratios.

Phytotoxins from Decomposing Rye Residues. Phytotoxicity of rye residue extracts was investigated using the same techniques as for corn. The results shown in Fig. 5 are representative of the bioassay results with extracts obtained after 5, 15, and 30 days' decomposition.

The toxic pattern of rye–soil extracts was quite different from that of corn–soil extracts. The toxicity built up rapidly at 5–15 days', and decreased after 20 days' decomposition. The toxicity was almost lost in the subsequent extracts.



FIGS. 5A,B. Phytotoxicity of decomposing rye residues as affected by decomposition period (5, 15, and 30 days) and serial extraction. The ratio of rye residue decomposed in soil was 1:1.

Identification of Phytotoxins Produced During Decomposition of Crop Residues in Soil

The ether fraction of the aqueous extracts was first analyzed by paper chromatography. The toxic spots were then eluted and purified by a method described by Chou (1971). The toxic spots were compared with known synthetic chemicals, using paper and gas chromatography. The results of the chromatographic analyses on both the unknown and the known compounds are shown in Table 1, in which most of the data are mean values from three replications. By using comparable data—such as the retention distance provided by gas chromatography, the R_f values of three solvent systems, and three color reactions obtained from paper chromatography—the unknown toxic compounds in the extracts could be identified. Thin-layer chromatography was also helpful in making further identification of some unknowns, such as phloroglucinol and resorcinol, and ferulic, *o*-coumaric, and caffeic acids.

Eighteen compounds were identified in the decomposing corn residues by using the techniques describe above (Table 2). Among them, butyric,

phenylacetic, and 4-phenylbutyric acids, and salicylaldehyde seem to be highly volatile. Compounds such as benzoic, *p*-hydroxybenzoic, vanillic, ferulic, *p*-coumaric, *o*-coumaric, *o*-hydroxyphenylacetic, salicylic, syringic, *trans*-cinnamic, and caffeic acids, and resorcinol, *p*-hydroxybenzaldehyde, and phloroglucinol are not volatile under test conditions.

The results of the phytotoxin identification agree with the bioassay results. The concentration of toxins reached a maximum at 15–25 days' decomposition. It was estimated that the concentration of each phytotoxin in decomposing corn residue was in the range of 5–100 μg/g soil.

The concentration of corn toxins was also related to the ratio of corn residue to soil. With the incorporation of more soil with the corn residue, fewer phytotoxins were found. *o*-Coumaric acid was present in soil after 5 serial extractions (Table 3), while the other compounds had disappeared after the second or third extraction.

The chromatographic bioassay revealed 4 toxic zones in which phloroglucinol, resorcinol, *trans*-cinnamic, *o*-coumaric, and *o*-hydroxyphenylacetic acids were identified. The phytotoxins such as benzoic, butyric, phenylacetic, and 4-phenylbutyric acids could not be detected by color spray reagents. Butyric, benzoic, phenylacetic, and 4-phenylbutyric acids were found in decomposing barley residues (Toussoun et al. 1968). Vanillic, *p*-coumaric, ferulic, syringic, and *p*-hydroxybenzoic acids were found ubiquitously in the crop vegetation of the world (Wang et al. 1967*b*, Börner 1971) and in stubble mulch fields (Guenzi and McCalla 1966). In addition to these 5 compounds, *o*-coumaric acid was also found in the litter and in soils of California chaparral vegetation (McPherson et al. 1971, Chou and Muller 1972), where they are ecologically significant by producing detrimental effects on the growth of grassland species.

In decomposing rye residues, 9 compounds were identified. These were salicylaldehyde, and vanillic, ferulic, phenylacetic, *o*-coumaric, 4-phenylbutyric, *p*-coumaric, *p*-hydroxybenzoic, and salicylic acids (Table 4). These toxins were most abundant in the 5- and 15-day decomposition periods.

Attempts were also made to determine the concentration levels of the various phytotoxins in the decomposing corn and rye residues. The results were inconclusive, but it appeared that the concentration ranged between 5 and 100 μg/g soil (see Table 2).

Bioassays with Known Synthetic Chemicals

Since the identities of the phytotoxins in the decomposing corn and rye residues were established with the aid of known synthetic chemicals, bioassays were also conducted using the synthetic chemicals to determine whether similar phytotoxic effects were produced. In the present study, 13 authentic

TABLE 1. IDENTIFICATION OF PHYTOTOXINS FROM DECOMPOSING PLANT RESIDUES IN SOILS
(The identity of the plant-derived phytotoxins was based on comparison with known synthetic compounds)

Compounds[a]	Gas chromatography[b]		Paper chromatography					
			R_f values (×100)			Color reaction[c]		
	Retention distance	(mm)	2% AA	BuAW	BzAW	s UV	DPNA	DQC
o-Hydroxyphenylacetic acid	35.5	87.5	82	88	70	ab	v-r	dk bl
Phytotoxin 1	34.5		81	88	66	ab	v-r	dk bl
p-Hydroxybenzoic acid	64		62	88	64	ab	r	dk bl
Phytotoxin 2	63		64	82	66	ab	r	dk bl
p-Coumaric acid	105		44 68	88	65	ab	dk bl	dk bl
Phytotoxin 3	106		41	88	62	ab	dk bl	bl
Vanillic acid	96		56	85	75	ab	v	pu bl
Phytotoxin 4	96		55	86	77	ab	v	pu bl
Ferulic acid	93.5		40 72	84	76	bl fl	sky bl	pu v
Phytotoxin 5	92		33 65	86	77	bl fl	sky bl	pu v
o-Coumaric acid	85	127	50 75	89	73 92	w fl	v-r	sky bl
Phytotoxin 6	85	127.5	47 75	89	67	NS[d]	v-r	sky bl
Syringic acid	110		49	83	39 75	ab	bl	pu bl
Phytotoxin 7	111		53	81	NS	ab	bl	pu bl
Phloroglucinol	175		60	70	10	ab	yel or	pk
Phytotoxin 8	176		61	72	16	ab	yel or	NS
Resorcinol	188		70	86	38	ab	yel or	gr pu
Phytotoxin 9	188		67	85	40	ab	yel or	gr pu

Caffeic acid	199.5		30	63	80	30	bl fl	yel gr	NS
Phytotoxin 10	200		28	60	78	29	bl fl	yel gr	NS
trans-Cinnamic acid	160		66		90	89	ab	NS	NS
Phytotoxin 11	162		67		89	NS	ab	NS	NS
p-Hydroxybenzaldehyde	172.5		70		90	67	ab	NS	NS
Phytotoxin 12	171		70		NS	NS	ab	NS	NS
Salicylaldehyde	33	59 68.5	NS		NS	NS	NS	NS	NS
Phytotoxin 13	32	59 68	NS		NS	NS	NS	NS	NS
Butyric acid	14		NS		NS	NS	NS	NS	NS
Phytotoxin 14	14.5		NS		NS	NS	NS	NS	NS
Phenylacetic acid	120		NS		NS	NS	NS	NS	NS
Phytotoxin 15	119.5		NS		NS	NS	NS	NS	NS
4-Phenylbutyric acid	132.5	144.5	NS		NS	NS	NS	NS	NS
Phytotoxin 16	132	145.5	NS		NS	NS	NS	NS	NS
Salicylic acid	149		NS		NS	NS	NS	NS	NS
Phytotoxin 17	150		NS		NS	NS	NS	NS	NS
Benzoic acid	100		NS		NS	NS	NS	NS	NS
Phytotoxin 18	100		NS		NS	NS	NS	NS	NS

[a] Plant-derived phytotoxins are designated as Phytotoxin 1, 2, 3, etc.
[b] The working conditions of the gas chromatograph are described in the text; the retention distance of each compound was measured by the distance from the sample peak to the solvent peak.
[c] Abbreviations: ab: absorption; bl: blue; dk: dark; fl: fluorescence; gr: gray; or: orange; pk: pink; pu: purple; r: red; v: violet; w: white; yel: yellow.
[d] NS: not sensitive.

TABLE 2. RELATIVE CONCENTRATIONS OF PHYTOTOXINS IN 3 TREATED CORN–SOIL MIXTURES AT 1:0, 1:1, AND 1:2 RATIOS AT VARIOUS DECOMPOSITION PERIODS[a]

	Decomposition periods								
	5 Days			15 Days			30 Days		
Compounds identified	1:0	1:1	1:2	1:0	1:1	1:2	1:0	1:1	1:2
Benzoic acid		+++			+++				+
Butyric acid	++	+++	++++	++++	++++	++++	+	++++	+
Caffeic acid							+		+
trans-Cinnamic acid	+	+							
o-Coumaric acid	++	++	+++	++++	++++	+	++++	++++	++++
p-Coumaric acid	++		+			+	+	+	+
Ferulic acid	++++	++			+				
p-Hydroxybenzaldehyde	+	+		+	+	+	+	+	
p-Hydroxybenzoic acid		+++			++	+	+	+	+
o-Hydroxyphenylacetic acid			+++		++++				
Phenylacetic acid	+			++++		++++	++++	++++	++++
4-Phenylbutyric acid	+			++	+		+		
Phloroglucinol	+	+	+	+	+	+	++		+
Resorcinol		+			+			+	
Salicylaldehyde			+	+++					++++
Salicylic acid	+		+	+		+	+	+	+
Syringic acid	+	+		+					
Vanillic acid	++	++	+	++					

[a] The quantitative comparison was determined by using the gas chromatograph; thus, the concentration was classified as more than 50 μg/g soil (++++), 25–50 μg/g soil (+++), 25–10 μg/g soil (++), and less than 5 μg/g soil (+). The working conditions of the gas chromatograph are described in the text.

TABLE 3. RELATIVE CONCENTRATIONS OF PHYTOTOXINS IN AQUEOUS EXTRACTS OF DECOMPOSING CORN RESIDUES FOLLOWING EACH OF 5 SERIAL EXTRACTIONS[a]

	Reextraction number														
	1			2			3			4			5		
Compounds identified	1:0	1:1	1:2	1:0	1:1	1:2	1:0	1:1	1:2	1:0	1:1	1:2	1:0	1:1	1:2
Benzoic acid	+	+		+											
o-Coumaric acid	++++	++++		++++	++++		+++	++		+++			++		
p-Hydroxybenzoic acid			+												
Phloroglucinol	+	+		+++			+								
Phenylacetic acid	+++	++++		++	++										
Salicylaldehyde		+		++++											

[a] The extracts used were at the 25-day decomposition period, and 5 reextractions were made. The corn–soil ratios were 1:0, 1:1, and 1:2. The quantitative comparisons were the same as those described in Table 2.

TABLE 4. IDENTITY OF PHYTOTOXINS IN EXTRACTS OF DECOMPOSING RYE RESIDUES IN SOILS AND THEIR RELATIVE CONCENTRATIONS[a]

					Reextraction number					
	Original extraction				1			2		
Compounds identified	0[b]	5	15	30	5	15	30	5	15	30
o-Coumaric acid		++	++++	++	+	+				
p-Coumaric acid	++									
p-Hydroxybenzoic acid	+									
Phenylacetic acid	+	++	++	+						
4-Phenylbutyric acid		+	+	+			+			
Salicylaldehyde		++	++		+++					
Salicylic acid	+	++	+	+	+	+		+		
Ferulic acid	++									
Vanillic acid	+	+	+	+						

[a] The extractions were made after 0, 5, 15, and 30 days' decomposition. The rye–soil ratio was 1:1. The quantitative comparisons were the same as those described in Table 2.

[b] Decomposition period in days.

TABLE 5. EFFECTS OF KNOWN COMPOUNDS IN AQUEOUS SOLUTION ON RADICLE GROWTH OF *Lactuca sativa* AFTER 72 HOURS AT 21–22°C

	Radicle growth (% of control)						
	Concentration (ppm)						
Compounds	25	50	75	100	200	300	400
Phenylacetic acid	55	23	14	0	0	0	0
4-Phenylbutyric acid	85[a]	39	27	0	0	0	0
Salicylic acid	39	31	0	0	0	0	0
Butyric acid				51	31	27	18
Benzoic acid	77	57	27	40	0	0	0
o-Hydroxyphenylacetic acid				30	26	10	0
o-Coumaric acid				30	23	25	21
trans-Cinnamic acid				43	20	24	25
p-Hydroxybenzoic acid[b]				46	40	37	34
Phloroglucinol				66	43	30	26
Resorcinol				63	49	41	25
Vanillic acid[b]				77	64	48	46
p-Hydroxybenzaldehyde				74	66	62	60
p-Coumaric acid[b]				89[a]	72	55	41
Syringic acid[b]				78	81	72	69
Ferulic acid[b]				83	88[a]	77	77
Caffeic acid				110[a]	92[a]	100[a]	82
Salicylaldehyde				93[a]	73	101[a]	82

[a] The results were not statistically significant from the control at the 5% level of confidence using the Student's *t-test*. The data without footnote (*a*) were significantly different from the control below the 5% level of confidence.

[b] Bioassay data of these chemicals are after Chou (1971).

chemicals (purchased from J.T. Baker Chemical Co.) were bioassayed by the chromatographic bioassay. The concentrations used were 25, 50, 75, 100, 200, 300, and 400 ppm, which are believed to be in the range of those found naturally.

The results (Table 5) expressed as the percent radicle growth of the control. The results showed that phenylacetic, 4-phenylbutyric, salicylic, benzoic, and *o*-hydroxyphenylacetic acids were highly inhibitory to the growth of lettuce, even at concentrations between 25 and 50 ppm. The rest of the test chemicals reduced growth significantly at 100 ppm. Butyric acid, a highly volatile substance, was bioassayed without direct contact with lettuce seeds, and produced considerable suppression of the growth of lettuce. The most toxic compounds were those in the R_f 0.37–0.97 zone, when developed

in 2% acetic acid solvent. In general, the results with the known synthetic compounds were similar to those obtained with the plant-derived phytotoxins, and the identities of the latter are thus confirmed.

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RESPONSE TO A MAMMALIAN PHEROMONE AND ITS GEOMETRIC ISOMER

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Abstract—Black-tailed deer (*Odocoileus hemionus columbianus*) discriminate between the geometric isomers of a lactone used as a pheromone by the species. They react strongly to (Z)-4-hydroxy-6-dodeceonic acid lactone, which occurs in their tarsal scent, but the response to the *E* isomer did not differ from the response to the solvent. The isomers were applied to one member of a group of freely interacting deer, and the sniffing, licking, and following responses were recorded. The synthesis of the geometric isomers of the lactone is described.

Key Words—Black-tailed deer, geometric isomers, (*E*) and (*Z*)-4-hydroxy-6-dodecenoic acid lactone, mammals, *Odocoileus hemionus columbianus*, olfactory discrimination, pheromones, stereoisomers, tarsal scent.

INTRODUCTION

Black-tailed deer (*Odocoileus hemionus columbianus*) possess a tarsal organ on the inside of their hocks. The organ consists of a cutaneous gland (composed of apocrine and sebaceous glands) and a conspicuous hair tuft. The latter carries the "scent"[2] due to glandular secretion and urine. The deer apply the urine with rubbing movements of their hindlegs ("rub-urination"). Both sexes have a tarsal organ, and group members sniff and lick each other's

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[2] The term "scent" is used here to mean everything that can be extracted from the tarsal hair tufts.

tarsal hair tufts. It has been shown experimentally that the tarsal scent serves in recognition of the sex of the individual, and of strange vs. familiar individuals (Müller-Schwarze 1971), and in subspecies recognition (Müller-Schwarze and Müller-Schwarze 1975). The main component that releases sniffing and licking responses is (*Z*)-4-hydroxy-6-dodecenoic acid lactone (see Fig. 1, structure 1) (Brownlee et al. 1969). In a previous study (Müller-Schwarze 1969), it was shown that the deer discriminate between this unsaturated lactone and saturated C_{10}–C_{12} lactones. We now report on a comparison of the responses to the naturally occurring *Z* lactone (structure 1) and its *E* isomer (structure 2).

METHODS AND MATERIALS

Test Animals

Two groups of black-tailed deer were used in these experiments, one consisting of 3 adult females (3–5 yr old during the test) the other of 6 captive-born fawns (5–8 mo old). The two groups were kept separately in two 40 × 17-m pens. All animals had been hand-reared to make them tractable as experimental animals, and were socially well integrated within their group. They were fed alfalfa hay, food pellets (50% alfalfa, plus barley, wheat bran, and minerals), salt, water, and, in addition, rolled oats in winter. Fresh grass and forbs were available to them in their pens throughout the growing season.

Preparation of Crude Tarsal "Scent"

Tarsal glands of male deer killed by hunters in Monterey County, California, were excised, and the tarsal hair was extracted in petroleum ether (30–60°C). One male gland can be expected to contain from 5 to 80 μg lactone (Müller-Schwarze 1971), with an estimated modal amount of 20 μg. To obtain the 2 μg needed for each test, one-tenth of the concentrated total extract from one gland was dissolved in 0.25 ml solvent.

Synthesis of the Z *and* E *Lactones*

The synthesis scheme (Fig. 1) was designed to yield both the *Z* (structure 1) and *E* (structure 2) isomers of the lactones. Furthermore, it was possible to resolve the enantiomers of the carboxylic acid (structure 4), and thus to prepare the enantiomers of the *Z* lactone, which will be used for behavioral studies. Yields were acceptable for all steps except the last, in which the best yield was 8%.

3-*Hydroxyadipic Acid γ-Lactone* (*Structure* 4). β-Hydromuconic acid

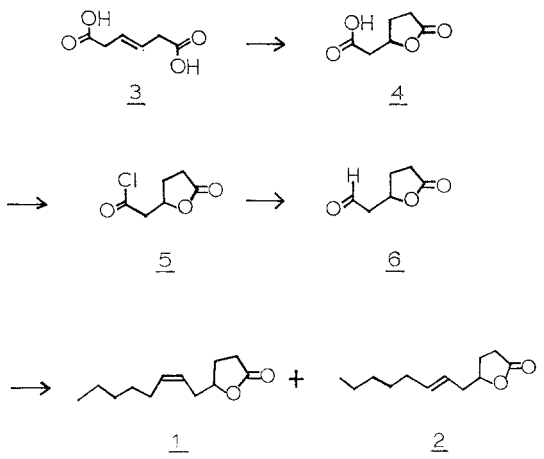

FIG. 1. Synthesis of (*Z*)- and (*E*)-4-hydroxy-6-dodecenoic acid lactone.

(structure 3), 100 g (0.69 mol), was refluxed with 500 ml glacial acetic acid and 250 ml concentrated hydrochloric acid for 1 week. The solution was concentrated at 100°C, 16 mm. Methylene chloride, 100 ml, was added, the mixture was left overnight at 10°C, and 4.6 g precipitated by-product was filtered off. The filtrate was concentrated, first on a rotary evaporator at 15 mm, then in a distillation apparatus at 100°C, 0.1 mm, to yield 91.2 g (91%) of viscous liquid residue, which, after a few days at room temperature, became a white solid, mp 46–55°C. A sample recrystallized from ether–methanol melted at 61–62°C (Elvidge et al. [1950] report 60–61°C); MS 144 (M), 85 (base); IR ($CHCl_3$) cm^{-1}, 1,770 (lactone C = 0), 1,724 (acid C = 0), 1,170 (C—0); NMR ($CDCl_3$, δ) 11.2 (COOH, s_1,1), 4.9 (CH—0, m,1).

Analysis: $C_6H_8O_4$; calculated: C, 50.00; H, 5.60
found: C, 49.85; H, 5.84

5-*Chlorocarbonyl*-4-*Hydroxyvaleric Acid Lactone* (*Structure* 5). The acid lactone (structure 4) (11.5 g, 0.08 mol) was mixed with 20 ml dry toluene (reagent grade, distilled and treated with 4A molecular sieves) and 20.3 g (0.16 mol) oxalyl chloride, in a flask protected with a $CaSO_4$ tube. The reaction proceeded at room temperature, bubbling as the acid dissolved, for 4 h; the solution was allowed to stand at room temperature for an additional 12 h, then concentrated at 40°C, 15 mm. Short-path distillation of the residue (80–90°C, 0.1 mm) gave 9.9 g (76%) of the acid chloride (structure 5). (Wessely et al. [1957]: bp 110–120°C (2 mm)); NMR ($CDCl_3$,δ) 4.92 (m,1); 3.33 (d,2).

4-*Hydroxy*-6-*Oxohexanoic Acid Lactone* (*Structure* 6). A mixture of

0.1 g 10% Pd on carbon catalyst and 0.05 mg tetramethylthiourea (Affrossman and Thomson 1962) in 20 ml dry toluene was stirred magnetically under N_2 for 15 min. Then 0.50 g (3.1 mmol) lactone acid chloride (structure 5) in 10 ml toluene was added, and, after flushing with N_2, H_2 was bubbled through the vigorously stirred reaction mixture at 10 ml/min. The effluent gases were passed through water to trap the HCl, and this aqueous solution was titrated with 0.20 N NaOH to follow the progress of the reaction. When the reaction was complete, after about 4 h of H_2 flow, the mixture was filtered through celite and concentrated on a rotary evaporator at 60°C, 15 mm. Short-path distillation of the residue (80–85°C, 0.05 mm) gave 0.16 (40%) of colorless liquid aldehyde (structure 6): IR (neat) cm^{-1}, 2,950 (CH), 2,740 (aldehyde CH), 1,767 (γ lactone C=0), 1,724 (aldehyde C=0); NMR ($CDCl_3$,δ) 9.80 (s,1), 4.95 (m,1).

n-*Hexyltriphenylphosphonium Bromide*. A solution of 10.0 g (0.038 mol) triphenylphosphine and 6.27 g (0.038 mol) redistilled 1-bromohexane in 50 ml chlorobenzene was refluxed for 24 h. A small amount of the viscous oil obtained by cooling a sample of the reaction mixture was washed with ether, and it solidified in a few hours. This solid was used to seed the cooling reaction mixture. The filtered crystals were washed with ether and dried in an evacuated desiccator over P_2O_5; the yield was 13.2 g (80%) of *n*-hexyltriphenylphosphonium bromide: mp 201–204°C (Hauser et al. [1963], mp 198–200°C), IR (KBr) cm^{-1} 1,422, 1,105, 742, 719, 687; NMR ($CDCl_3$,δ) 7.6–7.9 (m,15), 3.7 (m,2).

Analysis: $C_{24}H_{28}PBr$; calculated: C, 67.4; H, 6.6; P, 7.3
found: C, 67.67; H, 6.73; P, 7.12

4-*Hydroxy*-6-*Dodecenoic Acid Lactone* (*Structures* 1 *and* 2). A phenyllithium solution (3.3 ml, 6.6 mmol, 2.0 M in 70:30 benzene–ether) was added under N_2 to 2.81 g (6.6 mmol) *n*-hexyltriphenylphosphonium bromide. The mixture was stirred for 2 h at room temperature, and 5 ml of dimethylformamide (DMF) (distilled at reduced pressure from BaO and stored with 4A molecular sieves) was added. The red ylide solution was added dropwise under N_2 to a stirred solution, chilled to −40°C, of 0.85 g (6.6 mmol) freshly prepared aldehyde lactone (structure 6) in 5 ml purified DMF. After 0.5 h of stirring at −40°C, the reaction was allowed to warm to room temperature, and 0.1 ml 50% aqueous acetic acid was added. The mixture was concentrated on a rotary evaporator at 70°C, 15 mm; the residue was extracted 4 times with 2 ml ether, the ether was evaporated, and the residue was taken up in 5 ml petroleum ether (bp 30–60°C). The petroleum ether solution was concentrated at 40°C, 15 mm, and the residue was distilled in a short-path still at 105–120°C, 0.2 mm. The distillate was taken up in 5 ml cyclohexane and analyzed by GLC (5 m × 2.4 mm ID 10% DEGS on Chromosorb W 60–80-

mesh, 185°C, 40 ml/min N_2). The yield of *E* and *Z* lactones was 8%, and the ratio of *Z*:*E* was 8.7:1.

The *E* lactone (structure 2) had a retention time of 36.0 min on the DEGS column under the above conditions; MS 196 (M), 85 (base); IR (CCl_4) cm^{-1}, 1,783 (C=0), 1,175 (C—0), 1090 (*trans*=); NMR ($CDCl_3$) 5.4 (CH=CH, m,2), 4.5 (CH—0, m,1).

The *Z* lactone (structure 1) cochromatographed with the lactone isolated from the tarsal scent, retention time 38.3 min under the above conditions; ms *m/e* 196 (M), 85 (base), IR (CCl_4) cm^{-1}, 1,783 (C=0), 1,175 (C—0); NMR ($CDCl_3$,δ) 5.4 (CH=CH, m,2), 4.5 (CH—0, m,1).

Analysis: $C_{12}H_{20}O_2$; calculated: C, 73.43; H, 10.27
found: C, 73.38; H, 10.22

Stimulus Presentation

The crude tarsal extract and the *Z* and *E* isomers of the lactone were presented in random order to the experimental animals by spraying a sample dissolved in petroleum ether onto the outside of one shank of one female (odor carrier) in the group. Three concentrations (2.0, 0.2, and 0.02 μg lactone dissolved in 0.25 ml solvent) were sprayed onto the animals by means of a disposable plastic syringe (brand "TOMAC", 1-ml vol) without needle from a distance of 0.5–1 m. However, as the animals became less tolerant, a 1-m extension plunger was used, and finally the sample was applied through a hole in a shed by a person hidden inside. Even then, it became increasingly difficult to lure the animals near the hole by offering food. However, these difficulties affected only the time needed for application of the stimulus. Once the stimulus was applied, the behavior of the group toward the treated animal conformed to the same pattern throughout the experiment.

From 1 to 6 tests, separated by 0.5 to 1.5 h, were performed per day. Each female served as odor carrier 1–3 times per day. Samples were sprayed alternately on their right and left shanks. The deer's activity cycles and the weather determined the frequency of the tests and the time interval between them. The tests took place between November 1973 and March 1974. This period includes the reproductive season of both the adults and the fawns. Most of our does are bred in November, and the fawns reach the peak of their rut in January.

The behavioral responses of the deer to the odor stimuli were sniffing and licking the sample, and following the treated animal. The frequency of sniffing, licking, and following; the duration of licking; and the distance followed were recorded. One person reported the responses; a second person recorded these observations on prepared data sheets. The animals were observed for 15 min after each odor application. A test was started when at

TABLE 1. MEAN VALUES OF RESPONSES TO THE *Z* AND *E* ISOMERS AND THE SIGNIFICANCE LEVELS OF THEIR DIFFERENCES (*P*)

Sample		Duration of licking (sec)			Frequency of licking during 15-min period			Frequency of sniffing during 15-min period			Discriminant function (*P*)[c]
A	vs. B	A	vs. B	*P*	A	vs. B	*P*	A	vs. B	*P*	
2-Microgram											
Z (12[a])	*E* (12[a])	65.9	32.4	0.05	3.7	2.7	—	2.6	1.4	—	—
Crude (12)	*E*	91.0	32.4	0.025	7.6	2.7	0.025	2.8	1.4	—	—
Crude	*Z*	91.0	65.9	—	7.6	3.7	—	2.8	2.6	—	—
Crude	PE[b] (25)	91.0	23.5	0.001	7.6	2.1	0.001	2.8	0.5	0.001	0.001
E	PE	32.4	23.5	—	2.7	2.1	—	1.4	0.5	0.05	—
Z	PE	65.9	23.5	0.01	3.7	2.2	0.05	2.6	0.5	0.001	0.001
0.2-Microgram											
Z (5)	*E*(5)	29.6	14.0	—	4.2	2.4	—	2.0	1.0	—	—
Z	PE (25)	29.6	23.5	—	4.2	2.1	—	2.0	0.5	0.01	0.025
E	PE	14.0	23.5	—	2.4	2.1	—	1.0	0.5	—	—
0.02 Microgram											
Z (5)	*E* (5)	38.0	17.1	—	4.4	2.4	—	1.6	1.2	—	—
Z	PE (25)	38.0	23.5	—	4.4	2.1	0.05	1.6	0.5	0.01	0.05
E	PE	17.1	23.5	—	2.4	2.1	—	1.2	0.5	—	—

[a] Numbers in parentheses are numbers of tests per sample.
[b] PE: petroleum ether only (control).
[c] Probability of the difference between A and B as described by the discriminant function (multivariate F).

least 50% of the animals were active. If it was obvious that the animals were approaching a period of rest, no test was started.
Responses to the following four control stimuli were also recorded:

1) crude extract of male tarsal glands, containing all constituents
2) solvent alone
3) the untreated side of the treated animal (odor carrier)
4) the other members (males and females) of the group

The behavioral responses (frequency of sniffing, frequency of licking, and duration of licking) to pairs of samples were compared by one-way analysis of variance. In addition, the three responses were combined and a discriminant analysis (Morrison 1967) was carried out for each pair of samples (A vs. B), as listed in Table 1. As can be seen from the data, both methods yielded similar results.

RESULTS

For the final analysis of the results, three measurements were used: duration and frequency of licking and frequency of sniffing. Frequency and distance of following were not used because other factors, such as fences, caused "following behavior."

All three measurements are higher for the *Z* isomer than for the *E* isomer (Table 1). The most pronounced response differences were found at the 2-μg level, especially for the duration of licking. The duration of licking at this level is significantly different for the two isomers ($\bar{X}$ = 32.4 sec for *E*, 65.9 sec for *Z*, $P<0.05$, one-way analysis of variance; see Table 1). The frequency of sniffing and licking the *Z* isomer is not significantly different from the response to the *E* isomer. Although the discriminant function of all three measurements combined does not show a significant difference between the two isomers, the same function shows a significant difference for *Z* vs. petroleum either, but not for *E* vs. petroleum ether. Responses to the crude extract and to the *Z* lactone are high and not significantly different from each other. In contrast, the duration of sniffing and licking is significantly longer in response to the crude extract than to the *E* lactone.

In each category (Table 1), the crude extract gives a higher response value than the *Z* lactone. Although the differences are not statistically significant, the higher values may be due to other components of the crude extract (Müller-Schwarze 1969).

At the 0.2- and 0.02-μg levels, the responses to the *Z* lactone were still stronger than those to the *E* lactone, but did not reach the significance level of $P<0.05$. At both these concentrations, the difference between the *Z*

TABLE 2. CONTROL DATA: AVERAGE DURATION (SECONDS) AND FREQUENCY OF LICKING AND FREQUENCY OF SNIFFING OF UNTREATED GROUP MEMBERS AND UNTREATED SIDE OF "ODOR CARRIER" BY ALL OTHER GROUP MEMBERS COMBINED

Samples (2 μg)	Untreated side of test ♀			One side of untreated ♀♀			One side of untreated ♂♂		
	Licking		Sniffing frequency	Licking		Sniffing frequency	Licking		Sniffing frequency
	Duration	Frequency		Duration	Frequency		Duration	Frequency	
Z	0.00	0.25	0.00	0.37	0.08	0.00	0.02	0.03	0.00
E	1.14	0.25	0.42	0.73	0.08	0.14	0.00	0.00	0.03
Crude	0.78	0.25	0.00	0.00	0.00	0.00	0.16	0.03	0.06
PE	0.02	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.08

lactone and petroleum ether was significant, and the difference between the *E* lactone and petroleum ether was not.

Control data (Table 2) show low levels of duration and frequency of licking, and of frequency of sniffing in the shank area of the untreated side of the treated animal (odor carrier) and in the shank area of the untreated group members. For example, at the 2-μg level, the average control levels were roughly 100 to 1,000 times lower than the sample responses shown in Table 1.

DISCUSSION

The data support the conclusion that black-tailed deer respond more strongly to the naturally occurring *Z* lactone than to the *E* lactone; we do not know whether or not the *E* lactone is present in the scent.

The most pronounced response differences were found at the 2-μg level, especially for the duration of licking. Although the discriminant function for the difference between *Z* and *E* lactone does not show a significant difference, the same function is significantly different for the *Z* lactone vs. petroleum ether, but is not for the *E* lactone vs. petroleum ether. Responses to crude extract and to *Z* lactone are both high and do not differ from one another, but the crude extract vs. the *E* lactone shows a significant difference (Table 1).

Immature animals typically respond more strongly to components of the tarsal odor. This stronger response is true for the experiment reported here and also for previously reported work (Müller-Schwarze 1967, 1971). This raises the question of a possible function of the tarsal odor in maturation processes.

To eight persons in this laboratory, the odor of the *E* lactone was less intense than that of the *Z*, or even undetectable.

A number of insects discriminate between geometric isomers (see review, Silverstein and Young 1976). For example, the redbanded leafroller, *Argyrotaenia velutinana*, and the European corn borer, *Ostrinia nubilalis*, respond optimally to specific ratios of the *Z* and *E* isomers of 11-tetradecenyl acetate; the individual isomers, presented alone, evoke little, if any, response (Klun et al. 1973).

The present paper presents the first study of responses to geometric isomers of a mammalian pheromone. All tests were performed in outdoor pens under a wide range of often rapidly changing metereological conditions. Furthermore, the exact physiological state of the animals at the time of the tests could not be ascertained. Given these circumstances, the consistent response differences and the levels of their statistical significance are impressive.

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(*Z,E*)-3,5-TETRADECADIEN-1-OL ACETATE SEX ATTRACTANT FOR THE CARPENTERWORM MOTH, *Prionoxystus robiniae* (Peck) (LEPIDOPTERA: COSSIDAE)

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Abstract—Electroantennogram analyses of female gland extract and of male antennal responses to synthetic standards suggested that (*Z,E*)-3,5-tetradecadien-1-ol acetate is a pheromone component for the carpenterworm moth, *Prionoxystus robiniae* (Peck). The four 3,5-geometrical isomers were synthesized and bioassayed in the laboratory and the field in 1972, 1973, and 1974. The *Z,E* isomer was found to be active in the laboratory and a good attractant in the field. The synthesis of the *Z,E* isomer also produced considerable quantities of the *E,E* isomer, which is difficult to remove completely. The *E,E* isomer does not inhibit the response of males to the *Z,E* isomer when it is present in amounts up to 20% of the *Z,E* isomer. The addition of a keeper, a volatility modifier, or an antioxidant prolonged the activity of the attractant for as much as 43 days. (*Z,E*)-3,5-Tetradecadien-1-ol acetate may be a natural pheromone, but it has not been chemically defined from female extract. There is EAG evidence that a second pheromonal component may be present. The attractant nevertheless provides a tool for population survey, behavioral studies, evaluation of economic impact, and possibly control.

Key Words—carpenterworm moth, *Prionoxystus robiniae*, (*Z,E*)-3,5-tetradecadien-1-ol acetate, pheromone, electroantennogram.

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INTRODUCTION

The sex pheromone of the adult female carpenterworm, *Prionoxystus robiniae* (Peck), a destructive insect pest of southern hardwood forests that tunnels extensively in tree trunks, was first reported by Solomon and Morris (1966) and partially characterized by Solomon et al. (1972) as an acetate of a 14- or 16-carbon unsaturated alcohol. The pheromone is potentially valuable for population surveys and control of this pest. However, the insect is difficult to rear (Solomon, 1967; Leppla et al. 1974), which means that the classic isolation and identification methods used successfully to elucidate the chemical structures of the pheromones of many other species were not practical. Therefore, the electroantennogram (EAG) technique (Roelofs and Comeau, 1971*a*; Roelofs et al. 1971) was tried. In this procedure, retention times of active components in female pheromone gland extracts are determined by assaying the gas (liquid) chromatographic (GLC) effluent from polar and nonpolar columns by EAG analysis. A complete series of monounsaturated standards is then assayed by EAG analysis to determine possible positions and configuration of unsaturation for the compounds of the chain length and functional group suggested by the GLC–EAG analysis.

METHODS AND MATERIALS[5]

Electroantennogram Assays

EAG analyses were conducted as previously described (Roelofs and Comeau, 1971*b*; Roelofs et al., 1971). Moths used for these analyses were collected in the field in Mississippi and sent live to New York. Female abdominal tips were extracted with methylene chloride. The GLC columns (2 m × 4 mm ID, glass) employed were OV-1® (3% methyl silicone on 100–120 mesh Gas Chrom® Q) and CHDMS® (3% cyclohexanedimethanol succinate on 100–120 mesh Chromsorb® W-AW-DMCS). Pheromone component retention times were determined before the 1971 flight season, when very few insects were available, by injecting the methylene chloride extract of 10 female abdominal tips (10 FE) onto the OV-1 column (170°C) or the CHDMS column (180°C), and collecting 1-min fractions from each for EAG analysis. The response profiles measured with a series of nonunsaturated acetates were recorded during the 1971 season, when an abundance of males was available.

[5] Mention of a commercial or proprietary product in this paper does not constitute a recommendation or an endorsement by the USDA.

Laboratory and Field Bioassays

Laboratory bioassays were conducted as previously described (Solomon et al., 1972). Field bioassays were conducted by placing the candidate materials in traps in the forest and recording the number of males captured. Through 1972, these were screen traps (Solomon and Morris, 1966). A sticky trap (Solomon and Doolittle, 1976) was used for the 1973 and 1974 tests. All traps were suspended from branches 1.5 m above the ground and positioned about 300 m apart. Candidate chemicals were dissolved in 0.250 ml nanograde hexane, 99 mol% hexane, or spectroscopic grade isooctane, either with or without a keeper or preservative. The solution was placed on pieces of cotton dental roll (2 cm × 1 cm OD), which were placed in the traps during the afternoon, when the moths were most active. Test samples were assigned randomly to trap site, and rerandomized for each of 2–5 replicates. Catches per trap were recorded daily during the flight season, mid-May to early July, until the baits were no longer attractive. Tests were conducted with different quantities, isomeric mixtures, and preservatives.

Chemical Synthesis

Instrumentation used in the chemical synthesis included a Perkin–Elmer Model 137 infrared spectrophotometer, Varian Aerograph® Models 1522-B and 2100 gas chromatographs, Varian Associates Models HA-100 and T-60 NMR spectrometers, and a high-pressure liquid chromatographic system consisting of a Waters Model 6000 solvent delivery system, a septum injector, and a Lab Data Control refractive index detector.

Synthesis of (Z,Z) and (E,Z)-3,5-Tetradecadien-1-ol Acetates (VI and VII). The synthetic routes used are outlined in Fig. 1, scheme A. The first three steps in the synthesis of the *E,Z* and *Z,Z* isomers (scheme A) were essentially the same as those reported by Rodin et al. (1970) and Celmer and Solomons (1953), though there were some modifications. The lithium salt of 1-decyne in tetrahydrofuran (THF) was treated with acrolein to give the secondary alcohol I in 68% yield. Treatment of I with phosphorous tribromide in ether–pentane produced a mixture of primary bromides II in 55% yield. Cuprous cyanide in dimethylformamide with a catalytic amount of sodium cyanide converted II to a mixture of ene–yne nitriles III in 70% yield. Methanolic hydrogen chloride converted III to a mixture of the ene–yne methyl esters IV and V in a yield of 86%. The IR spectra and physical constants of I–III and the mixture of IV and V were in agreement with those reported earlier by Rodin et al. (1970). The pure *Z* and *E* isomers IV and V were separated by GLC on 45 × 0.80 cm ID aluminum columns containing 20% HiEFF-8BP® on 60/70 mesh Anachrom® ABS at 185°C with a N_2

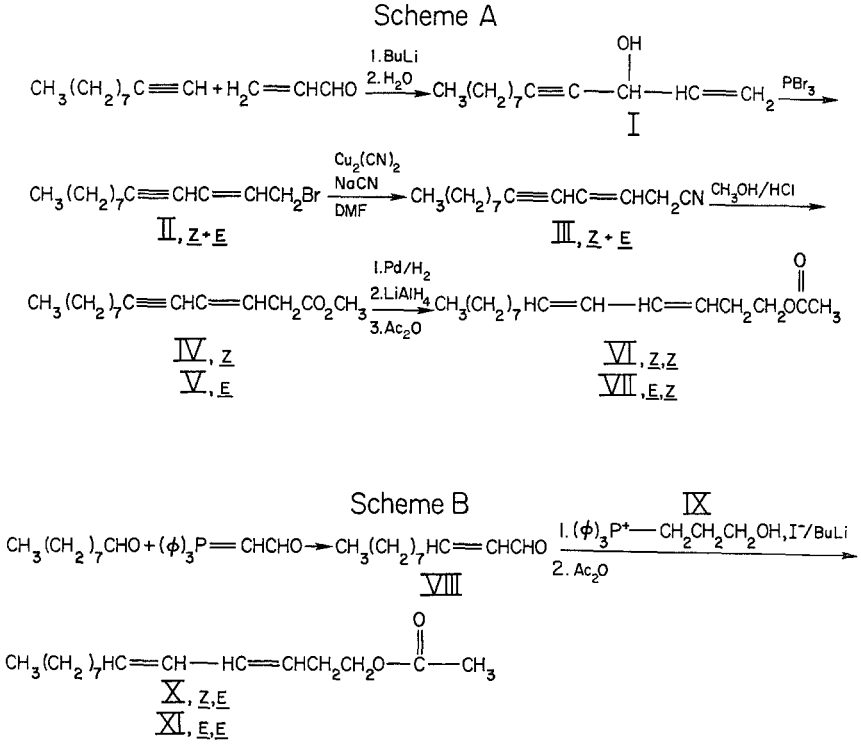

FIG. 1. Synthesis of the four isomers of 3,5-tetradecadien-1-ol acetate.

flow rate of 80 ml/min. The column effluent which was monitored by a thermal conductivity detector (250°C), was collected in 1.2-m lengths of Teflon tubing. From a total of 1.35 g mixed esters, 0.468 g IV (shorter retention time) and 0.675 g V (longer retention time) were obtained; accordingly, the ratio of *Z* to *E* isomers produced in the rearrangement of I to II was about 1:1.4. The IR spectrum of IV had bands at 3040 cm^{-1} (olefinic C–H), 2220 cm^{-1} (–C≡C–), 1745 cm^{-1} (ester C=O), and 1170 cm^{-1} (ester –C–O–R). The IR spectrum of V had bands at 3040 cm^{-1} (olefinic C–H), 2220 cm^{-1} (–C≡C–), 1745 cm^{-1} (ester –C=O), 1160 cm^{-1} (ester –C–O–R), and 960 cm^{-1} (*E*—HC=CH–). The esters were analyzed on a 1.8 m × 2 mm ID column of 3% HiEFF-8BP on 100/120 mesh Anachrom ABS at 175°C with a N_2 flow rate of 30 ml/min. The purity of IV determined by GLC was 96%; that of V was 90%.

The individual methyl esters IV and V were converted to the acetates VI and VII by semihydrogenation over Lindlar catalyst (Lindlar, 1952), followed by reduction with lithium aluminum hydride in ether. The crude

alcohols from the hydride reduction were converted to the acetates with acetic anhydride and pyridine in benzene at reflux. The diene esters VI and VII were purified by preparative GLC with a 60×0.80 cm ID column containing 10% HiEFF-8BP on 60/70 mesh Anachrom ABS at 185°C with a N_2 flow rate of 80 ml/min. The IR spectrum of the *Z,Z* isomer (VI) had bands at 3015 cm^{-1} and 3042 cm^{-1} (olefinic C–H), 1745 cm^{-1} (ester –C═O), 1240 cm^{-1} (acetate), and 1040 cm^{-1} (ester $-CH_2-O-C$). The IR spectrum of the *E,Z* isomer VII had bands at 3010 cm^{-1} and 3025 cm^{-1} (olefinic C–H), 1745 cm^{-1} (ester –C═O), 1240 cm^{-1} (acetate), 1035 cm^{-1} (ester $-CH_2-O-C-$), and 985 and 950 cm^{-1} (characteristic of a *Z,E* or *E,Z* conjugated diene). The UV spectra of the pure diene acetates had the following absorptions: VI (λ_{max} isooctane at 234 nm ε_{max} 35,000); VII, (λ_{max} isooctane at 233 nm, ε_{max} 25,000). The mass spectra of VI and VII had peaks at 192 ($M^+-CH_3CO_2H$), consistent with a molecular weight of 252. These analytical data are compatible with the assigned structures.

Synthesis of (Z,E) and (E,E)-3,5-Tetradecadien-1-ol Acetate. Initially the *Z,E* isomer was obtained by lithium aluminum hydride reduction of (*Z,E*)-3,5-tetradecadienoic acid in THF solution, followed by work-up with 1% sulfuric acid and extraction with hexane. The hexane extract was dried, the solvent was removed, and the residual oil was acetylated with acetic anhydride in the usual manner. The mixture of acetates was separated by preparative GLC on the same column described for the purification of the *Z,Z* and *E,Z* isomers. The IR spectrum of the product had bands at 1745 cm^{-1} (ester –C═O), 1235 cm^{-1} (acetate), 1040 cm^{-1} (ester $-CH_2-O-C-$), and 985 and 950 cm^{-1} (characteristic of a *Z,E* or *E,Z* conjugated diene).

The *Z,E* and *E,E* isomers were synthesized as described in Fig. 1, scheme B. 2-Undecenal was prepared by heating freshly distilled nonanal with (triphenylphosphoranylidene) acetaldehyde (Trippett and Walker, 1961; m.p. 178–180°C) at reflux in dry benzene under a N_2 atmosphere for 48 hr. The product was redistilled on a spinning band still to give pure 2-undecenal (b.p. 54°C/0.01 mm n^{20} 1.45625). The IR spectrum had bands at 2750 cm^{-1} and 2880 cm^{-1} (–C–H), 1715 cm^{-1} (conj.—C═O), and 985 cm^{-1} (*E*—HC═CH–).

(3-Hydroxypropyl) Triphenylphosphonium iodide (IX). The reaction of 3-iodopropanol with triphenylphosphine in benzene resulted in a 91% yield of white crystalline product m.p. 208–209°C (uncorrected) that was used without further purification.

(Z,E) and (E,E)-3,5-Tetradecadien-1-ol Acetate (X and XI). The reaction was run in a dry, N_2-swept 250-ml 4-neck flask fitted with a mechanical stirrer, thermometer, N_2 inlet, and septum stopper. Sodium hydride (1.32 g, 0.055 mol) in the form of 2.32 g of a 57% dispersion in mineral oil was added to the flask and washed free of mineral oil by repeated decantation with

pentane. Dry dimethyl sulfoxide (50 ml) was added via a syringe, and the flask was heated in a water bath with stirring to 60–65°C until foaming stopped. THF (15 ml) was added, and the reaction mixture was cooled to 0°C in an ice–salt bath. An Erlenmeyer flask (50 ml) containing 24.66 g (0.055 mol) IX was connected to one neck of the flask with a piece of Gooch tubing, and the phosphonium salt was slowly added with stirring while the temperature was kept below 5°C. After the salt had been added, 25 ml (0.055 mol) of a hexane solution of butyllithium was added dropwise with stirring to the reaction mixture while the temperature was kept as close to 0°C as possible. After addition of the butyllithium was complete and the dropping funnel was rinsed with 5 ml THF, the deep-red reaction mixture was held at 0°C for 1 hr; then 2-undecenal (8.41 g, 0.05 mol) was added dropwise. The reaction mixture was stirred and allowed to come to room temperature overnight. Then it was worked up by adding 3.5 ml (0.20 mol) ice water and extracting 4 times with pentane. The pentane extracts were washed with water and brine and then dried over sodium sulfate. The drying agent was removed by filtration, and the solvent was evaporated.

The residual oil was converted to a mixture of acetates (X and XI) in the usual fashion. GLC analysis showed 2 products in a ratio of about 2:1, either on a 1.8 m × 2 mm ID column packed with 3% HiEff-8BP on 100/120 mesh Gas Chrom Q: (175°C and a N_2 flow rate of 15 ml/min) or on a 1.8 m × 2 mm ID column packed with 3% Carbowax® 20 M on 120/140 mesh acid-washed and silanized Chromosorb W (175°C with a N_2 flow rate of 20 ml/min). The crude mixture of acetates was passed through a column of silica gel (J. T. Baker No. 3405) with 5–10% ether in hexane as eluant to remove any triphenylphosphine oxide remaining. When this reaction was run using two equivalents of sodium hydride to generate the phosphorane from IX, very little product was obtained, and no characteristic red color was observed during the reaction. This reaction is under further investigation.

The solvents were removed, and X and XI were separated several ways. Initially, the pure isomers were obtained by preparative GLC using the same columns and conditions reported for VI and VII. The IR spectrum of the isomer with the shorter retention time had bands at 3030 cm^{-1} (olefinic –CH), 1745 cm^{-1} (ester –C═O), 1235 cm^{-1} (acetate), 1040 cm^{-1} (ester –C–O–), and 950 and 985 cm^{-1} (*Z,E* conjugated diene), and the IR spectrum of the peak of longer retention time had bands at 3020 cm^{-1} (olefinic C–H), 1745 cm^{-1} (ester –C═O), 1240 cm^{-1} (acetate), 1035 cm^{-1} (ester –C–O–), and 990 cm^{-1} (*E,E* conjugated diene). The mass spectra of both X and XI had peaks at 192 (M^+-60), and the UV spectra showed for X (λ_{max} isooctane at 232 nm, ε_{max} 32,000) and XI (λ_{max} isooctane at 229 nm, ε_{max} 31,000). These data are consistent with the assigned structures X and XI. The isomers are produced in a ratio of 1:2, X:XI. Although distillation of the mixture of

acetates on a spinning band still failed to completely resolve the isomeric mixture, it was possible to produce one fraction (88–92°C/0.004 mm) that contained a minimum of 95% X and another (91–93°C/0.002 mm) that contained a minimum of 90% XI. Several intermediate fractions (90–91°/0.002 mm) contained mixtures of X and XI. The still-pot residue, after distillation in a short-path still, was found by GLC analysis to be 98% XI. High pressure liquid chromatography was used to prepare X 99+% pure. A column of 10-μm Lichrosorb® (50 cm × 0.44 cm ID) was developed with 3% ether in hexane flowing at 3.0 ml/min and 750 psig. Between 20 and 40 μl of material was injected in each run; fractions collected were freed of solvent by evaporation, and the residue was distilled (70°C/0.05 mm) in a short-path still to give X as a clear oil.

RESULTS AND DISCUSSION

EAG analysis of the fractions collected from the OV-1 column showed activity (1.2 mV vs 0 mV from other fractions) at 9–11 min. 1-Tetradecanol and 1-hexadecanol acetate on this column had retention times of 9.2 and 22.0 min, respectively. The portions collected from the CHDMS column (180°C) gave EAG activity at 9–10 min (1.4 mV vs 0.2 in previous fractions) and 14–17 min (2.0 mV); 1-tetradecanol and 1-hexadecanol acetate had retention times of 9.7 and 21.2 min, respectively. The major component had retention times that suggested either a 14-carbon chain acetate with more than one double bond or a 16-carbon aldehyde. Solomon et al. (1972) concluded that the pheromone is not an aldehyde.

Male antennal responses to the series of monounsaturated 12-, 14-, and 16-carbon chain alcohols and acetates showed good responses with 14-carbon chain acetates (Fig. 2), with unsaturation in the 3, 4, 5, and 9 positions. Interpretation of the GLC and the EAG data suggested that (*Z,E*)-3,5-tetradecadien-1-ol acetate could be one of the pheromone components. This result is in agreement with the earlier report that the pheromone is likely either a doubly unsaturated 14-carbon chain acetate or a monounsaturated 16-carbon chain acetate (Solomon et al., 1972).

(*Z,E*)-3,5-Tetradecadienoic acid (kindly furnished by W. Burkholder of the University of Wisconsin, Madison) was reduced and acetylated (see "Methods and Materials") to produce a mixture that was resolved by a combination of thin-layer and gas chromatography. The major product, presumably (*Z,E*)-3,5-tetradecadien-1-ol acetate, gave positive results in the laboratory and in field tests during the 1971 flight season. Consequently, all four isomers of 3,5-tetradecadien-1-ol acetate were synthesized.

The retention times of the four isomers of 3,5-tetradecadien-1-ol acetate on CHDMS relative to 1-tetradecanol acetate were: *Z,E*, 1.66; *E,Z*, 1.56;

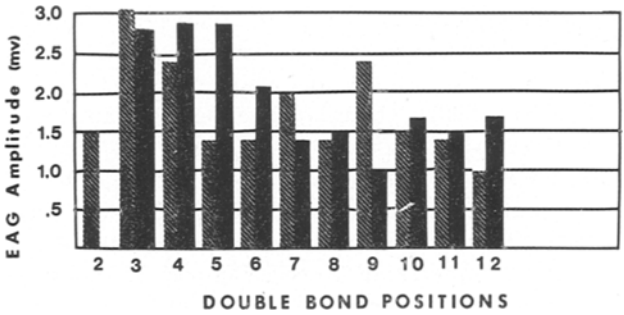

FIG. 2. Electroantennogram responses of male carpenterworm antennae to 10μg 14-carbon acetates on filter paper. Crosshatching represents the *Z* isomer; solid bars, the *E* isomer.

Z,Z, 1.76; and *E,E*, 2.00. The major active component of female extracts had a relative retention time of 1.44–1.75, which eliminated the *E,E* isomer. EAG analysis replicated 5 times produced the following order of activity: *Z,E* (3.3$\pm$1 mV); *Z,Z* (2.9$\pm$0.7 mV); *E,E* (2.4$\pm$0.9 mV); *E,Z* (2.4$\pm$0.4 mV); *Z*-3 (2.7$\pm$0.9 mV); and *E*-5 (2.0$\pm$0.9 mV). Therefore, the *Z*-3, *E*-5 isomer had the correct retention time and was the most active by response of the male antennae.

At concentrations of 1 μg/μl in the laboratory bioassay, of the four isomers, only (*Z,E*)-3,5-tetradecadien-1-ol acetate produced the characteristic responses (equal to or greater than 60%) of males to female extracts or to live virgin females: vibrating of wings, opening of genital claspers, and rapid crawling and dancing.

During the field bioassays conducted during the 1972 emergence season, 1–12 male carpenterworm moths were attracted to each screen trap (Solomon and Morris, 1966) in 6 experiments baited with 250 μg (*Z,E*)-3,5-tetradecadien-1-ol acetate, but no males were attracted to traps containing as much as 1250 μg of each of the other three isomers. Since geometrical isomers have been shown to increase or decrease attractiveness of the primary pheromonal component of many insect species, tests were conducted to measure the effect of the other three isomers on the attractiveness of the *Z,E* isomer.

Traps containing from 2.5 to 250 μg of either the *Z,Z* or the *E,Z* isomer in admixture with 250 μg of the *Z,E* isomer caught no moths, though 250 μg of the *Z,E* isomer or caged virgin females caught from 6 to 14 males/day for 1–4 days during this same period. Traps baited with 250 μg of the *Z,E* isomer plus 2.5 to 250 μg of the *E,E* isomer were as effective as traps baited with the *Z,E* isomer only. Thus, the *Z,Z* and the *E,Z*, isomers might be inhibitory, while the *E,E* isomer was not.

TABLE 1. ATTRACTION OF MALE CARPENTERWORM MOTHS TO MIXTURES OF (*Z*,*E*)- AND (*E*,*E*)-3,5-TETRADECADIEN-1-OL ACETATE [(*Z*,*E*)- AND (*E*,*E*)-TDDA]

Isomer mixture (μg)[a]		Number of males trapped (mean ± SD)	
(*Z*,*E*)	(*E*,*E*)[b]	June 6–30, 1973[c]	June 19–July 8, 1974[c]
250	25	13.0 ± 14.1	5.0 ± 3.0
250	50	22.0 ± 5.7	5.0 ± 3.0
250	125	94.5 ± 65.8	2.3 ± 0.6
250	250	39.5 ± 17.7	2.7 ± 0.6
250	500	38.0 ± 47.0	5.7 ± 4.0
500	50	11.5 ± 16.3	7.0 ± 6.2
500	100	181.0 ± 200.8	22.3 ± 16.0
500	250	101.0 ± 90.5	5.0 ± 5.6
500	500	17.5 ± 3.5	6.3 ± 4.5
500	1000	82.0 ± 67.0	5.0 ± 4.0
50	0	0.5 ± 0.7	1.0 ± 1.0
100	0	15.5 ± 21.9	0.3 ± 0.6
250	0	69.5 ± 92.6	2.3 ± 0.6
500	0	55.0 ± 69.3	1.7 ± 0.6
Virgin females		74.5 ± 7.8	15.0 ± 11.0

[a] Trioctanoin included as keeper at a ratio of 10× the total isomer mixture.
[b] Pure *E*,*E* isomer captured no males.
[c] Two replications in 1973 and 3 replications in 1974.

Additional tests were made during 1973 and 1974 to further assess the effect of the *E*,*E* isomer on the attractiveness of the *Z*,*E* isomer (Table 1). The striking differences among catches during the two test periods reflect differences in populations of moths. The large deviations within each test period reflect the influence of such variables as weather and trap placement. However, again, the addition of up to 20% of the *E*,*E* isomer did not appear to inhibit the attractiveness of the *Z*,*E* isomer. The absence of inhibition of the *Z*,*E* isomer by the *E*,*E* isomer is fortuitous, since the synthesis produces a mixture of the two isomers. Moreover, it is relatively easy to produce the *Z*,*E* isomer in up to 90% purity by distillation, but it would be expensive to produce it in a purity greater than 90%.

In 1974, 50–2000-μg quantities of the *Z*,*E* isomer plus a 10-fold amount of trioctanoin keeper were bioassayed for attractiveness. Although all amounts were attractive, quantities of 250 μg or more were most attractive (Table 2). Statistical analysis of variance indicated that the higher quantities were comparable with virgin females in both total catches and duration of attractancy. During the first several days, traps baited with 250 and 500 μg

TABLE 2. ATTRACTION OF MALE CARPENTERWORM MOTHS TO (*Z,E*)-3,5-TETRADECADIEN-1-OL ACETATE ON JUNE 4–12, 1974

Quantity of attractant[a] (μg)	Number of males trapped[b] (mean ± SD)	Number of days attraction (mean ± SD)
50	4.2 ± 1.2	1.2 ± 0.4
100	4.0 ± 1.6	1.6 ± 0.5
250	16.8 ± 3.2	3.2 ± 0.4
500	23.4 ± 5.0	5.0 ± 1.2
1000	20.4 ± 4.8	4.8 ± 1.3
2000	27.0 ± 7.4	7.4 ± 1.7
Virgin females	29.5 ± 4.5	4.5 ± 2.1

[a] Trioctanoin included as keeper at a ratio of 10× the total synthetic attractant.
[b] Each test was replicated 5 times.

were generally more attractive than those baited with 1000 and 2000 μg; however, the higher quantities remained attractive longer.

In a separate test, a volatility depressant containing an anti-oxidant was compared with trioctanoin, which only reduced the volatility of the attractant (Table 3). The food preservative, Sustane-6®, which contains 4% antioxidant (BHA and BHT) in vegetable oil, was added in ratios of 2:1, 5:1, 10:1, and 20:1 to various amounts of *Z,E* isomer (100, 250, and 500 μg). The mixtures of Sustane-6 with *Z,E* isomer were consistently more attractive than the mixtures with trioctanoin or no additive. For example, 500 μg *Z,E* isomer combined with Sustane-6 in ratios of 2:1, 5:1, 10:1, and 20:1 attracted means of 161, 132, 174, and 349 ♂♂, compared with means of 17, 39, 10, and 12♂♂ for corresponding samples containing trioctanoin. Moreover, the antioxidant greatly increased the duration of attractancy: 500 μg *Z,E* isomer plus antioxidant at the same ratios was attractive an average of 9, 14, 20, and 37 days, compared with 5, 5, 4, and 5 days for those containing trioctanoin. An analysis of variance indicated that the antioxidant was more effective than the trioctanoin at all three levels of attractant.

The effects on attractiveness of certain monounsaturated isomers suggested by the EAG analysis should be tested.

In summary, *Z,E*-3,5-tetradecadien-1-ol acetate with as much as 20% *E,E* isomer was a good attractant for male carpenterworm moths. The addition of Sustane-6 antioxidant helped to increase attractiveness and longevity of the lure.

The *Z,E* isomer has GLC retention times that correspond with those of

TABLE 3. COMPARISON OF TRIOCTANOIN AND SUSTANE-6 AS KEEPERS IN FIELD ATTRACTANCY OF (*Z,E*)-3,5-TETRADECADIEN-1-OL ACETATE TO MALE CARPENTERWORM MOTHS BETWEEN JUNE 6 AND JULY 16, 1974

Quantity of *Z,E*-TDDA (μg)	Quantity of keeper (μg)		Number of males trapped[a] (mean ± SD)	Number of days' attraction (mean ± SD)
	Trioctanoin	Sustane-6		
100	200	0	5 ± 0	1.0 ± 0.0
100	500	0	7 ± 4	1.0 ± 0.0
100	1,000	0	18 ± 22	2.0 ± 0.0
100	2,000	0	3 ± 4	0.5 ± 0.7
100	0	200	25 ± 16	1.5 ± 0.7
100	0	500	22 ± 9	2.5 ± 0.7
100	0	1,000	17 ± 11	4.0 ± 0.0
100	0	2,000	50 ± 0	7.0 ± 1.4
100	0	0	18 ± 21	1.0 ± 0.0
250	500	0	11 ± 10	2.5 ± 2.1
250	1,250	0	26 ± 11	2.5 ± 0.7
250	2,000	0	7 ± 3	2.5 ± 0.7
250	5,000	0	14 ± 8	2.5 ± 0.0
250	0	500	22 ± 15	4.5 ± 0.7
250	0	1,250	57 ± 48	7.0 ± 4.2
250	0	2,000	93 ± 76	11.0 ± 1.4
250	0	5,000	94 ± 8	14.0 ± 1.4
250	0	0	46 ± 40	1.5 ± 0.7
500	1,000	0	17 ± 10	5.0 ± 2.8
500	2,000	0	39 ± 48	5.0 ± 4.2
500	5,000	0	10 ± 11	3.5 ± 0.7
500	10,000	0	12 ± 3	4.5 ± 2.1
500	0	1,000	161 ± 76	8.5 ± 0.7
500	0	2,000	132 ± 76	14.0 ± 0.0
500	0	5,000	174 ± 112	19.5 ± 3.5
500	0	10,000	394 ± 115	36.5 ± 9.2
500	0	0	28 ± 21	3.0 ± 1.4
Virgin females			32 ± 26	4.8 ± 2.4

[a] Each test replicated twice; tests with virgin female replicated 8 times.

the primary pheromonal component in the extract of the female gland, it elicited the greatest EAG response of all the compounds tested, and it was itself active in laboratory and field behavioral tests. However, rigorous chemical proof is still needed to demonstrate that the material is the primary pheromonal component. A related compound, (*E,Z*)-3,5-tetradecadienoic acid, has been reported (Silverstein et al., 1967) as a pheromone of the black carpet beetle, *Attagenus megatoma* (F.).

The effectiveness of (*Z*,*E*)-3,5-tetradecadien-1-ol acetate as an attractant for male carpenterworms provides a potential tool for population survey, behavioral studies, evaluation of economic impact, and possibly control.

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OLFACTOMETER FOR ASSAY OF BEHAVIORAL CHEMICALS FOR THE SOUTHERN PINE BEETLE, *Dendroctonus frontalis* (COLEOPTERA: SCOLYTIDAE)[1]

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Abstract—A simple open-arena olfactometer to assay behavioral chemicals with pedestrian insects has been developed for the southern pine beetle. The olfactometer is constructed of Plexiglas and uses a single airstream. A feature of the olfactometer is the use of a clock-driven elution device that permits continuous release of liquid materials into the olfactometer airstream.

Key Words—olfactometer, assay, aggregation pheromones, *Dendroctonus*, Scolytidae, bark beetles.

INTRODUCTION

Behavioral chemicals offer a potential tool in southern pine beetle management in both population survey and pest suppression. Critical to identification of any behavioral chemical is an assay to monitor attraction during isolation and identification procedures. Various laboratory assay apparatuses and techniques have been developed for bark beetles, ranging from the open or closed

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single- to multiple-odor source pedestrian types (e.g., Wood and Bushing, 1963; Jantz and Rudinsky, 1965; Kangas et al., 1965; Kliefoth et al., 1964; Borden et al., 1968*a*,*b*; Moeck, 1970; Browne et al., 1974) to complex flight mills (Borden and Bennett, 1969; Hughes and Pitman, 1970). Previous behavioral assays of materials with the southern pine beetle have consisted primarily of field-trapping procedures (references included in Coulson et al., 1972). The successful use of a reliable laboratory assay procedure for the southern pine beetle has not been reported to date.

Field assay procedures, although necessary at some stage in the evaluation of a material, have inherent disadvantages as the sole source of assay. The ever-present, mostly uncontrollable and unknown, variables associated with a forest environment, such as competitive odor sources, beetle distributions, and seasonal availability, are restrictive to a continuous evaluation of materials. Laboratory assays can evaluate stimuli and test material quantities with far fewer insects than are required for field tests, and can be conducted throughout the year, even during the colder months, when beetle populations tend to be suppressed. Field assays must at some point be used in conjunction with laboratory assays to verify the effect of a given material on a natural population.

Three olfactometer designs used in pheromone studies with other bark beetle species (Wood and Bushing, 1963; Moeck, 1970; Browne et al., 1974) were evaluated in the present studies with the southern pine beetle. Various limitations of these assays made it necessary to develop an assay apparatus for the southern pine beetle that would provide consistent high levels of response to a standard source of attraction. Details of the design and results from its use are presented in this paper.

METHODS AND MATERIALS

Assay Apparatus

The assay apparatus was constructed of Plexiglas modified after the open-arena, pedestrian design described by Wood and Bushing (1963) (Fig. 1). The arena surface measured 22.5 × 28 cm, and conveniently accommodated standard letter-size typing paper, which was changed between each group of beetles assayed. Odor stimuli were delivered to the arena surface in a 1.5–2 liters/min airflow monitored by a Kontes Bantam® 3SA airflow meter. Airflow was supplied by a compressor and delivered through activated charcoal by way of 7-mm OD Teflon and glass tubing to the arena surface. Odor stimuli were eluted at the airflow outlet 9 cm from the release point for the beetles on the arena surface. Eluted odor stimuli were removed from the assay room through an exhaust hood attached to the assay apparatus (Fig. 1).

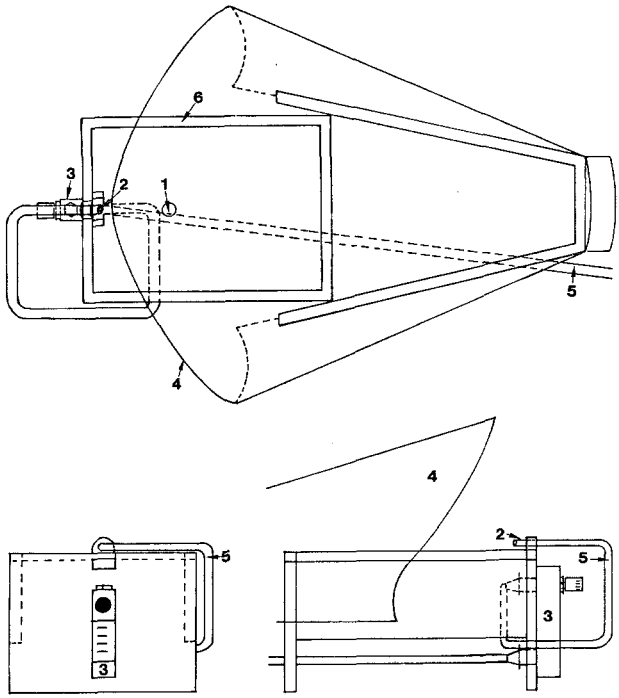

FIG. 1. Three-view schematic of the olfactometer for the southern pine beetle: 1, release point; 2, air outlet; 3, airflow meter; 4, exhaust hood; 5, air supply; 6, olfactometer surface.

Elution Device

Solutions containing candidate odor stimuli were eluted into the airstream at 1 μl/min from a 10-μl 700 Series Hamilton® syringe. The syringe plunger was continuously depressed by a rack-and-pinion gear-driven elution device modified after Browne (personal communication) (Fig. 2). The device was powered by a 30-min, spring-driven kitchen-timer motor mounted on a 3-mm-thick aluminum plate (16.5 × 30.5 cm) attached to a 2.5-cm angle-iron base. The brass pinion gear (5 cm diameter, 3 mm pitch; Boston Gear Y64128) was threaded to the shaft of the motor, which extended through the aluminum plate. A brass rack 10.5 cm long (Boston Gear Y64) was soldered to a 12.5-cm brass slide connected to a slide base, both of which were attached to the aluminum plate. The slide and slide base were made from a 15.2-cm-long brass surface bolt (National Manufacturing Co. No. V839). The forward end of the rack was filed flat to facilitate contact with the plunger. The syringe

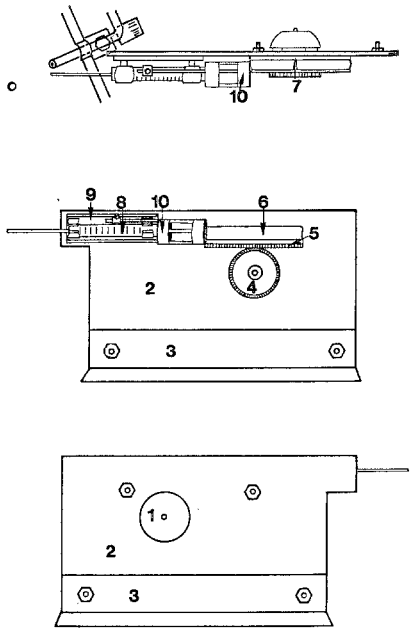

FIG. 2. Three-view schematic of the elution device: 1, timer motor; 2, aluminum plate; 3, angle-iron base; 4, pinion gear; 5, rack; 6, brass slide; 7, brass slide base; 8, syringe; 9, plastic base (syringe holders); 10, plunger guide.

was held in position on the aluminum plate by a plastic base section of a syringe shipping container. The plastic clips on the container base allowed for easy placement and removal of the syringe. The syringe was fitted with a Hamilton® WG plunger guide to prevent bending of the small-diameter plunger.

The timer motor, rack-and-pinion gears, and syringe holder were situated on the aluminum plate so that the tip of the syringe needle could be positioned immediately in front of the lower half of the orifice of the airstream.

For a given assay, about 5 μl of material was taken up in the syringe. The pinion gear on the elution device was turned to activate the timer motor and move the rack gear to provide room for the syringe with extended plunger to fit into the holder. The pinion gear was then advanced until the end of the rack made contact with the plunger.

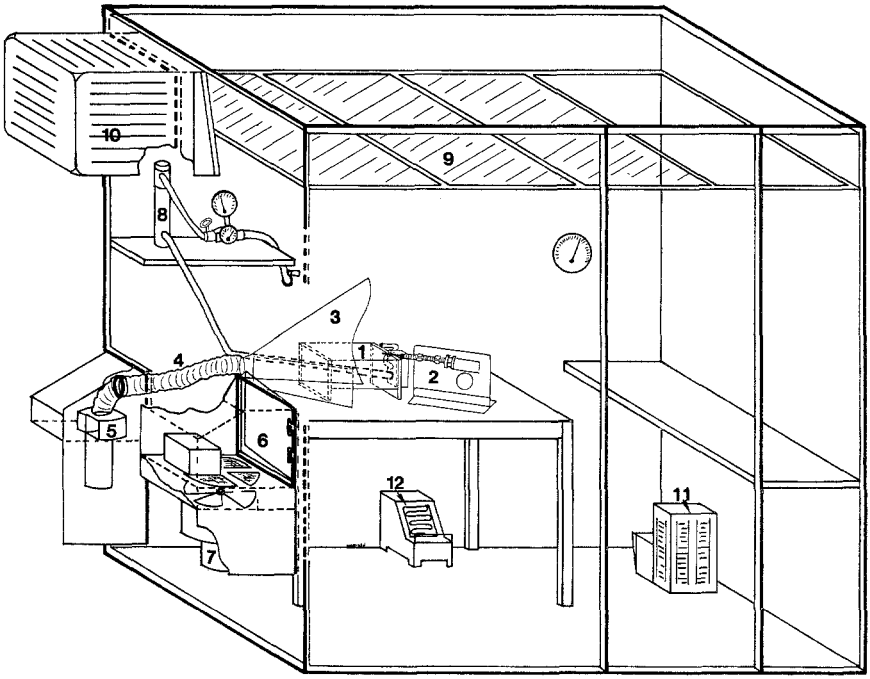

FIG. 3. Schematic of the assay room: 1, olfactometer; 2, elution device; 3, exhaust hood; 4, exhaust hose; 5, hood exhaust fan and motor; 6, chemical storage chamber; 7, storage chamber exhaust fan and motor; 8, activated charcoal filter; 9, suspended ceiling; 10, air conditioner; 11, dehumidifier; 12, space heater.

Assay Room

To provide for a controlled assay environment, the assay apparatus was maintained in a separate, windowless building measuring 183 cm long × 122 cm wide × 214 cm high (Fig. 3). Temperature in the assay room was maintained at about 20°C in order to prevent the beetles from taking flight from the arena surface. The relative humidity in the room was maintained at about 80% by means of a dehumidifier unit. A 1/25-hp fan, attached to the exhaust hood of the assay apparatus by an electric dryer hose, continuously removed the air passing over the arena surface. The chemical storage chamber on the exterior of the assay room provided for ready accessibility to the materials being evaluated, with a reduced possibility of contamination.

The room was fitted with an air diffuser suspended near the ceiling below the exhaust of an air conditioner. This diffuser minimized the air turbulence on the arena surface. The effectiveness of the suspended diffuser

was visualized with vapors generated with titanium tetrachloride placed on the arena surface. In addition, air velocities were measured throughout the room with a thermistor anemometer.

Assay Procedure

Beetles to be used in assays were collected daily from an emergence chamber modified after Browne (1972). They were sexed and scrutinized for the presence of all tarsi, both antennae, and for unencumbrance by mites. To prevent injury, beetles selected for assay were placed in individual #0 gelatin capsules. Beetles not used on the day of collection were discarded. In all cases, materials to be assayed were stored at −60°C. Materials being tested on a given day were maintained on dry ice in the chemical storage chamber attached to the assay room.

For each assay, 10 beetles of either sex were emptied from the gelatin capsules onto the release point of the arena surface (Fig. 1). Those beetles that walked to within 1 cm of the air outlet were counted for positive response. Beetles that did not respond, but left the airstream, were removed from the arena surface and retained for a second try. After the second try, all beetles were discarded. No time limits for the assay were established; however, the beetles usually responded within 1 min after release. After each assay, the paper surface was removed from the room and replaced.

Each day on which assays were to be conducted, separate groups of 10 males and 10 females were assayed in the morning and in the afternoon to a "solvent-only" stimulus, which consisted of pentane (the diluent used in all stimuli presented), and to a "standard" stimulus, which consisted of frontalin (Kinzer et al., 1969), *trans*-verbenol (Renwick, 1967), and loblolly turpentine at a ratio of 1:1:12. This "standard" has been shown to be attractive to field populations of both male and female southern pine beetle in the forest (Payne and Coster, unpublished). The solvent-only and standard assays were carried out as a measure of the activity level of the beetles and as an indicator of possible contamination. When response levels to pentane exceeded 20%, or when response to the standard was below 50% for either sex, further assays were not performed. The apparatus was thoroughly cleaned with acetone or pentane to eliminate any contamination. Assays of candidate materials resumed when responses to the solvent and the standard returned to the acceptable levels.

The evaluation of any material in the assay consisted of a minimum of 5 assays per sex (50 beetles per sex = 100 beetles total) at no fewer than 3 elution rates for each material. For known materials, the 3 rates varied from 1×10^{-5} to 1×10^{2} μg/min. To establish a dilution–response curve for the standard, assays were run at each $\log_{10}$ interval between these rates. However,

when other materials were assayed, 1 μg/min of the standard was used and compared against the dilution–response curve for relative activity. The 1-μg/min rate elicited about 50% response from both sexes when the response levels were corrected for response to the control. The assay was used to establish the presence of pheromone activity in collected, naturally produced volatiles and subsequent fractionation leading toward eventual isolation and identification of specific compounds. Assay rates ranged from 0.01 to 1.0 beetle min/min (Browne et al., 1974) for volatiles collected on Porapak (Byrne et al., 1975) and from beetles boring in host material.

RESULTS AND DISCUSSION

The assay apparatus was used successfully to establish a dosage–response curve for the attractive mixture of frontalin, *trans*-verbenol, and loblolly turpentine (Fig. 4). In general, percentage response increased with increased elution rate of the mixture from 0.5×10^{-5} to 500 μg/min. Response by females was generally lower than that by males. The BR_{50} level was about 0.1 and

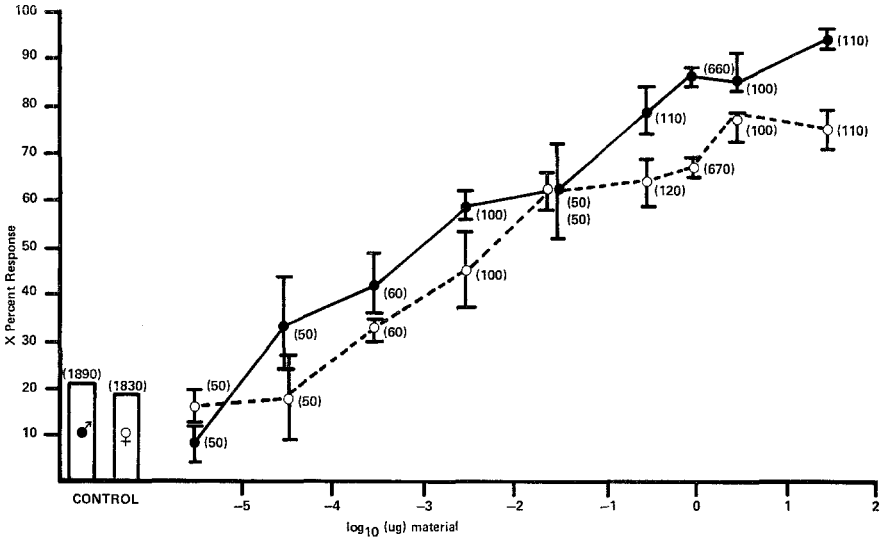

FIG. 4. Mean percentage response of male and female southern pine beetles to the standard (1:1:12 mixture of frontalin, *trans*-verbenol, and loblolly turpentine). Data collected from January 1 through July 1, 1975. Dilutions are based on the amount of frontalin in the mixture. ●, Male; ○, Female; ( ), number assayed; vertical brackets, standard error.

1.0 μg/min for males and females, respectively, when adjusted for response to the solvent.

Behaviorally, the beetles appeared to disperse quickly at random out of the airstream at low rates of the standard, and only a few beetles gave a positive response. At the intermediate rates, fewer beetles quickly dispersed at random, and the majority tended to remain in the airstream for a few minutes before dispersing or giving a positive response. Most beetles responded to the higher rates by direct orientation toward the odor source and contact with the air outlet. At these rates, beetles that began to leave the air-stream on release would usually stop after a few centimeters, pivot around with their antennae raised, and walk directly toward the odor source.

Currently, the apparatus is used on an operational basis to assay the chemical fractionation of Porapak-collected volatiles from pine bolts artificially and naturally infested with southern pine beetles.

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BENZOYL CYANIDE AND MANDELONITRILE IN THE CYANOGENETIC SECRETION OF A CENTIPEDE[1]

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Abstract—Centipedes of the order Geophilomorpha produce a viscid odorous secretion that is effectively defensive against ants (*Formica exsectoides*). The secretion is proteinaceous and cyanogenetic. In *Geophilus vittatus*, the secretion contains two cyanogenetic compounds, mandelonitrile (I) and benzoyl cyanide (II), as well as two products derived from these compounds as a result of hydrogen cyanide production [benzaldehyde (III) and benzoic acid (IV)]. Benzoyl cyanide has not been reported previously from any natural source.

Key Words—cyanogenesis, benzoyl cyanide, mandelonitrile, defensive secretion, geophilomorph centipede.

INTRODUCTION

The centipedes of the order Geophilomorpha produce a viscous and odorous defensive secretion, which they discharge in response to disturbance, from segmental glands that open ventrally along the length of the body (Brade-Birks and Brade-Birks, 1920; Koch, 1927). Schildknecht et al. (1968), working with the European species *Pachymerium ferrugineum*, found the secretion to be proteinaceous and cyanogenetic. They speculated that the hydrogen cyanide might be generated by dissociation of a cyanohydrin such as mandelonitrile, but they identified no such cyanogenetic compound. We here report on the chemistry and defensive effectiveness of the secretion of the North American *Geophilus vittatus*. The fluid of this centipede is shown to be

[1] This is Paper No. 48 in the series *Defense Mechanisms of Arthropods.*

also proteinaceous and cyanogenetic, and to contain two cyanogenetic compounds, mandelonitrile (I) and benzoyl cyanide (II). Other geophilomorphs of the genera *Geophilus* and *Strigamia* appear to produce comparable secretions.

METHODS AND MATERIALS

The species studied were *Geophilus vittatus*, *G. cayugae*, *Strigamia bothriopa*, *S. chionophila*, and *S. icterica*. They were collected in the environs of Ithaca, New York, under bark of fallen trees and in other cryptic habitats. *G. vittatus* was the most common. To prevent the animals from discharging prematurely, they were not handled, but were coaxed gently into individual containers, within which they were maintained until needed for experimentation. They were fed small pieces of freshly killed insects. The specimen of *Orphnaeus brasilianus* (see Fig. 2) was taken on Lignum Vitae Key, Florida.

The centipedes were "milked" of secretion by holding them in forceps and pinching them along the length of the body with a second pair of forceps, causing the fluid to be discharged from the various segmental sites stimulated. The secretion was taken up as needed for the various tests, on glass surfaces, filter paper, or chromatoplates.

Hydrogen cyanide emission was detected by the copper acetate–benzidine acetate test (Feigl, 1966). Quantitative assay of the compound was effected by the technique previously used with cyanogenetic millipeds and described in detail by Eisner *et al.* (1967). This technique involves placement of the animal in a closed system within which it is electrically stimulated and caused to discharge, and in which the emitted hydrogen cyanide is trapped in ammoniacal silver iodide solution for subsequent analysis.

Protein determinations were made using a modified Lowery procedure developed for the detection of protein in the presence of interfering substances (Murthy and Leroux, 1975).

Thin-layer chromatograms of fresh, undiluted secretion, and of ethereal extracts of secretion, were made on silica gel 6060 thin-layer plates (Eastman Kodak), and developed in benzene–chloroform (5:1) and in petroleum ether. Visualization was by I_2 vapor or by ethanolic 2,4-dinitrophenylhydrazine.

Gas-chromatographic analyses were carried out with a Varian 2100 gas chromatograph equipped with a flame ionization detector, using a 2.4 m $\times$ 2 mm column packed with 5% OV-1 on Gaschrom Q. Mass spectra were obtained using the same column in a Finnigan Model 3300 gas chromatograph/mass spectrometer coupled with a System Industries Model 150 computer.

The ants (*Formica exsectoides*, from Ithaca, New York) used in the predation tests were kept in a large artificial colony, equipped with a Lucite-

covered foraging arena within which the encounters with the centipedes were staged.

RESULTS

Chemistry of the Secretion

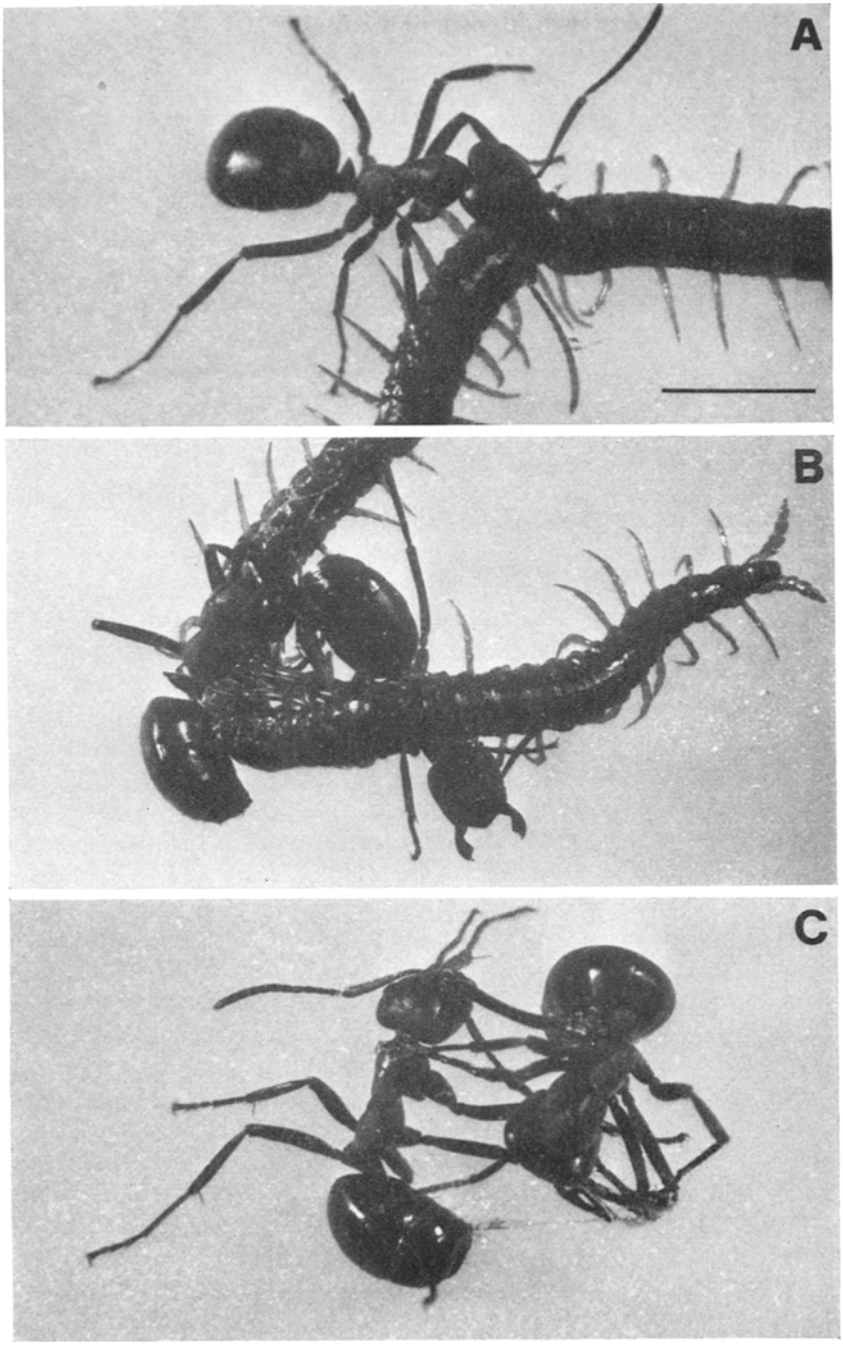
Most of the chemical work was done with *G. vittatus*. This species ejects its clear, viscous secretion promptly in response to manipulation or pinching. The fluid visibly contaminates the body of the animal, as well as the substrate on which it walks. The pH of the fluid was found to range from 6.0 to 6.5 (6 determinations). Emission of hydrogen cyanide from disturbed centipedes was readily demonstrated by the blue color that developed on strips of filter paper impregnated with copper acetate–benzidine acetate reagent held close to their bodies. Papers held beside discharged secretion also turned blue. No discoloration occurred in papers held next to undisturbed centipedes.

Only a single specimen was assayed quantitatively for hydrogen cyanide emission. The 35-mg specimen produced 6.6 μg of the compound.

For protein analyses, secretion samples from 3 specimens were collected on pieces of filter paper. The samples contained 25, 37, and 40 μg protein. Given the inefficiency of the collection technique, it is doubtful that even the highest of these values is indicative of maximal protein output of *G. vittatus*.

Gas-chromatographic analysis of an ethereal extract of fresh secretion showed two peaks of comparable intensity. The first had a gas-chromatographic retention time and a mass spectrum identical to those of authentic benzaldehyde (III), while the second peak showed a mass spectrum suggestive of benzoyl cyanide (II) [*m/e* 132 (6), 131 (67), 105 (100), 77 (90), 56 (63), 55 (41)]. An authentic sample of benzoyl cyanide, m.p. 30–31°C was prepared as described by Oakwood and Weisgerber (1965); direct GC/MS comparison confirmed its identity with the natural product.

The observation of both hydrogen cyanide and benzaldehyde in this secretion suggested that the native effluent should contain mandelonitrile (I) (benzaldehyde cyanohydrin). Since mandelonitrile appeared to dissociate instantaneously to give benzaldehyde under our gas-chromatographic conditions, we turned to thin-layer chromatography to search for the undissociated cyanohydrin. Thin-layer chromatography revealed the presence of a third and a fourth component. As expected, one of these had R_f values, in both elution systems, matching those of authentic mandelonitrile. The other had R_f values corresponding to those of benzoic acid (IV), which could be expected to be formed from benzaldehyde autoxidation, as well as from benzoyl cyanide hydrolysis.


A
B
C

Only few individuals of the other Geophilomorpha studied (*G. cayugae*, *S. bothriopa*, *S. chionophila*, *S. icterica*) were available for testing. However, all produced sticky, cyanogenetic secretions (positive copper acetate–benzidine acetate test). A single 13-mg specimen of *Strigamia* (probably *bothriopa*) produced 4.0 μg hydrogen cyanide. The presence of protein and benzoyl cyanide (GC/MS) was verified in this species.

Effect of the Secretion on Ants

Several *G. vittatus* and *S. bothriopa* that had not been previously milked of secretion were introduced one at a time into the ants' foraging arena. They were immediately attacked by one or more ants, and the results were dramatic (Fig. 1). Within seconds after having clamped themselves to the body of a centipede, the ants were seen to relinquish their hold, and to be left behind as the centipede scurried away. Visibly contaminated with the sticky secretion, the ants performed intensive cleansing activities, vigorously at first, and then more and more awkwardly, as the secretion hardened on exposure to air. Ants that contacted one another after being wetted with secretion, or that were wetted simultaneously as they attacked adjacent sites of a centipede, frequently became stuck together and virtually immobilized. Some became stuck to the substrate. Although many of the ants that had been only lightly contaminated with secretion seemed eventually to recover, those more massively wetted frequently died. The centipedes sustained no injuries as long as their secretory supply lasted. Their body wall is resilient and rubbery and they were only rarely pierced by the ants' bites. Once depleted of secretion, they were vulnerable. A single *S. bothriopa* that was introduced into the arena after having been milked exhaustively was overrun by a swarm of ants, doused in the ants' acid spray, and eventually dismembered and killed.

Geophilomorph females may also employ their secretion defensively when they guard their eggs. Figure 2 shows the response of one such female to prodding with forceps. The animal did not abandon the eggs when disturbed, but merely discharged secretion from its ventral surface, which was appropriately oriented outward.

FIG. 1. Stages in the attack of ants (*Formica exsectoides*) upon *Strigamia bothriopa*: A, single ant, initiating its attack; B, pair of ants that bit adjacent sides of a centipede, visibly contaminated with secretion, and stuck to one another as well as to the centipede; C, same pair of ants as in B, photographed moments later, after the centipede escaped, stuck together and to the substrate. The reference bar in A represents 3 mm.

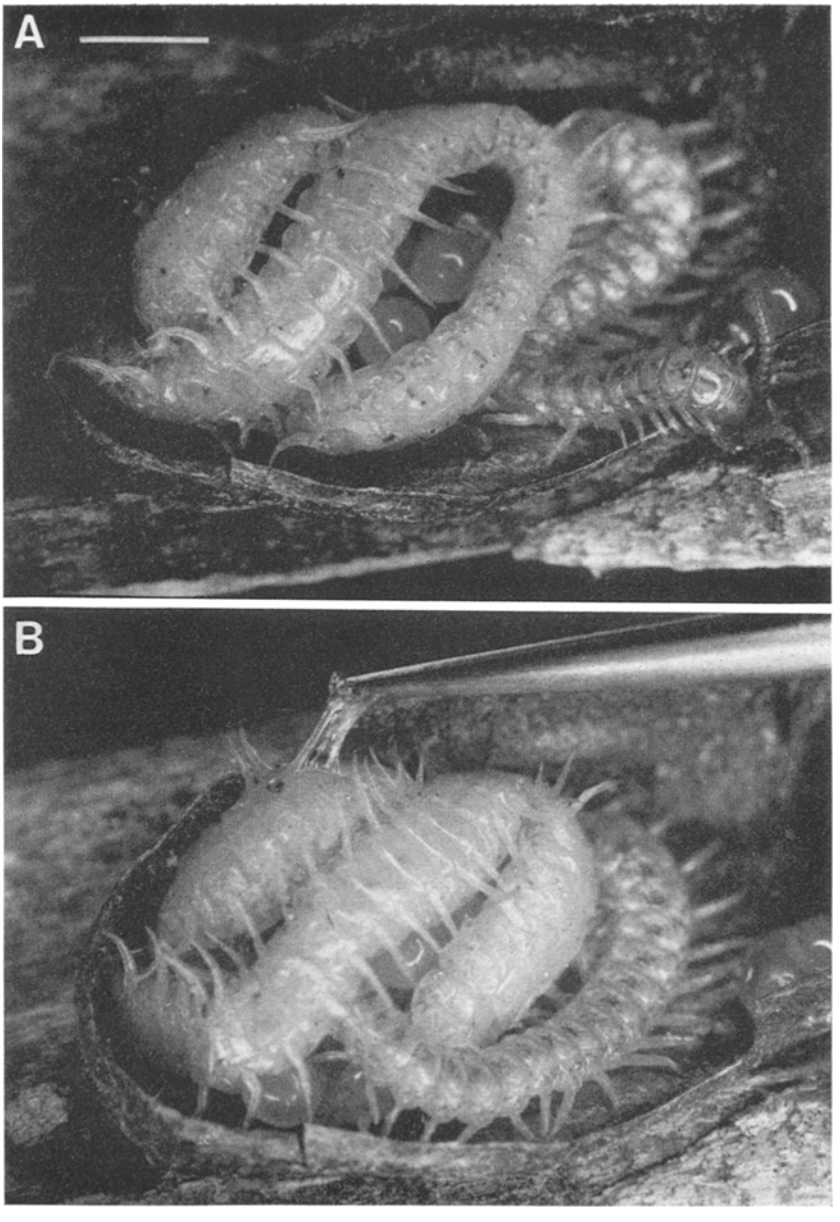


FIG. 2. A, Female of *Orphnaeus brasilianus*, coiled around its eggs in typical guarding posture; the centipede was sheltered in an empty pupal cocoon of a moth (*Megalopyge opercularis*), which was cut away to expose the animal for photography. B, Same, responding to prodding with forceps by discharging some of its sticky defensive fluid from the site stimulated. The reference bar in A represents 3 mm.

DISCUSSION

The two cyanogenetic components in the secretion of *G. vittatus*, mandelonitrile and benzoyl cyanide, are the obvious sources of the hydrogen cyanide liberated at discharge, as well as of the other two aromatic compounds in the mixture, benzaldehyde and benzoic acid. Mandelonitrile dissociates directly to benzaldehyde and hydrogen cyanide, while benzoyl cyanide gives benzoic acid and hydrogen cyanide on hydrolysis. It seems probable that these processes are catalyzed, as cyanogenesis is known to be in polydesmoid millipeds (Eisner et al., 1963).

The most interesting chemical feature of the secretion of *G. vittatus* is the presence of benzoyl cyanide, an active acylating agent as well as a cyanide precursor, which has not hitherto been isolated from cyanogenetic animals or plants, or for that matter from any other natural source. The other cyanogenetic compound in the mixture, mandelonitrile, is commonly present in cyanogenetic organisms, including even certain arthropods. It has been identified as such in polydesmoid millipeds (Barbetta et al., 1966; Eisner et al., 1963), and it appears to occur as a glycoside in certain Australian chrysomelid beetle larvae (Moore, 1967). In polydesmoid millipeds, mandelonitrile is synthesized from phenylalanine (Towers et al., 1972). This might also hold for *Geophilus*, which might then make its benzoyl cyanide simply by dehydrogenation of mandelonitrile. The possible chemical relationships of the *Geophilus* compounds are shown in Fig. 3.

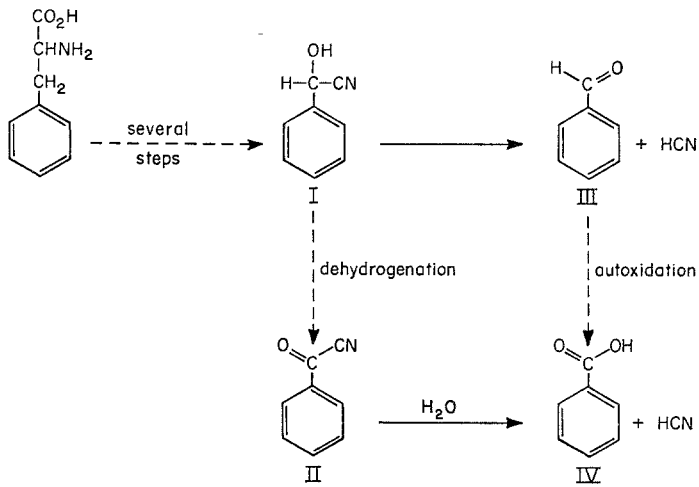


FIG. 3. Aromatic components of *G. vittatus* (I–IV) and their possible relationships. The presumed biosynthesis of I from phenylalanine is also indicated.

Other investigators had noted both in the field and in the laboratory that ants are deterred by geophilomorph secretion (Brade-Birks and Brade-Birks, 1920; Schildknecht et al. 1968), and our findings certainly confirm this. The secretion appeared to affect the ants primarily mechanically by virtue of the viscidness that it derives from its proteins, but it could obviously have acted also in chemical ways. Some of its components might have acted as topical irritants, perhaps inducing the prompt cleansing motions that the secretion elicited on contact, while others such as hydrogen cyanide could have acted as toxins, and contributed to the death of ants that were heavily contaminated with secretion. Although no tests were done with other predators, the secretion might well be deterrent to arthropods other than ants.

One wonders whether hydrogen cyanide production is of general or restricted occurrence within the Geophilomorpha. Generalized occurrence appears to be ruled out, since there are some families within the order that reportedly lack the glands (Attems, 1926; Crabill, 1952). The three genera in which cyanogenesis has so far been demonstrated, *Pachymerium*, *Geophilus*, and *Strigamia*, belong to only two families (Geophilidae and Dignathodontidae) of the several that do have glands. What chemical diversity prevails among these gland-bearing families remains to be seen.

The secretion of some geophilomorphs has been said to be luminescent (Brade-Birks and Brade-Birks, 1920; Koch, 1927). We found this to apply also to the secretion of *G. vittatus*, which when freshly discharged emits a faint but distinct blue-green glow that lasts for some seconds. Neither the chemical basis nor the defensive function (if any) of this luminescence is understood.

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OVIPOSITIONAL BEHAVIOR OF *Bracon mellitor* SAY (HYMENOPTERA: BRACONIDAE), A PARASITOID OF BOLL WEEVIL (*Anthonomus grandis* Boh.) I. ISOLATION AND IDENTIFICATION OF A SYNTHETIC RELEASER OF OVIPOSITOR PROBING[1]

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Abstract—Female *Bracon mellitor* Say responded to the frass of diet-reared boll weevil larvae by probing with the ovipositor. Similar responses were elicited by the hexane and chloroform–methanol fractions after differential extraction of boll weevil feces. The active component of the chloroform–methanol fraction was identified as methyl *p*-hydroxybenzoate (methyl parasept). Because the methyl parasept is an artificial component of the diet, the results suggest that the parasitoid response may be associatively learned. The response to methyl parasept decreased with time in the absence of reinforcement. Results demonstrate that certain chemicals may release behavior normally evoked by kairomones, and may interfere with the isolation of natural kairomones.

Key Words—boll weevil, cotton insects, parasitoid behavior, *Bracon mellitor*, *Anthonomus grandis*, ovipositor probing behavior, kairomone, methyl *p*-hydroxybenzoate.

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INTRODUCTION

Host-searching, location, and recognition by entomophagous parasitoids involve a sequence of behavioral responses triggered by various stimuli. The types of clues utilized by parasitoids in host selection have been reviewed by Vinson (1975). Chemicals appear to play an important role as the primary stimuli for host-finding by parasitoids. These transpecific chemical messengers have been referred to as *kairomones* (Brown et al., 1970; Whittaker and Feeny, 1971).

Bracon mellitor Say is an ectoparasitoid (Pierce, 1910; Willard, 1927) of many coleopterous larvae of the family Curculionidae and several species of Lepidoptera (Cross and Chesnut, 1971). The biology (Adams et al., 1969) and ovipositional behavior (McGovern and Cross, 1974) of *B. mellitor* have been described. *Bracon mellitor* has been referred to as the most important parasite of the boll weevil in the southwestern United States (Cross and Chesnut, 1971; Cross, 1973). However, very little work has been done toward elucidating the factors important in host selection by *B. mellitor*. Adams et al. (1969) suggested that females detected the presence of hosts by IR radiation. Folsom (1936) indicated that the parasitoid responded with tactile sensitivity.

Oviposition of boll weevil females in flower buds (squares) and fruits (bolls) of cotton has been described by Cushman (1911). In squares, immature weevils continue to develop until about the molt from second to third larval instar, at which time the squares either completely abscise and fall to the ground or incompletely abscise and "hang" on the plants. Supposedly, the parasitoids use some chemical stimulus elicited by the plant during this abscission to locate areas housing potential hosts. Hunter and Hinds (1905) and Pierce (1908) were among the first to note the significance of these "hanging squares" in consistently containing a higher percentage of parasitized weevil larvae.

Bracon mellitor females must locate boll weevil–susceptible stages inside these plant structures. Realistically, such host location has to be considered in light of the female parasitoid's response to the cotton plant. Bottrell and Walker (unpublished data) indicated a significant response of *B. mellitor* females to freshly abscised cotton squares independent of weevil infestation.

The current study was not intended to mimic the complexity of the cotton ecosystem or to determine long-range host-finding cues used by *B. mellitor*. We were interested in determining whether contact chemicals are a factor important in host selection by *B. mellitor*.

METHODS AND MATERIALS

Insects

The host, *Anthonomus grandis* Boheman, was reared according to the method of Vanderzant and Davich (1958), modified by the addition of 0.2% methyl *p*-hydroxybenzoate in the diet to retard fungal growth. A modification of the procedure of Adams and Cross (1967) was used to rear the parasitoid, *B. mellitor*.

Bioassays

From 5 to 10 third-instar boll weevil larvae were placed in the lid of a 9-cm plastic petri dish and covered with a 9-cm circle cut from a Kim Wipe.® The Kim Wipe was forced into contact with the larvae for 20 min by placing the petri dish bottom on top of the Kim Wipe. The Kim Wipe–covered larvae were then exposed to 20 adult female and male parasitoids for 2 or 3 hr in a 30 × 30-cm-high Plexiglas cage. Two 4-cm holes at each end of the cage for air flow were covered with organdy cloth to prevent the escape of the parasitoids. Adults had access to a solution of 1 part honey and 4 parts water. All insects were held at 26–28°C on a 14:10, L:D circadian cycle and a relative humidity of about 45%.

Initial studies were designed to identify the source of an odor or contact chemical that would result in ovipositor probing by *B. mellitor*. From 6 to 10 small (0.5 cm) squares of diet, frass caps produced by the diet-reared larvae, third-instar larvae, pupae, or legless adults (legs removed to prevent their escape) were placed in a petri dish. In some tests, the samples were covered with a 9-cm Kim Wipe circle, which was contaminated by placing it in contact with the samples. In several tests, the Kim Wipes were elevated 2 cm above the samples, thus preventing their contamination.

Bioassays to monitor the active components during isolation were carried out by applying 10 μl of the frass extract or the purified and isolated chemicals to the center of one half of a 9-cm Whatman® No. 1 filter paper disc. Ten μl of the appropriate solvent was applied to the other half of the filter paper disc as a control. The extracts resulted in a 0.5-cm-diameter spot, which was outlined lightly in pencil. After solvent evaporation, the filter paper disc was placed in a cage with 20 females and 20 males. The responses of females were recorded in two ways: In tests used to monitor the isolation and identification of the active material, the number of females probing the treated spot with their ovipositors at 5-min intervals for 25 min was recorded. In later tests, which examined female response to different concentrations of the identified material, the activity was scored by recording the number of ovipositor probing responses of females and the total number

of female contacts with the test material during 30 min of constant observation. The females used in all tests, with the exception of those in Table 4, cage B, were laboratory-reared, and had been exposed to diet-reared weevil larvae. Some of the females used to obtain the data in Table 4, cage B, were field-collected, and were presumably exposed to field hosts. All tests were replicated and the results expressed as the percentage of contacts by females that resulted in ovipositor probing.

Chemical Isolation and Characterization

From 1 to 2 g of frass from diet-reared third-instar boll weevil larvae were extracted in a solvent series of hexane, chloroform, methanol, and water. In later extractions, the frass was homogenized in cold chloroform:methanol (2:1 vol/vol), and the homogenate was extracted according to Folch et al. (1957). The chloroform lower phase was dried with Na_2SO_4 applied to a florisil column, and eluted with 12 25-ml volumes of chloroform, followed by an increasingly more polar series of solvents. Chloroform fractions 6–8 were the most active, and were further fractioned on silica gel TLC plates developed with chloroform:acetone (90:10 vol/vol). The various bands were scraped off and eluted with methylene chloride. The active band after additional purification by TLC was subjected to mass spectral analysis.

RESULTS AND DISCUSSION

The response of female *B. mellitor* to the diet and various stages of the boll weevil revealed that the artificial diet was not active, while the frass or exudates from boll weevil larvae were active in eliciting ovipositional probing (Table 1). Although a large number of antennal responses to naked boll weevil larvae were observed, very few ovipositional attempts were made. However, female parasitoids were observed to probe the areas around the larvae. Kim Wipes contaminated with boll weevil exudate elicited ovipositor probing by a number of females, while uncontaminated Kim Wipe held 2 cm above the larvae did not elicit such response.

Bioassays of the boll weevil frass extracts (Table 2) revealed the major activity to be present in the hexane, methanol, and chloroform–methanol fractions. Further separation of the chloroform layer of the chloroform–methanol fraction on a florisil column disclosed several active fractions (Table 2). Fractions 6–8 were further separated by TLC. Since the greatest activity was present in a discrete band at R_f 0.06, this band was subjected to further analysis. Thin-layer chromatography of the active band in several systems yielded a single spot. Mass spectral analysis of this fraction yielded a mass spectral fragmentation pattern with M+ 152, *m/e* 121, 39, 65, 93,

TABLE 1. RESPONSE OF FEMALE *B. mellitor* TO DIFFERENT MATERIALS ASSOCIATED WITH BOLL WEEVILS

Material	Total number of females responding at 5-min intervals for 30 min		
	Antennal touching	Ovipositional probing	Control[a]
Media	3	1	0
Frass	27	18	2
Third-instar BW[b] larvae	36	8	1
BW pupae	0	0	3
BW adults	0	0	2
KW[c]-covered media	8	2	3
KW-covered larvae	23	21	1
KW-covered BW pupae	2	0	3
KW-covered BW adults[d]	0	0	1
KW-covered BW larval frass	13	13	2
KW 2 cm above BW larvae[e]	3	3	0

[a] A solution of 1 part honey and 4 parts water on a sponge served as a control.
[b] BW: boll weevil.
[c] KW: A Kim Wipe that covered and touched the material, thus being contaminated.
[d] Legs were removed to prevent movement.
[e] Kim Wipe was held 2 cm above the boll weevil larvae, so that only odors or sound would pass through, eliminating contact chemicals.

and 152. This suggested the methyl ester of *p*-hydroxybenzoic acid (methyl parasept) as the component. Cochromatography of the active material with an authentic sample of methyl parasept yielded a single spot. When authentic samples of methyl parasept were exposed to females, the characteristic ovipositor probing was elicited.

The responses of female parasitoids to different amounts of methyl parasept are shown in Table 3. The maximal percentage of females responding occurred at 100 ng/spot. The range of responses by *B. mellitor* to methyl parasept declined rapidly below 10 ng/spot and above 1 μg/spot. Although many females did not respond by ovipositor probing at the lowest amount, a number of females did respond briefly with antennal examination of the treated spot. The amount of methyl parasept that elicited a maximal response by *B. mellitor* was slightly greater than the amounts of kairomone necessary to maximally stimulate *Orgilus lepidus* (Hendry et al., 1973) and *Microplitis croceipes* (Jones et al., 1971).

Methyl parasept is a component of the host diet; thus, its isolation as an active factor eliciting ovipositor probing by *B. mellitor* suggested a number of interesting questions. Methyl parasept is often added to insect diets to

TABLE 2. OVIPOSITIONAL PROBING RESPONSE OF FEMALE *B. mellitor* TO VARIOUS FRACTIONS OF THE FECES OF DIET-REARED BOLL WEEVIL LARVAE

Feces fraction	Number of females responding at 5 5-min intervals
Hexane extract	10
Chloroform extract	18
Methanol extract	23
Water extract	8
Chloroform-methanol homogenate	17
Chloroform layer	16
Methanol layer	6
Chloroform fractions 1–5[a]	0
Chloroform fractions 6–8	8
Chloroform fractions 9–12	1
Chloroform:methanol (90:10)	5
Chloroform:methanol (80:20)	2
Chloroform:methanol (70:30)	0
Chloroform:methanol (50:50)	1
Methanol	5

[a] Eluates from a florisil column.

TABLE 3. RESPONSE OF FEMALE *B. mellitor* TO DIFFERENT AMOUNTS OF METHYL PARASEPT APPLIED IN ACETONE

Amount of methyl parasept applied (ng)[a]	Contacts	Percentage responding[b]
10^6	24	16.6
10^5	24	25.0
10^4	18	33.3
10^3	26	84.6
10^2	22	90.9
10	20	30.0
1	14	14.2
0.1	18	11.1

[a] Applied to Whatman® #1 filter paper, resulting in a 0.5-cm circular spot.

[b] Only those that raised their abdomens and began ovipositor probing were recorded as responding.

prevent fungal growth (Singh, 1974), but has rarely been reported in nature. One exception is the production of methyl parasept by the giant water beetle, *Dytiscus latissemus*, and several related species (Schildknecht, 1968). The function of methyl parasept in the giant water beetle was presumed to be antimicrobial (Schildknecht et al., 1964).

Because females did not respond to the diet containing methyl parasept, but did respond to frass (Table 1), the levels of methyl parasept in the diet and frass were determined by gas chromatography (3% QF-1). There was essentially no difference in the percentages of water in the diet (average of 71.7%) and in the frass (average of 73.7%). There was also little difference in the amount of methyl parasept in the diet or frass, as the methyl parasept ratio of frass to diet equaled 1.05.

These results were surprising in view of the observation that females probed the frass, did not probe the diet, and yet responded to the methyl parasept fraction. However, in our rearing procedure, the parasites were exposed to boll weevil larvae removed from the diet, placed in a petri dish, and covered with a Kim Wipe. As the Kim Wipe above the weevil larvae become contaminated with frass containing methyl parasept, the methyl parasept would act as an orienting cue. The concentration of methyl parasept in the diet and frass was determined to be 0.4 μg/mg wet weight, which was close to the amount that gave a maximal response in our bioassays (Table 3).

Because methyl parasept gave a response, we field-collected several grams of both weevil-infested squares and weevil larvae. These specimens were extracted and carried through the same procedure used to isolate and identify methyl parasept in the diet. No methyl parasept was detected. The absence of methyl parasept in the field-collected boll weevil larvae and infested squares suggests that *B. mellitor* females may learn to cue on certain chemicals associated with the host. Several species of parasitoids have been reported capable of associative learning (Arthur 1966, 1971; Taylor 1974). Because females exposed to diet in earlier studies had not responded, the responses of females to weevil larvae covered with a Kim Wipe and to methyl parasept were recorded during their repeated exposure to weevil larvae in the diet (Table 4). Females responded to methyl parasept following exposure to Kim Wipe–covered weevil larvae, but did not respond to the diet or frass. However, after repeated exposure of females to plates of diet containing weevil larvae, responses to the frass were observed. When females that were responding to the frass in media plates were reexposed to methyl parasept, the response to the methyl parasept greatly decreased (Table 4). The initial low response of females to the media even though it contained methyl parasept may be due to the presence of components that are slight repellent. Further, because the contraction of methyl parasept was approximately equal in the diet and the frass, the methyl parasept would not serve

TABLE 4. PROBING RESPONSE OF FEMALE *B. mellitor* TO A SEQUENCE OF MATERIALS OVER A PERIOD OF DAYS

Exposure sequence	Exposed materials	Cumulative number of females probing at 5-min intervals for 25 min in each cage		
		A	B	C
1	Kim Wipe–covered larvae	25	19	31
2	Methyl parasept[a]	16	14	17
3	Diet plate[b]			
	Diet	1	0	1
	Frass	2	1	4
10	Diet plate[c]			
	Diet	2	3	1
	Frass	14	9	18
11	Methyl parasept[a]	1	2	2
12	Kim Wipe–covered larvae	12	16	9

[a] Methyl parasept, 100 ng, applied to 9 spots equidistant from one another on a 9-cm filter paper disc.
[b] Responses by females to frass and diet were recorded independently.
[c] Responses recorded as in sequence 3 after 6 previous 8-hr exposures/day to diet plates.

in orienting the female parasitoid to the host or as a cue to host presence. As can be seen in Table 4, the response of females to the frass after being exposed to diet infested with weevil larvae increased after a number of exposures, but the response to methyl parasept decreased. However, the response to boll weevil larvae covered with the Kim Wipe continued. These results, while speculative, can be interpreted if the response to methyl parasept is a learned response in association with a second compound, possibly an unidentified component in the hexane fraction from the frass or weevil larvae.

To determine whether the response to methyl parasept would continue if female parasitoids were not continually reinforced by being exposed to methyl parasept–contaminated frass, a test was designed to expose females only to methyl parasept. Female parasitoids were first exposed to Kim Wipe–covered weevil larvae for 8 hr a day for several days to ensure a strong response to methyl parasept. The females were then held for 2 days with no exposure. On the third day, they were exposed to Kim Wipe–covered weevil larvae for 1 hr, then exposed at different times to filter paper discs containing 9 approximately equally placed 0.5-cm spots, each containing 100 ng methyl parasept. The results (Table 5) show that females responded to methyl parasept through the first 8 hr, with a drop-off in response in 24–36 hr.

TABLE 5. PROBING RESPONSE OF FEMALE *B. mellitor* TO METHYL PARASEPT AT DIFFERENT TIMES AFTER INITIAL EXPOSURE TO DIET-REARED BOLL WEEVIL LARVAE

Time after initial exposure	Cummulative number of females probing at 5-min intervals[a]		
	A	B	C
1 hr	10	11	16
4 hr	9	7	18
8 hr	2	1	5
24 hr	1	1	3
36 hr	0	0	1

[a] Exposed to 100 ng methyl parasept applied to 9 equally placed spots on a 9-cm filter paper disc and placed in cage for 25 min.

The results suggest that the response to methyl parasept decreases in the absence of reinforcement, and is similar to the loss of avoidance behavior exhibited by *Drosophila melanogaster* to certain odors after removal of shock reinforcement (Quinn et al., 1974) or habituations.

The results of this study suggest that *B. mellitor* may associatively learn certain chemical cues, although a more detailed study is necessary before such conclusions can be reached. The study also points up a serious problem in the isolation and identification of kairomones involved in host location and selection by insect parasitoids with a wide host range. Although a very specific bioassay may be developed, it can lead to the isolation of chemicals other than naturally occurring kairomones that are capable of eliciting the behavior under conditions of rearing or the bioassay procedure.

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EVIDENCE FOR METABOLIC ADAPTATION TO FLOODING IN *Leavenworthia uniflora*

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Abstract—*Leavenworthia uniflora* (Cruciferae) is a winter annual that is restricted to shallow, limestone soils that are subject to waterlogging from late autumn to early spring. To determine its responses and adaptations to waterlogged soil, the effect of flooding on growth and alcohol dehydrogenase (ADH) activity was studied. During a 31-day growth period, the average relative growth rate of plants grown in flooded soil was 54 mg g^{-1} d^{-1}, and that of plants grown in unflooded soil was 68 mg g^{-1} d^{-1}. Flooding did not cause an increase in ADH activity, implying that ethanol did not accumulate, and that *L. uniflora* is metabolically adapted to growing with its roots under anaerobic conditions.

Key Words—metabolic adaption, helophyte, flooding, anaerobiosis, winter annual, *Leavenworthia uniflora*.

INTRODUCTION

Leavenworthia is a small genus of the Cruciferae consisting of seven species and several varieties (Rollins, 1963), all of which are restricted to shallow, calcareous soils underlain by limestone. These shallow soils are often waterlogged during late summer, winter, and early spring, but are subject to long periods of drought during summer. Thus, in order to persist in this habitat, a plant must be adapted to extremes of moisture conditions. Members of the genus *Leavenworthia* are winter annuals, and complete their life cycle between mid-September–mid-October and mid May (Rollins, 1963; Caudle and Baskin, 1968; Baskin and Baskin, 1971, 1972). This type of life cycle allows *Leavenworthia* to pass the hot, dry season in the drought-resistant seed stage. However, since the soil in its habitat is often waterlogged from late autumn to early spring, the plants must be adapted to grow with their roots under anaerobic conditions.

Dr. R. M. M. Crawford and his associates in Great Britain have studied the metabolic adaptations of roots of plants that grow in habitats where the soil is waterlogged (helophytes) (Crawford, 1966, 1967*a*,*b*, 1969, 1972; Crawford and McManmon, 1968; Crawford and Tyler, 1969; Tyler and Crawford, 1970; McManmon and Crawford, 1971; Garcia-Novo and Crawford, 1973). On being flooded, nonhelophytes accumulate ethanol, which is toxic to the plant (Fulton and Erickson, 1964; McManmon and Crawford, 1971), whereas helophytes accumulate malate or some other nontoxic metabolite, but not ethanol (Crawford, 1969, 1972; McManmon and Crawford, 1971).

Acetaldehyde resulting from anaerobic respiration has been shown to be an inductive stimulus of alcohol dehydrogenase (ADH) in roots of nonhelophytes (Hageman and Flesher, 1960; Crawford and McManmom, 1968). An increase in ADH activity is accompanied by an increase in ethanol accumulation. The amount of ethanol accumulated in roots can be inferred by measuring the activity of ADH, the adaptive enzyme that catalyzes the interconversion of acetaldehyde and ethanol (Racker, 1950, 1955). Thus, in nonhelophytes, anerobiosis causes an increase in acetaldehyde, ADH activity, and ethanol. On the other hand, in helophytes, anaerobiosis does not cause an increase in production of acetaldehyde; therefore, ADH activity and ethanol do not increase.

The purpose of this investigation was to compare growth and ADH activity in flooded and unflooded plants of *Leavenworthia uniflora* (Michx.) Britton, the most widely distributed taxon of the genus. In a comparison of ADH activity in helophytes and nonhelophytes, Crawford (1967*a*) and McManmon and Crawford (1971) found that when the plants were flooded, ADH activity increased greatly in the nonhelophytes, but showed no increase in helophytes. Thus, our initial hypothesis was that since *L. uniflora* is tolerant of waterlogging in its natural habitat, there would be no difference in ADH activity in flooded and unflooded plants. As a check on our technique, we compared ADH activity in roots of flooded and unflooded corn (*Zea mays* L.), a nonhelophyte known to show an increase in ADH when flooded (Marshall et al., 1973).

METHODS AND MATERIALS

Leavenworthia uniflora

Seeds of *L. uniflora* were collected in Simpson County in south central Kentucky and germinated on moist soil in a greenhouse in early October 1973. On October 19, 1973, when the seedlings had 2 or 3 foliage leaves plus the cotyledons, 3 seedlings were transplanted into each of 50 250-ml Styrofoam cups filled with greenhouse potting soil. On November 12, 1973, the soil in

25 of the cups (75 plants) was flooded. The cups containing the seedlings were placed in plastic cups of larger diameter, but of approximately the same depth. When the plastic cups were filled with tap water, the soil was waterlogged to the surface, but the shoots were not submerged. Water was added as needed during the experiment to keep the plastic cups filled. The other 25 cups of *L. uniflora* seedlings were free-draining, and were watered as needed to keep the soil moist. Plants were grown in a heated greenhouse under natural photoperiods. Mean daily maximum and minimum temperatures from November 12 to December 20, calculated from thermograph records, were 20.9 and 15.6°C, respectively.

On December 20, the plants were harvested, and protein content and ADH activity were determined, using crude extracts obtained from roots of flooded and unflooded *L. uniflora* plants. Roots from flooded and unflooded plants were each divided into 2 batches of approximately 3 g wet weight, and separate extractions and measurements of protein and ADH activity were made on the 4 batches of roots.

The root extracts were prepared in an ice bath by grinding in a 0.1 M Tris buffer solution (pH 8), to which 1 ml of a 1 mg/ml solution of bovine serum albumin had been added. The extracts were ground with a mortar and pestle, and were then separated from the root material by filtering through muslin and centrifuging at 12,000*g* for 20 min at 7°C. Protein was determined spectrophotometrically (Layne, 1957):

$$\text{Protein concentration (mg/ml)} = 1.55\ OD_{280} - 0.76\ OD_{260}$$

Optical density readings for protein determination were made on a 100-fold dilution of the crude extract, 2 determinations being made on each of 3 aliquots from each of the 4 extracts.

Determination of ADH activity was carried out using a slightly modified version of the procedure of Racker (1950, 1955). The reaction mixture consisted of 0.6 ml 0.05 M $Na_4P_2O_7$, pH 8.5, 1.1 ml distilled water, 0.8 ml 0.002 M NAD, 0.4 ml 1.0 M ethanol (the substrate), and 0.1 ml plant extract. In the control cuvette, the 0.1 ml extract was replaced by distilled water. After addition of the plant extract, OD readings were taken at 340 nm every 15 sec for 2 min.

The OD readings were plotted against time, and the change in OD was determined from the linear portion of the graph, which was usually between 15 and 45 sec. The change in OD for the 30 sec was multiplied by 2 to give ΔOD/min. To obtain micromoles of NAD reduced per minute, the ΔOD/min was divided by the extinction coefficient of NADH, 6.22×10^3 at 340 nm. Specific activity of ADH was obtained by dividing micromoles of substrate converted per minute by the amount of protein in the reaction mixture.

To determine the effect of flooding on growth of *L. uniflora*, we grew

plants under flooded and unflooded conditions, and compared the dry weights of roots and shoots. On October 19, 1973, 3 seedlings were transplanted into each of 45 250-ml Styrofoam cups filled with greenhouse potting soil. On November 3, at the beginning of the flooding experiment, seedlings in 5 of the cups (15 seedlings) were harvested, dried to constant weight at 80°C, and weighed to the nearest 0.1 mg. On this same date, the root systems of 60 seedlings (20 cups) were flooded as previously described, and kept flooded until the end of the experiment. The other 20 cups of seedlings were free-draining, and were watered as needed to keep the soil moist. Plants in flooded and unflooded soil were grown in a warm greenhouse under natural photoperiods for 1 month. Mean daily maximum and minimum temperatures during this period were 21.5 and 15.4°C, respectively. On December 3, the flooded and unflooded plants were harvested, dried at 80°C, and weighed. Growth rates of flooded and unflooded plants were calculated using the following equation (Radford, 1967):

$$RGR = \frac{\log_e W_2 - \log_e W_1}{t_2 - t_1}$$

where RGR is the relative growth rate, W_2 is the mean dry weight of plants at time t_2, W_1 is the mean dry weight of plants at time t_1, and $t_2 - t_1$ is the interval of time between harvest dates.

Zea mays

Seeds of a commercial variety of corn were planted on December 28, 1973, in each of 4 greenhouse flats containing vermiculite. Plants were grown in a growth chamber on a 14-hr photoperiod and 30°C (day)/15°C (night) daily thermoperiod. The light intensity during the day was approximately 21,500 lx of "cool white" fluorescent light. On January 7, 1974, the root systems of plants in 2 of the flats were flooded by placing the flats of seedlings in pans without drainage holes. The other 2 flats of seedlings were free-draining, and were watered as needed to keep them moist. On January 11, the plants were harvested and the root systems washed and separated from the shoots. The extraction procedure and determination of protein concentration and specific activity of ADH were carried out as described for *L. uniflora.*

RESULTS AND DISCUSSION

At the beginning of the flooding experiment on November 3, 1973, the mean oven-dry weight ($\pm$SE) of the *L. uniflora* seedlings was 7.9 ± 0.9 mg. One month later, the mean dry weight of flooded seedlings was 42.1 ± 2.3 mg,

and the mean dry weight of unflooded seedlings was 65.2 ± 2.8 mg. Shoots of flooded and unflooded plants weighed 33.3 ± 1.8 and 54.1 ± 2.3 mg, respectively, and roots weighed 8.8 ± 0.5 and 11.3 ± 0.6 mg, respectively. The differences between roots, shoots, and whole plants of flooded and unflooded plants were all significant at the 1% level. Although plants grew better in unflooded soil, good growth also occurred in the plants that were continuously flooded for 1 month. The average relative growth rate of flooded plants was 54 mg g^{-1} d^{-1}, and the average relative growth rate of unflooded plants was 68 mg g^{-1} d^{-1}.

Flooding caused only a small increase in ADH activity in *L. uniflora*, but a very large increase in roots of corn (Table 1 and Fig. 1). These results mean that in waterlogged soil, ethanol does not accumulate in roots of *L. uniflora*, but does accumulate in roots of corn. The lack of ethanol accumulation in *L. uniflora* is interpreted to mean that the end product of anaerobic respiration is some nontoxic compound(s), as has been found in other flood-tolerant species (McManmon and Crawford, 1971; Crawford, 1972). These compounds may include malate (Crawford and Tyler, 1969; McManmon and Crawford, 1971; Crawford, 1972), shikimate (Tyler and Crawford, 1970), and glycerol (Crawford, 1972).

Recent studies have shown that there are multiple forms (isozymes) of ADH (Marshall et al., 1973; Marshall et al., 1974), and that within species, differences in metabolic responses to flooding occur. Plants of *Veronica*

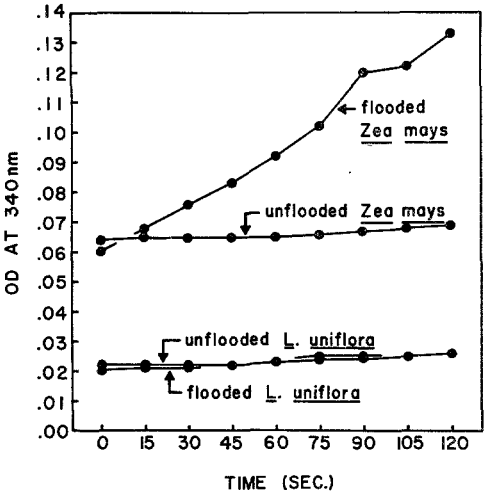

FIG. 1. Reduction of NAD to NADH by root extracts from flooded and unflooded *Zea mays* and *L. uniflora*.

TABLE 1. EFFECT OF FLOODING ON SPECIFIC ACTIVITY OF ADH EXTRACTED FROM ROOTS OF *Zea mays* AND *Leavenworthia uniflora*

Species	Ethanol converted (μmol/min per mg protein)	
	Flooded	Unflooded
Zea mays	13.0×10^{-3}	0.8×10^{-3}
L. uniflora	5.5×10^{-3}	4.2×10^{-3}

peregrina L. growing in the center of a season pool accumulated more malate than those growing at the periphery. This implies that plants at the center of the population had less malic enzyme activity, which allowed malate to accumulate, rather than being converted to pyruvate and then to ethanol (Linhart and Baker, 1973). Francis et al. (1974) compared the effect of flooding on ADH activity of several varieties of three subspecies of *Trifolium subterraneum* L. Subspecies *yanninicum* was the most flood-tolerant and had significantly lower ADH activity than subspp. *brachycalycinum* and *subterraneum*. Under flooded conditions, growth of *Zea mays* L. plants with the gene for fast ADH activity was reduced more than the growth of plants with the gene for slow ADH activity. Alcohol dehydrogenase activity was much greater in flooded plants with the fast gene, presumably because a higher amount of ethanol accumulated in these plants (Marshall et al., 1973).

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FEMALE SEX PHEROMONE OF THE GERMAN COCKROACH, *Blattella germanica* (L.) (ORTHOPTERA: BLATTELLIDAE), RESPONSIBLE FOR MALE WING-RAISING II. 29-HYDROXY-3,11-DIMETHYL-2-NONACOSANONE

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Abstract—One component of the sex pheromone responsible for wing-raising in courtship behaviors of *Blattella germanica* males was isolated from cuticular wax of the mature females and identified as 29-hydroxy-3,11-dimethyl-2-nonacosanone. This component showed the activity at a concentration as low as 3 μg/ml in carbon tetrachloride on the behavioral bioassay.

Key Words—German cockroach, *Blattella germanica*, sex pheromone, isolation, identification, 29-hydroxy-3,11-dimethyl-2-nonacosanone.

INTRODUCTION

Male adults of the German cockroach, *Blattella germanica* (L.), show a characteristic wing-raising posture in the sequential courtship behavior. The chemical factor responsible for the wing-raising is contained in the cuticular wax of sexually mature females and transmitted to males by direct contact chemoreception via the antennae (Roth and Willis, 1952; Ishii, 1972). This sex pheromone is composed mainly of two components, compounds A and B, each of which elicits independently the wing-raising response from males. Compound A was isolated and characterized as 3,11-dimethyl-2-nonacosanone (Ia) (Nishida et al., 1974, 1975). We now report the isolation and identification of compound B as 29-hydroxy-3,11-dimethyl-2-nonacosanone (Ib).

METHODS AND MATERIALS

The bioassay used in these experiments was essentially that described for the isolation of compound A (Nishida et al., 1974, 1975).

Spectral data were obtained with a Shimazu IR-400 spectrometer, Hitachi R-22 NMR spectrometer (90 MHz), Hitachi RMS-4 mass spectrometer coupled with Hitachi K-53 gas chromatograph, and JASCO ORD Model J-5 spectropolarimeter. Every gas–liquid chromatogram was run with a Yanaco GCG-550F gas chromatograph, using a stainless steel column (3 mm × 75 cm) packed with 2% OV-17 on Chromosorb W (60–80 mesh) at 220°C with a He flow rate of 25 cc/min. Column chromatography was carried out on silicic acid (Mallinckrodt Chemical Works, 100 mesh), unless otherwise mentioned.

Isolation of Compound B

Blattella germanica females were segregated from males after adult ecdysis and reared for 1–2 weeks at about 28°C until their sexual maturation. The mature females (1000–3000) in a rearing batch were anesthetized with carbon dioxide and rinsed 3 times with *n*-hexane (a total of 1 ml per insect). The combined rinse of 224,000 females was passed through a short column packed with a mixture of Celite-545 (20 g) and anhydrous sodium sulfate (70 g), and evaporated under reduced pressure to give a waxy material (67 g).

This material was fractionated by column chromatography on silica gel (500 g, Wako gel C-200, 65 × 350 mm) with the following solvent systems into 18 fractions of 1 liter each: Nos. 1 and 2, *n*-hexane; Nos. 3 and 4, *n*-hexane: benzene (2:1); Nos. 5–10, benzene; Nos. 11 and 12, benzene:ethyl acetate (30:1); Nos. 13 and 14, benzene:ethyl acetate (10:1); Nos. 15 and 16, benzene:ethyl acetate (2:1); and Nos. 17 and 18; ethyl acetate. Of these fractions, only fraction 7 was active on bioassay and gave, on isolation, compound A (m.p. 45–46°C, fine needles, 239 mg), but a preliminary study of other fractions by combination of TLC separation and bioassay revealed that fractions 15 and 16 contained the active component, which proved to correspond to the fraction B described in the previous papers (Nishida et al., 1974, 1975). Thereupon, the isolation of the active component from fractions 15 and 16 was made with the aid of bioassay as follows: First, the inactive oil (1.25 g) given after evaporation of fraction 15 was chromatographed twice (20 g and 16 g) on columns of silicic acid by eluting with *n*-hexane containing ether in stepwise-increasing mixing ratio. The first chromatography gave an active oil (220 mg) in the eluate of *n*-hexane:ether

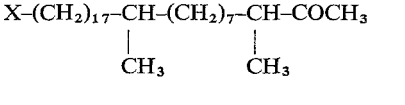
(10:1–5:1), which was subjected to the second chromatography to give an active oil (33 mg), tentatively named "No. 15A," in the eluate of *n*-hexane: ether (10:1).

Evaporation of fraction 16 gave an oil (480 mg), the ethereal solution of which was shaken successively with dilute hydrochloric acid and sodium hydroxide solution to separate the active neutral component (345 mg) from inactive acidic and basic fractions. The neutral component was chromatographed again on a silicic acid column (20 g) and fractionated into 10-ml fractions by eluting with *n*-hexane containing ether in stepwise-increasing mixing ratio. Thirty fractions eluted with *n*-hexane:ether (5:1) showed remarkable activity. Of these fractions, on evaporation, the middle several ones gave a crystalline mass (3.8 mg), the others an oil (31 mg), which were tentatively named "No. 16A" and "No. 16B" respectively.

Numbers "15A" and "16B" were combined and rechromatographed on a silicic acid column (7.5 g), using benzene containing ethyl acetate in increasing mixing ratio. The eluate of benzene:ethyl acetate (50:1) gave a highly active material (4.2 mg), which was combined with "No. 16A" and subjected to preparative TLC (20 × 20 cm, 0.75 mm thick, Silica gel GF, E. Merck) developed with a mixture of benzene:ethyl acetate (5:1). The activity was found in the band around R_f 0.4, which was scraped off and extracted with ethyl acetate to give an active crystalline mass (4.6 mg). This mass was pure enough for general spectrometric analyses and chemical conversions to the derivatives for the structural elucidation, and further purification was carried out by recrystallization from a small amount (less than 1 ml) of *n*-hexane to give compound B (1.7 mg, colorless fine needles, m.p. 42–43°C). On GLC analysis, this compound showed a single peak at R_t 17.5 min. Under the same conditions, compound A and hexatriacontane were found at R_t 5.4 and 13.2 min, respectively.

Conversions of Compound B to Its Derivatives for Structural Elucidation

Oxidation of Compound B to the Carboxylic Acid (Ic) and Its Methyl Ester (Id). To the acetone solution (0.5 ml) of compound B (~1 mg), 1 ml of the stock solution of the oxidizing reagent [a mixture of chromic anhydride (500 mg), water (1 ml), acetone (3 ml), and concentrated sulfuric acid (1 ml)] was added and left to stand overnight at room temperature. The reaction mixture was extracted with benzene under acidic condition to yield the carboxylic acid (Ic). The acid was methylated, without further purification,



Ia; X = H_3C–
Ib; X = $HOCH_2$–
Ic; X = HOOC–
Id; X = H_3COOC–

by refluxing for 2 hr in benzene (1 ml) and 5% methanolic hydrogen chloride solution (4 ml). The usual work-up gave the methyl ester (Id) as an amorphous solid: MS *m/e* 494 (M^+ for $C_{32}H_{62}O_3$), GLC R_t = 18.5 min.

29-Hydroxy-3,11-dimethylnonacosane (*IIa*). The Wolff-Kishner reduction of compound B was achieved as follows: A mixture of compound B (1.5 mg), hydrazine hydrate (0.12 ml), and potassium hydroxide (80 mg) was dissolved in diethylene glycol (0.8 ml), and was refluxed for 5 hr in an oil bath (185°C). It was then diluted with water and extracted with benzene. The extract was dried over anhydrous sodium sulfate and evaporated to give the alcohol (IIa) as a crystalline mass, m.p. 41–43°C, GLC R_t = 7.5 min. The trimethylsilyl ether of the alcohol gave MS *m/e* 524 (M^+), GLC R_t = 6.5 min.

29-Bromo-3,11-dimethylnonacosane (*IIb*). The alcohol (IIa) (1.5 mg) was added to 47% hydrobromic acid (1 ml) and concentrated sulfuric acid (0.8 ml), and was heated in an oil bath (120°C) for 24 hr with vigorous stirring. The reaction mixture was extracted with *n*-hexane, the crude product was

$$\mathrm{X\text{-}(CH_2)_{17}\text{-}\underset{\displaystyle CH_3}{\underset{|}{CH}}\text{-}(CH_2)_7\text{-}\underset{\displaystyle CH_3}{\underset{|}{CH}}\text{-}CH_2\text{-}CH_3}$$

IIa; X = $HOCH_2$–
IIb; X = $BrCH_2$–
IIc; X = CH_3–
IId; X = CH_2D–

purified through a silicic acid column (3 g), and the eluate was evaporated to yield the corresponding bromide (IIb) as an oil: GLC R_t = 9.8 min, NMR ($CDCl_3$) δ 3.40 (Br–CH_2CH_2–, triplet, J = 6.5 Hz).

3,11-Dimethylnonacosane (*IIc*). The bromide obtained as described above (~1 mg) was dissolved in tetrahydrofuran (3 ml) and reduced with lithium aluminum hydride (30 mg) by refluxing for 4 hr with stirring. To the resultant mixture, dilute hydrochloric acid was added and extracted with *n*-hexane. Evaporation of the extract gave an oil, which was purified through a silicic acid column (3 g, Wako gel C-200) to give the hydrocarbon (IIc): MS *m/e* 436 (M^+ for $C_{31}H_{64}$), GLC R_t = 2.4 min.

29-Deutero-3,11-dimethylnonacosane (*IId*). The bromide (IIb) (~1 mg) was reduced with lithium aluminum tetradeuteride (E. Merck) according to the procedure described above. The product obtained was identical with IIc, except for the mass spectrum, which was *m/e* 437 (M^+ for $C_{31}H_{63}D$).

RESULTS AND DISCUSSION

Two chemical factors responsible for male wing-raising in the sequential courtship behavior of *B. germanica* were found in the female cuticular wax. Of these two factors, compound B was contained in so small an amount that its isolation was inevitably made from a large number of females, as com-

pared with the case of compound A. At the early stage of isolation, however, there was unexpected trouble that had not been encountered in the previous work with a small number of females. It was likely due to some chemical factor(s) that accompanied compound B on the initial column chromatography and masked its activity. As described in the previous papers (Nishida et al., 1974, 1975), compound B was of more polar nature than compound A. On the column chromatography of the crude extract, however, eluates more polar than the one containing compound A did not show any activity. Further fractionation of the polar eluates by subsequent chromatography, however, revealed distinct activity, probably due to removal of the masking factor(s) from the fraction containing compound B. Finally, compound B was isolated as crystalline form melting at 42–43°C and showing a positive optical rotation $[\alpha]_D^{18} = +7.1°$ and a small positive Cotton effect $[\alpha]_{305}^{18} = +50°$ and $[\alpha]_{275}^{18} = 0°$ ($c = 0.35$ in *n*-hexane).

The molecular formula of compound B proved to be $C_{31}H_{62}O_2$, from the following data: Its mass spectrum showed the molecular ion peak at *m/e* 466 shown in Fig. 1, and absorption bands at 3635 and 1715 cm^{-1} in the IR spectrum (in carbon tetrachloride) suggested hydroxyl and carbonyl group(s), respectively. The presence of the hydroxyl group was also confirmed by formation of the monotrimethylsilylated derivative (M^+: *m/e* 538). In the NMR spectrum (in $CDCl_3$) of compound B, the signal for $-OCH_2-CH_2-$ at δ 3.56 (2H, triplet, $J = 6.5$ Hz) coupling with the adjacent methylene at δ 1.5 indicated the hydroxyl to be primary. The structural moiety of $-CH_2-CH(CH_3)-CO-CH_3$ was concluded from the 3H signal for the acetyl at δ 2.08 (singlet) and the 1H signal for the methine at δ 2.45 (sextet, $J = 6.8$ Hz) coupling with the 3H signal for the methyl at δ 1.03 (doublet, $J = 6.8$ Hz). The presence of the moiety was also supported by the mass spectrum shown in Fig. 1, in which a most abundant fragment ion peak was observed at *m/e* 72 and assigned to the radical ion $CH_3-CH=\overset{+\cdot}{C}(OH)-CH_3$ resulting from McLafferty rearrangement of compound B. In the NMR spectrum of compound B, besides these functional groups, a virtually coupled 3H signal

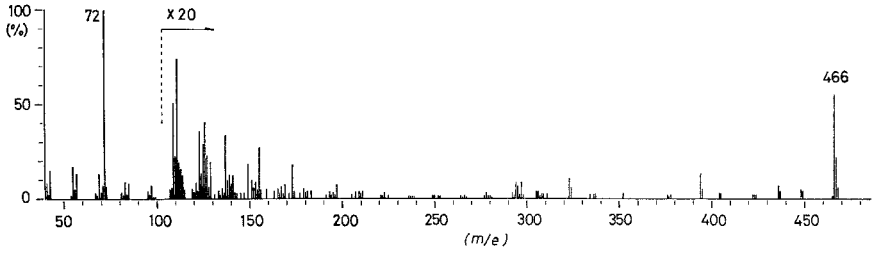


FIG. 1. Mass spectrum of compound B (direct inlet system).

for a secondary methyl at δ 0.80 (broad doublet, J = 6.0 Hz) attached to a methine in a long methylene chain (about 50 protons around δ 1.2) was observed.

These findings from the spectral data were consistent with the result of oxidation of compound B to the corresponding carboxylic acid (Ic), which was identified as its methyl ester (Id). The mass spectrum of the ester showed the molecular ion at *m/e* 494 (for $C_{32}H_{62}O_3$), as well as two McLafferty rearrangement peaks at *m/e* 72 (100%) and 74 (15%) from each terminal structural moiety, $-CH(CH_3)-CO-CH_3$ and $CH_3O-CO-CH_2-$, respectively.

The data described above give compound B the provisional structure $HO-CH_2-(CH_2)_m-CH(CH_3)-(CH_2)_n-CH(CH_3)-CO-CH_3$ [$m+n$ = 24], but any conclusive information as to the position of the methyl group in the long chain of methylenes was not obtained, even from the mass spectrum of either compound B itself or that of the methyl ester (Id). To solve this problem, compound B was subjected to reductive reactions to the corresponding ω-deutero-alkane (IId). The Wolff-Kishner reduction of compound B gave the alcohol (IIa), which was converted to its bromide (IIb), followed by treatment with lithium aluminum tetradeuteride to afford the deutero-alkane (IId). In its mass spectrum (Fig. 2b), there were two prominent peaks, at *m/e* 183 and 282, only the latter of which showed one mass shift from the corresponding ion peak at *m/e* 281 in the mass spectrum of the alkane (IIc)

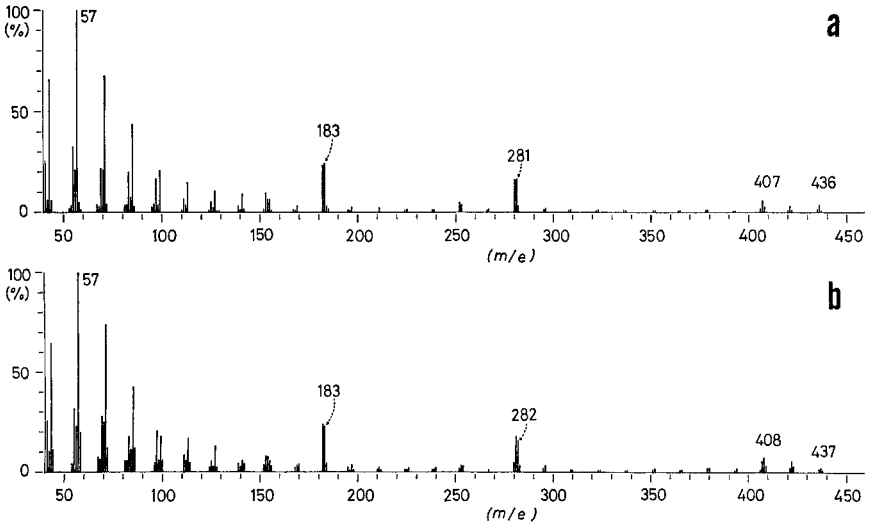
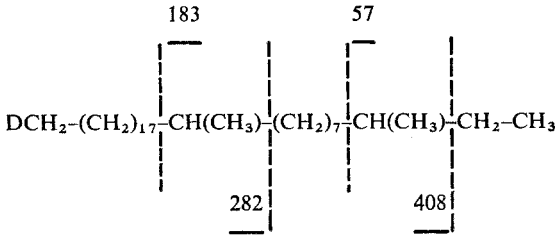


FIG. 2. Mass spectra of the hydrocarbon (IIc) (a) and the deuterated hydrocarbon (IId) (b) (GC–MS).

(see Fig. 2a). The difference between the mass spectra of alkanes IIc and IId indicated the fragmentation of the deutero-alkane (IId) as follows:



These experimental data gave unequivocal evidence that the structure of compound B is 29-hydroxy-3,11-dimethyl-2-nonacosanone (Ib), except absolute configuration on the 3- and 11-carbons. This structure was also confirmed by its synthesis, details of which will be presented in the next report in this series (Nishida et al., 1976).

Compound B was much more active than compound A, eliciting the wing-raising response from all males tested at a concentration of 3 μg/ml in carbon tetrachloride.

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PORAPAK-Q COLLECTION OF PHEROMONE COMPONENTS AND ISOLATION OF (*Z*)- AND (*E*)-14-METHYL-8-HEXADECENAL, SEX PHEROMONE COMPONENTS, FROM THE FEMALES OF FOUR SPECIES OF *Trogoderma* (COLEOPTERA: DERMESTIDAE)

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Abstract—A major sex pheromone component of each of four *Trogoderma* species was isolated by aeration of the female beetles and absorption of the volatiles on Porapak-Q. (*Z*)-14-Methyl-8-hexadecenal was identified as the major component in *T. inclusum* and *T. variabile*, and (*E*)-14-methyl-8-hexadecenal was identified in *T. glabrum*. Both (*Z*)- and (*E*)-14-methyl-8-hexadecenal were found in *T. granarium* (Khapra beetle), in the ratio 92*Z*:8*E*. In laboratory bioassays, male beetles exhibited arousal and mating responses to the aldehydes, and could discriminate between the geometric isomers. The daily production of the aldehyde was calculated for each species, and other active components were detected. These aeration–absorption studies contrast with earlier studies on macerated beetles, in which the aldehyde was not detected. The efficacy of the aeration–absorption system for collection of the sex pheromones is also described. The absorbent (Porapak-Q) efficiently collected the active pheromone; only minor amounts of activity were left in the other parts of the system.

Key Words—Coleoptera, Dermestidae, *Trogoderma granarium* (Khapra beetle), *T. variabile*, *T. glabrum*, *T inclusum*, (*Z*)- and (*E*)-14-methyl-8-hexadecenal, aeration, Porapak-Q, sex attractant

INTRODUCTION

Aeration of living insects with collection of the volatiles on Porapak-Q[4] has several advantages over the usual process of extracting glands or whole insects (Byrne et al., 1975). Our earlier studies of *Trogoderma inclusum* and *T. glabrum* were done on extracts of whole insects (Rodin et al., 1969; Yarger et al., 1975), and in both cases, the most important component of the sex attractant was missed, even though these studies apparently met the criteria (Silverstein, 1970) for a "rigorous" isolation and identification study. Furthermore, a mixture of methyl oleate, ethyl oleate, ethyl linoleate, ethyl stearate, and ethyl palmitate, recovered from homogenized *T. granarium*, was claimed to be the "assembling scent" of this species (Ikan *et al.*, 1969; Yinon et al., 1971); however, it seemed to be a rather nonspecific attractant (Levinson, 1975). It seems likely in these insects, and in many others, that one or more of the components of a pheromone may be produced either intermittently or continuously but slowly, and thus may be present only in very small amounts at any one time. Hill et al. (1975) identified a pheromone component collected by aeration that was present in only minute amounts in the gland extract of *Argyrotaenia citrana*. Pearce et al. (1975) and Peacock et al. (1975) reported that frass extracts from the female elm bark beetle, *Scolytus multistriatus*, were not active in field bioassays, although the volatile material collected by aeration of beetles boring in logs was active and furnished the three components of the aggregation pheromone.

We now report that aeration of female *Trogoderma* beetles of four species, *T. inclusum* LeConte, *T. glabrum* (Herbst), *T. variabile* Ballion, and *T. granarium* Everts (Khapra beetle),[5] with collection of the volatile material on Porapak-Q, led in each case to the discovery of a highly active pheromone component, (*Z*)- or (*E*)-14-methyl-8-hexadecenal, which was missed in earlier studies. We determined the distribution of the volatiles of *T. granarium* by monitoring (GLC) extracts of the Porapak-Q, of the aerated insects, and of the filter paper on which the insects resided during the aeration.

[4] Mention of a proprietary product does not imply endorsement by the USDA.

[5] Rearing, aeration, and bioassay of *T. granarium* were carried out exclusively in Seewiesen, Federal Republic of Germany; *T. granarium* cannot be reared in the United States because of quarantine restrictions.

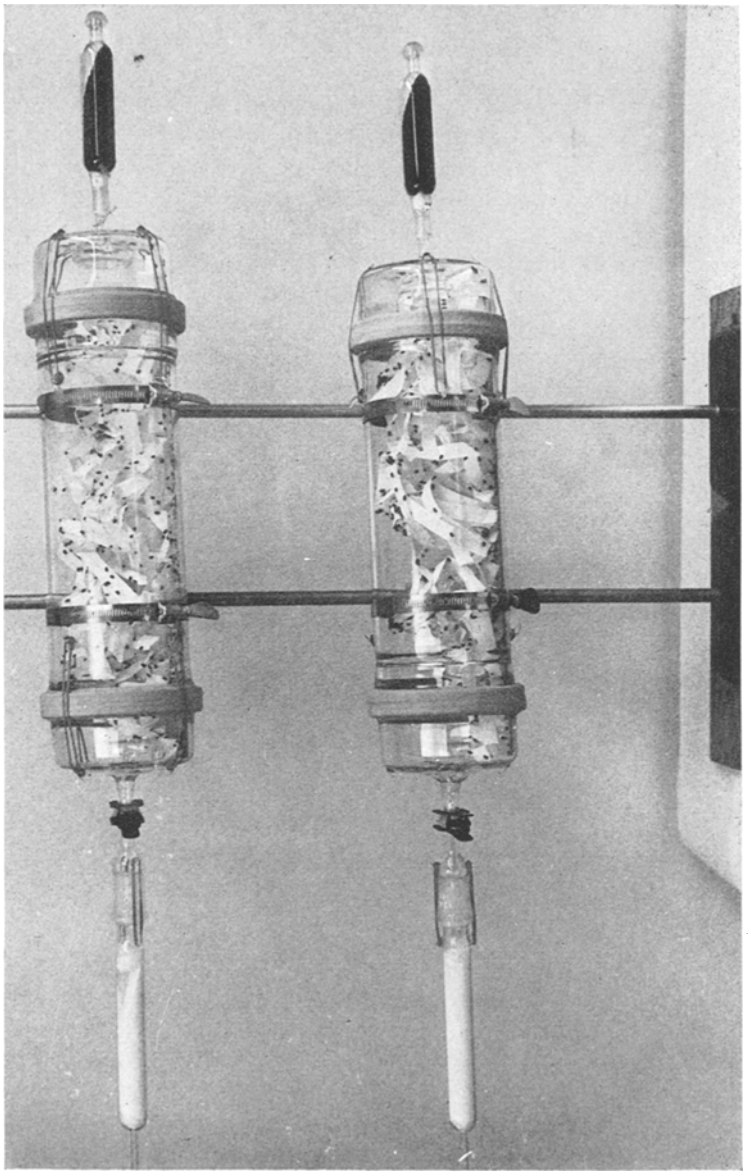


FIG. 1. Apparatus used to aerate female *Trogoderma* beetles and to collect pheromone components on Porapak-Q. Air flow is from charcoal tube (black) to Porapak-Q tube (white).

METHODS AND MATERIALS

Collection of Attractant Samples

Unmated female beetles of the four *Trogoderma* species (approximately 2000/batch) were maintained on strips of filter paper within glass chambers (Fig. 1) while air was drawn over them by vacuum applied downstream of the Porapak holder. The air was filtered through charcoal (and, for the Khapra beetles, dried over calcium chloride) upstream of the aeration chamber. Porapak-Q (ethylvinylbenzene–divinylbenzene copolymer, 60/80 mesh, Waters Associates, Inc., Framingham, Massachusetts, approximately 16 g/batch of beetles) was conditioned by extraction with redistilled hexane for 24 hr in a Soxhlet extractor prior to use. Preliminary studies (see the Appendix, page 466) demonstrated an effect of air flow on collection. *Trogoderma granarium* females were aerated at 3 liters/min for 28–30 days; the other three species were aerated at 2 liters/min for 14 days. During aeration, all beetles were kept at 20–28°C on a 16:8 light:dark photocycle. After aeration, the Porapak and filter paper samples were extracted into redistilled hexane with a Soxhlet extractor (300-ml capacity) for 24 hr. The female *T. granarium* were macerated with a Waring blender once with a mixture of acetone and hexane (1:1) and 4 times with hexane alone. The resulting macerates were filtered free of solid matter. The extracts from the Porapak, the filter paper, and the female beetles were concentrated by distillation through a 20-cm glass bead–packed column, and either chromatographed directly (as described below) or diluted to known volumes for bioassay.

Isolation of (Z)- and (E)-14-Methyl-8-hexadecenal

The porapak extracts from three species were chromatographed without further purification on column A, 10% Carbowax 20M on Chromosorb W 60/80 mesh, 2.4 m × 6 mm OD glass, 60 ml/min He flow rate, 125°C initial temperature for 6 min, then temperature-programmed at 4°/min to 200°C and held there for 50 min. Under these conditions, methyl palmitate had a retention time of 40 min. The peak with the retention time (42 min) of synthetic (*Z*)- and (*E*)-14-methyl-8-hexadecenal was collected and rechromatographed on column B, 5% SE-30 on Chromosorb G 60/80 mesh, 5 m × 6 mm OD glass, 60 ml/min He flow rate at 190°C (retention time, 73 min; retention time of methyl pentadecanoate, 64 min). The geometric configuration of the natural aldehyde was determined by coinjection of the natural compound and one of the geometric isomers of the synthetic aldehyde on column C, 15% SP-2340 on Chromosorb P, 60/80 mesh, 2.5 m × 6 mm OD glass, 50 ml/min He flow rate at 185°C (retention times: *E* aldehyde, 22.3 min; *Z*, 23.7 min).

The Porapak extract from *T. granarium* was chromatographed on

column D, 6% Carbowax 20M on Chromosorb G 60/80 mesh, 5 m × 6 mm OD glass, 75 ml/min He flow rate, 123°C initial temperature for 12 min, then temperature-programmed at 2°/min to 220°C and held for 60 min (retention time: hexanoic acid, 53 min; *Z* and *E* aldehydes, 84 min). The geometric configuration of the Khapra beetle aldehyde was determined by coinjection of the isolated compound with synthetic *Z* aldehyde on column E, 15% SP-2340 on Chromosorb P, 60/80 mesh, 3.7 m × 6 mm OD glass, 45 ml/min He flow rate at 185°C initial column temperature for 24 min, then programmed at 10°/min to 220°C and held there (retention time: *E* aldehyde, 28 min; *Z* 29 min). All columns and solid supports were acid-washed and treated with dichlorodimethylsilane. The fractions from the Varian Model 204B gas chromatographs 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 spectrophotometer equipped with beam condensers and Barnes Engineering 4 μl cavity cells. Mass spectra were obtained on a Hitachi RMU-6 low-resolution electron impact (70 eV) mass spectrometer, a modification of which allowed introduction of the glass capillaries used with the gas chromatograph, or on a Finnegan model 3300 quadruple mass spectrometer with a gas chromatographic inlet. Microozonolyses were performed in hexane at −65°C, and the ozonide was decomposed with triphenylphosphine (Beroza and Bierl, 1968).

By comparing the area under the aldehyde peak on column A with that of a known quantity of the synthetic aldehyde, we could estimate the quantity of aldehyde produced. Synthetic aldehyde was prepared by oxidation of (*Z*)- and (*E*)-14-methyl-8-hexadecen-1-ol (Rodin et al., 1969; obtained commercially from Farchan Division, Storey Chemical Co.) with Collins' reagent (Ratcliffe and Rodehorst, 1970). Pure (*Z*)-14-methyl-8-hexadecenal was also prepared for the determination of the geometric configuration of the natural aldehyde.

Bioassay samples were prepared by collection of Porapak extract from column A. We could collect either all the peaks eluting within 75 min ("Porapak extract," column in Table 1), only the aldehyde ("Aldehyde"), or all the peaks except the aldehyde ("Porapak extract minus aldehyde"). Samples were diluted in redistilled hexane for quantitative bioassay. The method of Greenblatt et al. (1976) was used for *T. glabrum* and *T. variabile*, that of Vick et al. (1970) was used for *T. inclusum*, and that of Levinson and Bar Ilan (1970) was used for *T. granarium*. The activity of the *T. granarium* samples was determined by establishing dosage–response curves at levels ranging from 10^{-7} to 1 female equivalent.

RESULTS AND DISCUSSION

We determined the distribution of the pheromone by measuring the minimum amounts of the Porapak, filter paper, and beetle extracts needed in the bioassay to elicit a threshold (25%) response from *T. granarium*. The relative activity of each sample was then obtained as the inverse of the 50% response amount. The amounts in female equivalents were: Porapak, 1×10^{-5}; filter paper, 1×10^{-2}; macerated females, 1×10^{-1}. The corresponding relative activities are: Porapak, 10,000; filter paper, 10; macerated females, 1. The degree of attraction of the three sources after 28 days of aeration for male Khapra beetles (3–6 days after pupal–adult ecdysis) is shown in Fig. 2. The greatest amount of bioactivity was detected on the Porapak; only minor

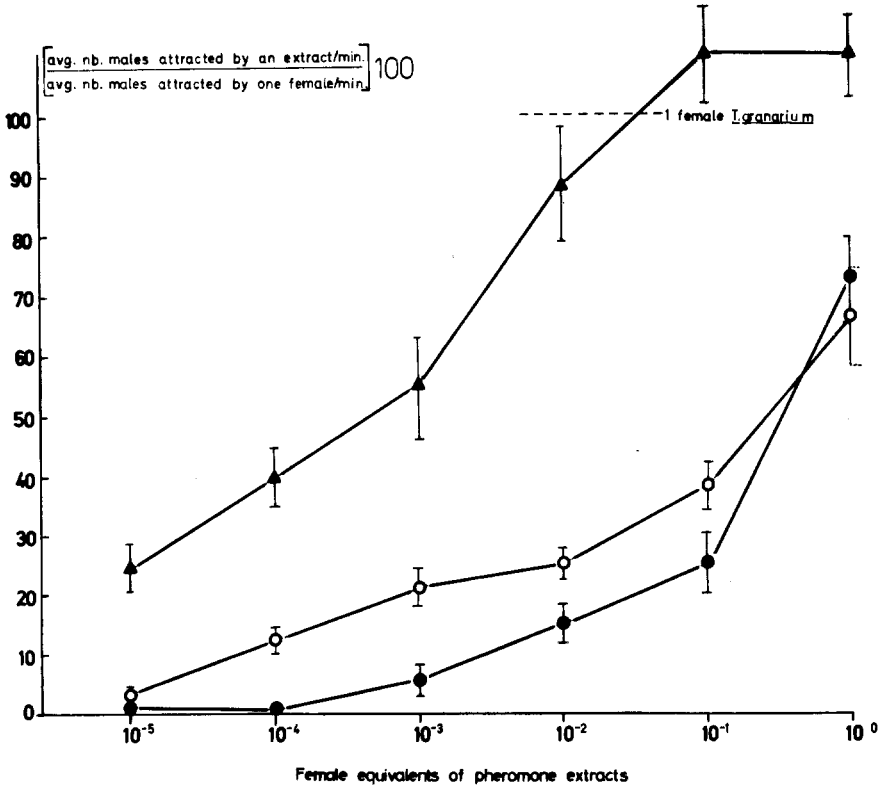
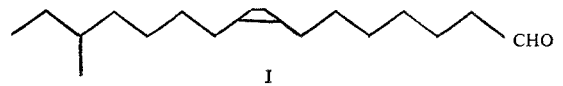


FIG. 2. Quantitative distribution of *T. granarium* pheromone among Porapak-Q, filter paper, and female beetles following 28 days of aeration. ▲——▲ Porapak-Q, ○——○ filter paper, ●——● macerated female beetles. Each point represents 6 or 7 repetitions.

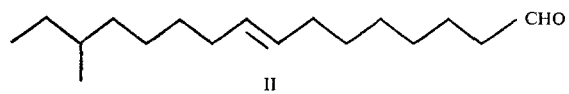
bioactivity was detected in the females themselves and in the filter paper on which they resided during the aeration.

The behavior pattern shown by male Khapra beetles to the scent emanating from unmated female beetles (Levinson and Levinson, 1973) is the same as that shown to the Porapak extract. This identical behavior suggests that all of the important pheromone components of this species are trapped by the Porapak during the aeration; this result is in agreement with data obtained from the collection of model pheromone compounds by Byrne et al. (1975).

Bioassays of the GLC fractions of the Porpak extract from aeration of *T. variabile* lead to the isolation of an extremely active component, which was identified as (*Z*)-14-methyl-8-hexadecenal (I) by NMR, IR, and mass spectrometry and microozonolysis. The spectra, ozonolysis results, and GLC retention times were congruent with those of an authentic sample. This *Z* aldehyde was also found to be the most important component in the Porapak extract of *T. inclusum*. The corresponding *E* aldehyde (II) was found to be the most important component in the Porapak extract from *T. glabrum*. Both isomers of this aldehyde (92*Z*:8*E*) were identified in the *T. granarium* Porapak extract.



I



II

The NMR, IR, and mass spectra obtained from (*Z*)-14-methyl-8-hexadecenal (I) isolated from *T. variabile* are shown in Figs. 3 and 4. The aldehyde group was detected by the IR absorptions at 1720 cm^{-1}, 2720 cm^{-1}, and 2870 cm^{-1}, and by the NMR absorption at 9.62 δ (1H, t, $J = 2$ Hz). The NMR absorption at 5.36 δ (2H, m) and IR absorption at 3010 cm^{-1} are due to a double bond, which is *Z*, since no sharp band is present at 975 cm^{-1}. The mass spectrum showed a molecular ion at *m/e* 252. Other information was provided by NMR absorption at 2.42 δ (2H, doublet, $J = 2$ Hz, of triplets, $J = 7$ Hz, CH_2–C(=O)–H), 2.02 δ (4H, broad m, CH_2–C=C). The position of the double bond and the presence of a methyl branch at C_{14} were shown by microozonolysis to 1,8-octanedial and 6-methyloctanal, which were identified by GLC matching with authentic samples. Chromatography on column C proved that only the *Z* isomer was present. The *E* aldehyde was identified by coinjection with the authentic aldehyde on column C, and by congruence of mass spectra.

The biological activities of the aldehyde relative to the total Porapak

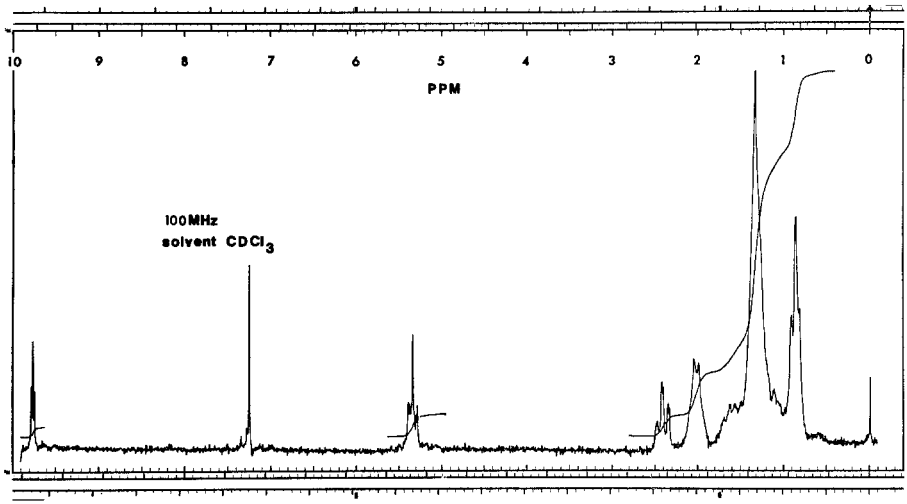


FIG. 3. Nuclear magnetic resonance spectrum of (*Z*)-14-methyl-8-hexadecenal from *T. variabile* Ballion.

extract and the Porapak extract minus the aldehyde are given in Table 1.[6] In adequate amounts, the aldehyde elicits arousal in males of the four *Trogoderma* species, and mating responses in males of *T. glabrum* (Greenblatt et al., 1976) as well as *T. granarium* (Levinson et al., unpublished). The *Z* aldehyde, found in *T. variabile* (cf. Cross et al., submitted for publication), is a far more effective pheromone for *T. variabile* males than the *E* isomer. The relatively small difference (10×) in biological activity between the *Z* aldehyde and the *E* aldehyde for male *T. granarium* is not surprising, since the females of this species produce both isomers. The ability of these dermestids to distinguish geometrical isomers is in accord with the findings on other insects of other investigators (e.g., Kaissling and Preisner, 1970).

Greatly decreased but distinct biological activity remains in the Porapak extract after the aldehyde is removed. The roles of the other active components have been partially elucidated for *T. glabrum* (Greenblatt et al., 1976). These compounds may increase the species specificity of the pheromone, or they may be involved in mediating other types of behavior.

[6] The 95% confidence intervals of these activities were calculated for *T. glabrum*, *T. inclusum*, and *T. variabile*. They indicated agreement between the biological activities of the aldehyde and the Porapak extract, and a significant difference between the activities of the aldehyde and the Porapak extract minus the aldehyde.

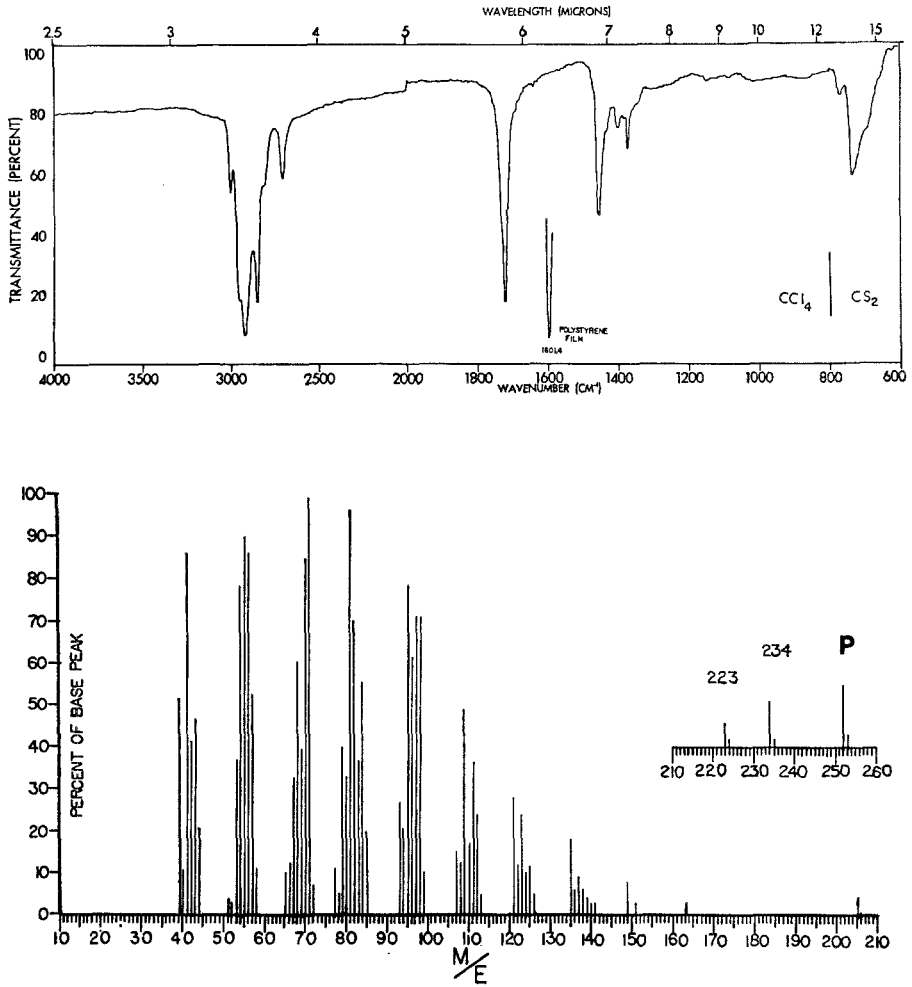


FIG. 4. Infrared and mass spectra of (*Z*)-14-methyl-8-hexadecenal from *T. variabile* Ballion.

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TABLE 1. BIOLOGICAL ACTIVITY OF THE PORAPAK EXTRACT, THE ALDEHYDES ISOLATED FROM THE PORAPAK EXTRACT, AND THE SYNTHETIC ALDEHYDES, (*Z*)- AND (*E*)-14-METHYL-8-HEXADECENAL

Species	Amounts of 14-methyl-8-hexadecenal isolated (ng/beetle day) (isomer)	Biological activity: Isolated material (beetle days)			Biological activity: Synthetic 14-methyl-8-hexadecenal (μg)	
		Porapak extract	aldehyde	Porapak extract minus aldehyde	*Z*	*E*
T. glabrum[a]	28.3 (*E*)	10^{-3}	3×10^{-4}	9×10^{-2}	2×10^{-4}	2×10^{-6}
T. inclusum[a]	1.65, 2.35 (*Z*)	6×10^{-3}	2×10^{-2}	1	4×10^{-6}	2×10^{-4}
T. variabile[a]	35.66 (*Z*)	8×10^{-5}	2×10^{-5}	8×10^{-3}	5×10^{-8}	4×10^{-3}
T. granarium[b]	1.64, 2.34 (92*Z*:8*E*)	5×10^{-3}	10^{-2}	5×10^{-1}	10^{-3}	10^{-2}

[a] Bioassay method of Greenblatt et al. (1976), 50% arousal response.
[b] Bioassay method of Levinson and Bar Ilan (1970), 50% attraction.

John Gorman, Department of Entomology, University of Wisconsin, for rearing *T. glabrum*, *T. inclusum*, and *T. variabile*; Dr. T. E. Czeschlik, Max-Planck Institut, for help with *T. granarium*; Mrs. Hazel Jennison and Mr. Larry McCandless, C.E.S.&F., for their help in obtaining mass and NMR spectra; and Dr. J. D. Henion of the Mass Spectroscopy Facility, Cornell University, for obtaining GLC/MS spectra. The Varian XL-100 NMR spectrometer was obtained through a grant from the National Science Foundation.

APPENDIX

Porapak Flow Rate Study

Preliminary data from the collection of the attractant showed a dependence of the activity of the Porapak extract on the rate of air flow through the aeration chamber. The air flow rate was consequently adjusted to yield the highest activity. Bioassays of crude extracts of the female beetles, the filter paper, and the absorbent showed the highest relative activity in the Porapak extract at an aeration rate of 3 liters/min (see Table 2).

The absorbent extract was most attractive at an aeration rate of 3 liters/min. The female beetles called normally in air flows up to 3 liters/min. The number of calling insects decreased, however, at the 5 liters/min flow rate. The aeration rate of 2–3 liters/min was thus used for subsequent studies with the aeration–absorption system.

TABLE 2. PORAPAK FLOW—RATE STUDY

(Conditions: 27°C, 70% RH, 14 hr light/10 hr dark, aeration time 24 hr)

Air flow rate (liters/min)	Percentage response to ♀ equivalents		
	10^{-3} ♀ eq.	10^{-2} ♀ eq.	10^{-1} ♀ eq.
0.5	0	100	100
1.0	40	—	100
3.0	100	—	100
5.0	0	0	100

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ALLELOPATHIC EFFECTS OF FESCUE ON THE GROWTH OF SWEETGUM

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Abstract—The height growth of field-planted sweetgum (*Liquidambar styraciflua* L.) seedlings was reduced where residual fescue (*Festuca arundinacea* Shreb. Var. Ky. 31) from previous agricultural experimentation was present. Interference (competition + allelopathy) effects of fescue on the growth of sweetgum were tested in the greenhouse. Fescue seeded into pots containing sweetgum seedlings resulted in dry weight reduction of sweetgum from 29 to 95%. Elimination of the competitive effect through the use of a stairstep apparatus implicated an allelopathic mechanism in the interference of sweetgum growth by fescue. Leachates from the rhizosphere of live fescue, dead fescue roots, and dead fescue leaves resulted in reduction up to 60% in dry matter production of sweetgum seedlings. Chemical analysis of sweetgum seedlings from the stairstep experiment suggested impaired adsorption of phosphorus and nitrogen by seedlings treated with fescue leachates.

Key Words—allelopathy, competition, fescue, sweetgum growth, plant leachates, mineral adsorption.

INTRODUCTION

The height growth of 10-year-old sweetgum (*Liquidambar styraciflua* L.) growing in plots containing fescue (*Festuca arundinacea* Shreb. Var. Ky. 31) was found to be less than that of trees in adjacent plots that were devoid of fescue. Results from chemical and physical soil analysis did not account for differences in height of seedlings grown in fescue and nonfescue plots. While sweetgum growth was correlated with residual phosphorus and magnesium, this correlation was achieved across all experimental plots without respect to the presence or absence of fescue. These results suggested that the

presence of fescue resulted in an interference reaction on sweetgum over and above the response of sweetgum growth to environmental variation.

For the purposes of this study, interference is defined in the sense of Rice (1974) as the combined deleterious effects of competition and allelopathy of one plant on the growth of another. Competition represents that aspect of interference that occurs when one or more growth factors are removed or reduced by one plant from the environment of another plant sharing the same habitat. Allelopathy is defined as any direct or indirect harmful effect by one plant on another through the production of chemical compounds released from it into the environment.

Inhibitors may be present in any part of the plant, although leaves and roots are the most consistent source, since they contain large amounts and a diversity of plant metabolites (Al-Naib and Rice, 1971; Rovira, 1969; Tukey, 1971). Plant metabolites are released in a variety of ways. Water-soluble phytotoxins may be leached from roots or aboveground plant parts (Bell and Koeppe, 1972) or actively exuded from living roots on environmental stimulus (Bell and Koeppe, 1972; Vancura, 1964; Wilson and Rice, 1968). Leachates from decaying residues of several plants have been shown to have phytotoxic effects on the growth of associated species (Bell and Koeppe, 1972; McCalla, 1971; Norstadt and McCalla, 1968).

Several studies have shown allelopathic inhibition of woody species by herbaceous and woody understory (Brown, 1967; Jameson, 1968; Peterson, 1965). Specific accounts of interference by fescue have also been documented. For example, Peters (1968) tested extracts of fescue leaves and roots in sand cultures, and found that these extracts significantly reduced both root and shoot growth of rape (*Brassica nigra*) and birdsfoot trefoil (*Lotus corniculatus*). Rietveld (1975) found that extracts of green foliage of Arizona fescue reduced germination of ponderosa pine seeds, and retarded the speed of elongation and mean radicle length.

The results of a study to determine the nature of interference by fescue on the growth and mineral nutrition of sweetgum seedlings are reported in this paper.

METHODS AND MATERIALS

Single-Pot Experiment

A single-pot, mixed-culture experiment was conducted in the greenhouse to determine whether interference of fescue was operative on the growth of sweetgum.

Fescue seeds were placed in 12 19-cm-diameter pots containing pure washed quartz sand, and allowed to grow for 2 months. At the end of this

period, 8 sweetgum seeds of southern Illinois origin were planted radially around each pot containing the fescue and in 36 additional pots containing quartz sand. Of these 36 pots, 12 were seeded with fescue at the time sweetgum was planted, 12 were seeded with fescue 2 months after sweetgum was planted, and the remaining 12 pots were not planted with fescue (controls).

All pots were watered daily with half-strength Hoagland's solution and received a 16-hr photoperiod using fluorescent Gro-lux lamps. The experiment was terminated at the end of 4 months, and differences in total height and oven-dry weight of leaves, stems, and roots were statistically analyzed using Student's *t* test.

Stairstep Experiment

A stairstep apparatus (Fig. 1) was constructed in the greenhouse to separate the effects of competition from those of allelopathy (Bell and Koeppe, 1972; Wilson and Rice, 1968). Full-strength Hoagland's solution was supplied to the uppermost series of pots from the supply reservoir. The solution then filtered through the quartz sand growing medium into a funnel and into the next series of pots below it. After the solution had filtered through the test pots and into the collection reservoir, it was pumped to the supply reservoir to begin the cycle again.

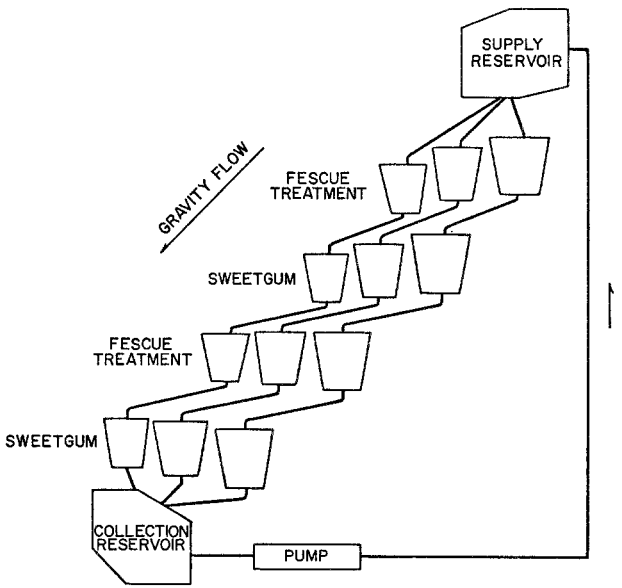

FIG. 1. Schematic diagram of one treatment line of the stairstep apparatus.

Each treatment line of the stairstep consisted of 3 pots per step. The control series contained 3 sweetgum seedlings per pot in each pot in the series. Test series contained alternating fescue treatments and sweetgum seedlings. The stairstep was designed to contain a total of 4 treatment lines.

Sweetgum seedlings of southern Illinois origin were grown in the greenhouse from seed for 1 month, at which time seedlings of uniform vigor and height were chosen and transplanted, 3 per pot, into the stairstep pots.

Treatments consisted of leachates from the rhizosphere of (1) live mature fescue, (2) air-dried fescue leaves, and (3) air-dried fescue roots. Roots and leaves were cut up and incorporated into the sand of the stairstep pots at the rate of 30 g air-dry tissue/pot. The quantity of tissue was determined from the amount contained in a 19-cm-diameter plug from the field, which corresponded to the diameter of the pot used in the study. All plants received a 16-hr photoperiod using fluorescent Gro-lux lamps and ultraviolet sun lamps. Leachate solutions were cycled for 4 hr/day and monitored for pH to ensure uniform growing conditions. Hoagland's solution was added every 2 to 3 days to compensate for evaporational losses.

At the end of 30 days, the seedlings were harvested and plant height and dry weight of leaves, stems, and roots determined. Leaf and root samples were composited by block and treatment to provide an adequate amount of tissue for chemical analysis.

The bottom row of seedlings received a lower light intensity than the upper row; therefore, the heights, dry weight, and nutrient concentrations of the seedlings were analyzed as a randomized complete block, with treatments divided into two blocks based on light intensity. The initial height of seedlings was used as a covariate to adjust the final values for any difference that might have existed in seedling vigor at the beginning of the experiment.

Sweetgum leaf and root tissues were dried at 65°C and then ground. Leaf and root tissues were analyzed for nitrogen by the semimicro Kjeldahl method (Bremner, 1965). Separate samples of leaf and root tissues were ashed at 450°C, and the ash was dissolved in 3 N HCl. Potassium, calcium, and magnesium were determined in this ash solution by atomic absorption, and phosphorus by the molybdate method using ascorbic acid (Murphy and Riley, 1962). Nutrient contents were statistically analyzed at the 95% confidence level using Duncan's multiple range test.

RESULTS

Single-Pot Experiment

The effect of fescue on the germination and survival of sweetgum seedlings is shown in Table 1. Sweetgum seeded into mature fescue exhibited

TABLE 1. INTERFERENCE EFFECTS OF FESCUE ON GERMINATION AND SURVIVAL OF SWEETGUM SEEDLINGS

Treatment[a]	Germination (%)	Survival (%)
A	75	100
B	67[b]	50[b]
C	52[b]	0[b]
Control	82	100

[a] Fescue seeded in association with sweetgum 2 months after sweetgum seedlings were established; B: fescue seeded simultaneously and in association with sweetgum for 4 months; C: Sweetgum seeded into mature fescue.
[b] Significantly different from the control at the 95% confidence level.

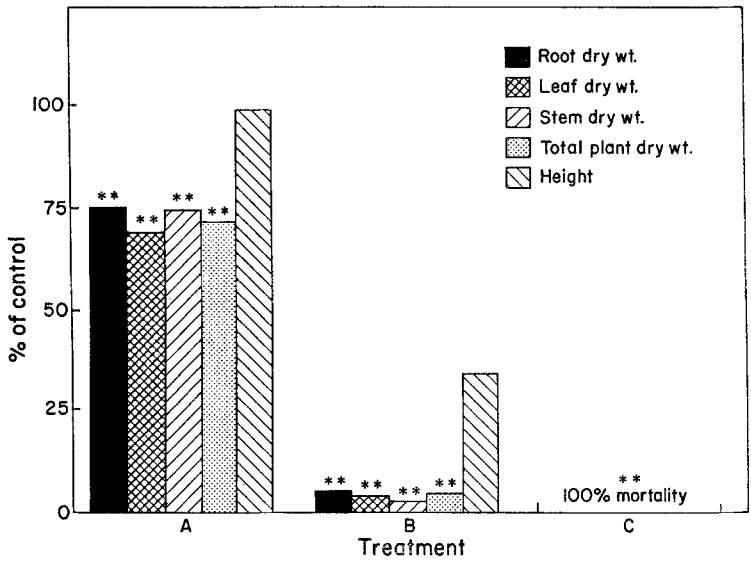

FIG. 2. Interference effects of fescue on the dry weight of sweetgum. Treatments are fescue seeded in association with sweetgum for 2 months (A) and 4 months (B), and sweetgum seeded into mature fescue (C). **Indicates significant difference from the control at the 99% confidence level.

low germination percentage, and all seedlings died 1 week after germination. Sweetgum seeded simultaneously and grown for 4 months with fescue exhibited significantly higher germination and survival than those seedlings in mature fescue. Seedlings that had been established for 2 months before fescue was seeded into the pots had the highest survival percentages of all treatments.

The dry weight of seedlings decreased with increasing time of association with fescue (Fig. 2). Root, leaf, and stem dry weights were significantly different from the controls, for those seedlings growing with fescue for 2 and 4 months, with dry weight reductions of 28% and 95%, respectively. The height of seedlings growing with fescue for 2 months was equivalent to that of the controls. During the course of the experiment, the fescue exhibited no ill effects.

The results of the single-pot experiments indicate that interference effects are operative in the greenhouse, but the experiment fails to separate allelopathic from competition effects.

Allelopathic Effects of Fescue

The allelopathic effects of fescue on the dry weight and height of sweetgum seedlings are shown in Fig. 3. Treatments consisting of leachates from live fescue roots, dead fescue roots, and dead fescue leaves resulted in reductions of 19%, 48%, and 60%, respectively, in the total dry weight of the seedlings.

Seedlings grown in contact with leachates from dead fescue roots and leaves exhibited the greatest reduction in growth and leaf dry weight production of the sweetgum seedlings. Twelve days after the start of the experiment, leaves of the seedlings associated with the dead root and leaf treatments became chlorotic.

There was no significant difference in the root weight, stem weight, or height of seedlings grown in contact with leachate from the rhizosphere of mature fescue. The leaf weight of live fescue root–treated seedlings, however, decreased significantly, and became the contributing factor in reducing the total weight of seedlings.

Covariance analysis indicated no significant difference in the initial height of the seedlings used on the stairstep. Therefore, no adjustment of the final data was necessary.

The nitrogen concentrations in sweetgum leaves did not differ significantly between treatments when expressed as milligrams per gram dry weight (Table 2). But when nitrogen was expressed on the basis of milligrams per plant top, the means of seedlings treated with dead fescue roots and leaves were 1/3 as large as the control. The phosphorus concentrations in

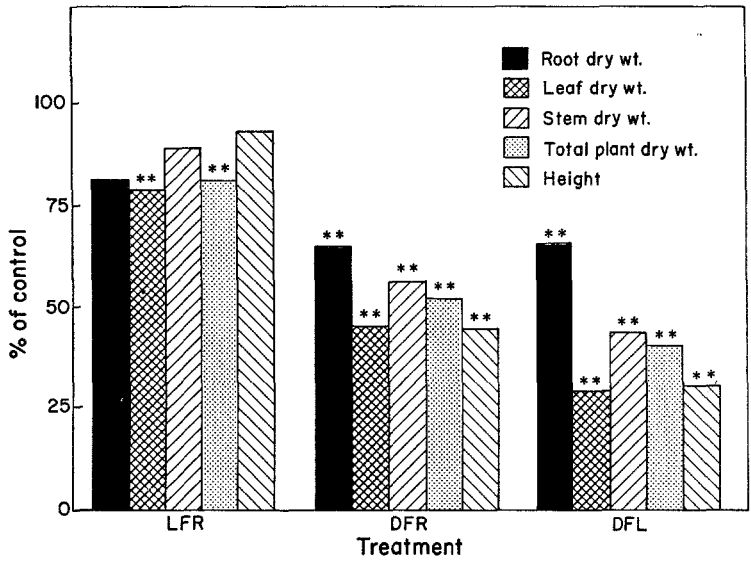

FIG. 3. Allelopathic effects of fescue on the dry weight of sweetgum. Treatments are 1-month-old sweetgum grown in contact with materials leached from the rhizosphere of live mature fescue roots (LFR), dead fescue roots (DFR), and dead fescue leaves (DFL). ** Indicates significant difference from the control at the 99% confidence level.

leaves were markedly reduced in seedlings treated with leachates from dead fescue leaves and roots. There were no significant differences from the control in the phosphorus concentrations of leaves subject to leachates from live fescue roots, although roots of seedlings in this treatment had phosphorus concentrations $\frac{1}{2}$ of the control, and were significant at the 95% confidence level (Tables 2 and 3).

The potassium concentrations in leaves of seedlings treated with dead fescue roots and leaves showed no differences, but both groups of treated seedlings had significantly higher concentrations than the control or live fescue root–treated seedlings when compared on a dry weight basis. But when compared as total uptake in seedling tops, only those seedlings treated with dead fescue leaves were significantly different from the control (Table 2). The root concentrations of potassium were not different from the control in seedlings treated with live and dead fescue root leachates (Table 3). The only significant difference in root potassium concentrations was between live fescue root and dead fescue root treatments.

The magnesium concentrations in the roots were not significantly different. All leaf concentrations of magnesium in fescue-treated seedlings

TABLE 2. LEAF NUTRIENT CONTENTS OF SWEETGUM SEEDLINGS TREATED WITH LEACHATES FROM FESCUE

Treatment[a]	N	P	K	Mg	Ca
	(mg/g dry weight)[b]				
Control	20.40a	2.09a	30.46a	2.09a	6.42a
LFR	20.83a	1.89ab	37.75b	3.34b	7.32ab
DFR	15.55a	1.29b	52.08c	4.54c	8.37b
DFL	17.71a	1.28b	51.91c	4.62c	8.07b
	(mg/plant top)[b]				
Control	13.63a	1.41a	20.31a	1.98a	4.27a
LFR	11.11a	1.01ab	20.05a	1.76a	3.86ab
DFR	4.67b	0.39bc	15.64ab	1.36ab	2.52bc
DFL	3.38b	0.25c	9.95b	0.88b	1.54c

[a] LFR, DFR, DFL: Leachates of live fescue roots, dead fescue roots, and dead fescue leaves, respectively.
[b] Any two means in the same column not followed by the same letter are significantly different from each other at the 95% confidence level.

TABLE 3. ROOT NUTRIENT CONTENTS OF SWEETGUM SEEDLINGS TREATED WITH LEACHATES FROM FESCUE

Treatment[a]	N	P	K	Mg	Ca
	(mg/g dry weight)[b]				
Control	11.57a	6.31a	24.93ab	3.70a	13.18a
LFR	12.64a	3.58b	29.43a	4.87a	12.23a
DFR	9.15a	1.51c	24.37ab	5.37a	9.01b
DFL	12.30a	1.70c	16.90b	5.18a	10.44ab
	(mg/plant roots)[b]				
Control	3.20a	1.73	6.73a	1.11a	3.61a
LFR	2.85a	0.80a	6.67a	1.02a	2.77ab
DFR	2.18a	0.31a	4.30a	0.95a	1.85ab
DFL	1.62a	0.27a	3.04a	0.92a	1.60b

[a] LFR, DFR, DFL: Leachates of live fescue roots, dead fescue roots, and dead fescue leaves, respectively.
[b] Any two means in the same column not followed by the same letter are significantly different from each other at the 95% level of probability.

were higher than the control. Those seedlings treated with dead fescue leaf and dead fescue root leachates had magnesium concentrations exceeding the control by a factor of approximately 1.5.

The calcium concentrations in the leaves were not significantly different among the fescue-treated seedlings. Only dead fescue root– and dead fescue leaf–treated seedlings had calcium concentrations different from the control at the 95% confidence level. The calcium concentrations in the roots differed only slightly.

DISCUSSION

The results of the single-pot experiments indicate that interference effects are operative in the greenhouse. The mortality of sweetgum seeded into mature fescue and the reduced growth observed in seedlings associated with fescue for 4 months can be attributed to extreme root competition and low light intensity caused by overtopping of seedlings by fescue. Those seedlings associated with fescue for 2 months had well-established root systems and had reached sufficient height so that competition for light was negligible. Although nutrient solution was applied daily, differential uptake of nutrients may have caused the decrease in the growth of seedlings associated with fescue. Allelopathic effects may also have been present.

Through the use of the stairstep apparatus, the competitive aspect of interference was eliminated, and an allelopathic mechanism has been implicated in the interference of sweetgum growth by fescue.

Allelopathic inhibition of sweetgum biomass by live fescue was less severe than inhibition associated with interference effects of live fescue in the single-pot experiment. Through the use of the stair-step apparatus, the effect of allelopathic interaction was tested in the absence of fescue competition. Sweetgum biomass was significantly reduced, although the degree of reduction was not so great as in the single-pot experiments (19–69% reduction, as compared with 28–95% reduction). This finding suggests that while competetive pressure may result in some biomass reduction, the predominant mechanism in fescue interference is allelopathy.

Dead fescue leaf and root tissue leachate treatments of the stairstep exhibited the most severe effects on sweetgum growth. Apparently the allelopathic substance was released at much higher concentrations when the fescue tissue was broken down. This same effect has been shown with leachates of foxtail (*Setaria faberii*) on corn (*Zea mays*) (Bell and Koeppe, 1972). The increased phytotoxicity of dead fescue leachates may have been due to the breakdown of membranes and subsequent release of higher concentrations of toxins as the tissue decomposed (Rice, 1974; Rietveld, 1975). There is a possibility that by-products from microbial decomposition of fescue tissue

are responsible for the toxic effects exhibited on sweetgum. Many higher plants have shown the ability to alter soil microorganisms and exudates through the production of phytotoxins (Rice, 1968).

Nutrient analyses of sweetgum used in the stairstep apparatus gave some insight into the possible mechanism of allelopathic inhibition of sweetgum by leachates of fescue. Increases in potassium, magnesium, and calcium in fescue-treated seedlings may be attributed to unhindered absorption of these elements coupled with an inhibition of growth. Decrease in phosphorus concentrations indicates a deficiency in the ability of fescue-treated seedlings to absorb phosphorus that might be attributed to a decrease in mycorrhizae in roots of the leachate treated seedlings. Gray and Gerdemann (1967) showed that uptake of phosphorus by sweetgum is less in seedlings without mycorrhizae than in those with mycorrhizae. Although nitrogen concentrations were not significantly different, seedlings of the dead fescue root and dead fescue leaf treatments had similar reduced nitrogen concentrations, suggesting impaired absorptive capacity of the roots for nitrogen. Allelopathic interactions affecting mineral uptake have been documented in specific cases involving phosphorus and nitrogen (Buchholtz, 1971; Chambers and Holm, 1965).

The chlorotic nature of the leaves of dead fescue root– and dead fescue leaf–treated seedlings was possibly due to a deficiency of chlorophyll. Phosphorus and nitrogen deficiencies may have inhibited the ability of these seedlings to synthesize chlorophyll and subsequently increase their biomass.

Plass (1968) reported that sweetgum growth was reduced when sweetgum was planted in association with fescue on strip mine spoil banks, and attributed the reductions in growth to competition between fescue and sweetgum for water. His data were contrary to the results of the field part of this experiment, as soil moisture was consistently higher on fescue plots than on non-fescue plots throughout the 1974 growing season (Walters, 1975). Although the agent of allelopathic inhibition of sweetgum by fescue has not been identified, the results suggest that fescue exhibits a noncompetitive influence on the growth and mineral nutrition of sweetgum.

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DETERMINING RELEASE RATES OF 3-METHYL-2-CYCLOHEXEN-1-ONE ANTIAGGREGATION PHEROMONE OF *Dendroctonus pseudotsugae* (COLEOPTERA: SCOLYTIDAE)

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Abstract—A method of analysis using high-pressure liquid chromatography (HPLC) with an ultraviolet detector was developed for determining release rates of the antiaggregation pheromone of *Dendroctonus pseudotsugae* Hopkins, 3-methyl-2-cyclohexen-1-one (MCH), in controlled-release formulations. The pheromone released by the formulations was swept from the test apparatus, trapped on Porapak-QS packing, and eluted with solvent. Aliquots of the eluate were analyzed by liquid chromatography. Release rates of 5–400 μg MCH/hr per g formulation were determined from 5-g samples of the controlled-release formulations.

Key Words—antiaggregation pheromone, controlled-release formulation, high-pressure liquid chromatography, Douglas fir beetle, *Dendroctonus pseudotsugae*.

INTRODUCTION

The existence of the Douglas fir beetle pheromone, 3-methyl-2-cyclohexen-1-one (MCH), was first reported by Kinzer et al. (1971) (Fig. 1). Rudinsky et al. (1972) and Furniss et al. (1972) found that MCH showed promise as an antiaggregation pheromone. MCH can be formulated in controlled-release forms that will emit the pheromone over a period of 60 days or more at a rate of 10 μg/hr per g formulation (J. W. Young, unpublished report).[1]

[1] This report is on file at the Zoecon Corporation, Palo Alto, California.

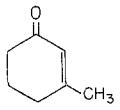

FIG. 1. Structure of 3-methyl-2-cyclohexen-1-one (MCH).

In recent field studies, entomologists needed a way of ascertaining the efficacy of MCH for protecting susceptible forest trees from infestation by the Douglas fir beetle, *Dendroctonus pseudotsugae*, over a period of 30–90 days. A method of analysis was needed for determining the release rates of MCH from several controlled-release formulations that were exposed for different time intervals. It is possible to measure release rates of tritium-labeled MCH over periods of months, but the measurement of minute quantities of unlabeled MCH in field formulations by means such as gas chromatography is, at best, difficult. It was extremely difficult to analyze samples containing less than 0.1 μg MCH/10 μl solution by gas chromatography. Larger aliquots of the samples caused quenching of the flame ionization detector and masking of the MCH peak by the solvent peak on the chromatogram. Concentration of the samples to remove solvent inevitably led to the loss of MCH.

Reported here is a fast and efficient method of measuring MCH by high-pressure liquid chromatography (HPLC) with an ultraviolet detector.

METHODS AND MATERIALS

The pheromone 3-methyl-2-cyclohexen-1-one was purchased from the Aldrich Chemical Co., Inc., Milwaukee, Wisconsin[2] as 98% MCH. The absorbing medium was Porapak-QS/80–100 mesh (Waters Associates, Milford, Massachusetts). The solvents were dioxane (Mallinckrodt 4951) and trimethylpentane (Mallinckrodt 6051).

A Waters Associates Series ALC-200 liquid chromatograph was used. It was equipped with a Model 6000A solvent delivery system, a Model 440 absorbance detector set at 254 nm, and a U6K sample injector. The stainless steel columns (4 ft $\times \frac{1}{8}$ in) were prepacked with Corasil-1 (Waters Associates No. 98157) and operated at ambient temperature. The mobile phase was a mixture of dioxane–trimethylpentane (3:7) pumped at a flow rate of 2 ml/min. The UV detector setting for most analyses was varied from 0.5 to 0.05 AUFS. Sample sizes were usually 10 μl, but varied sometimes to compensate for quantities of MCH.

[2] Trade names and commercial products or enterprises are mentioned solely for information. No endorsement by the USDA is implied.

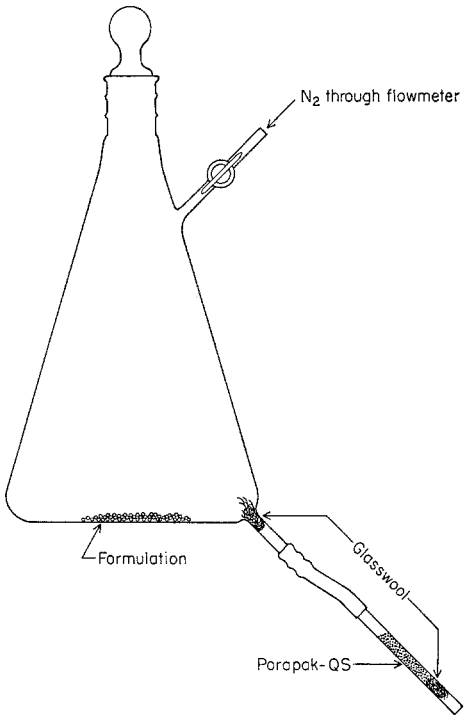

FIG. 2. Elution flask.

At room temperature, dry nitrogen was passed at 175 ml/min through a modified 2-liter filter flask fitted with a glass stopper and a bottom outlet (Fig. 2). This apparatus provided a flat surface on which as much as 20 g of formulation could be spread in a thin layer. A smaller flask can be used for smaller samples. Approximately 5 g of weighed formulation was placed on the bottom of the flask. (Formulations that were stored for any length of time in a closed container were first aired in an open dish for 1–2 hr to dissipate surface materials that had accumulated. Abnormally high release rates have resulted when this was not done.) The bottom outlet was loosely plugged with glass wool. A glass tube (125 × 8 mm) was filled with 0.25 g Porapak-QS, 50/80 mesh, held in place with a glass wool plug; the tube was attached to the bottom outlet with a piece of Tygon tubing. Sweeping with nitrogen was continued for 1–2 hr, depending on estimated release rate. Afterward, the Porapak tube was removed and eluted with enough trimethylpentane to fill a 10-ml volumetric flask.[3] An aliquot of the eluate was injected into the

[3] This method of trapping pheromone on and eluting from Porapak-QS is similar to that described by Byrne et al. (1975).

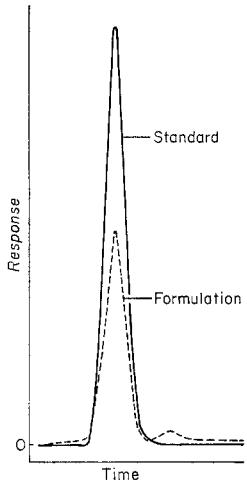

FIG. 3. Liquid chromatogram of MCH formulation.

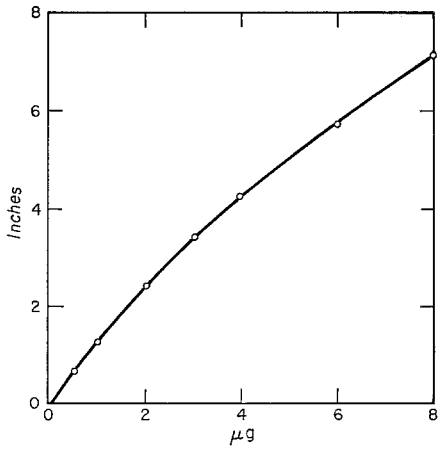

FIG. 4. Relationship of peak height to μg MCH on 10-mV, 10-in recorder at 0.5 in/min.

liquid chromatograph. The peak height of the MCH peak on the chromatogram was compared, and the quantity of MCH in the sample was calculated from standard curves determined by triplicate injections of known quantities of MCH (see Figs. 3 and 4). In samples in which a large aliquot size (50–100 μl) was necessary, more precise measurements were made by dilution of the trimethylpentane eluate with dioxane (7:3) to match the mobile phase in order to cancel refractive index effects in the UV detector. Solvents were

first tested for presence of UV-absorbing materials at the high sensitivity of the HPLC detector.

RESULTS AND DISCUSSION

The release rates of field-exposed MCH formulations differing in matrices were on the order of 5–400 μg/hr per g (Table 1). For the analyses, 5–25 gm of formulation was used and swept for 1–2 hr with nitrogen. By injecting 10–100-μl aliquots of the trimethylpentane eluates, it was found that this method was applicable for analyzing these formulations. Previous tests here and in another laboratory (J. W. Young, unpublished report) using formulations with release rates of 0.1–150 μg tritium-labeled MCH/g formulation per hr showed that MCH is retained by the Porapak and eluted by trimethylpentane to within 99% by this technique. Moreover, no residual MCH was found to be absorbed by the apparatus, and varying sweep rates of 50–600 ml/min of nitrogen had insignificant effects on the release rates.

The use of HPLC in analyses proved quicker and more accurate for samples with low release rates of MCH than gas chromatography. In addition, it offered two advantages: (1) It was possible to inject larger samples (hence larger total amounts of MCH) into the liquid chromatograph; large samples caused flame quenching in the gas chromatograph with a flame ionization detector. (2) Solvent peaks often masked the MCH peak on the gas chromatogram, whereas solvent peaks were not encountered, except with large aliquots, on the liquid chromatogram.

The MCH appeared on the chromatogram at a retention time of 2.6 min under the given conditions. In a blank run using this procedure, no UV-absorbing compounds were found on the chromatogram with the same retention time.

Due to varying conditions encountered in the field, release rates measured

TABLE 1. MCH RELEASE RATES[a] OF SEVERAL FIELD FORMULATIONS

Formulation No.[b]	Days of exposure							
	1	9	14	21	28	35	42	49
9	114	77	105	31	9	17	13	10
10	150	149	92	23	24	15	—	7
11	120	146	106	36	14	20	26	12

[a] Rates are expressed in μg/hr per g formulation and are the averages of 3 measurements.

[b] Dimeracid–polyamide matrix formulations as described by Furniss et al. in preparation.

by this method under laboratory conditions will probably not be the same as those in the field. However, the method is valid for comparing release rates of one sample with those of another.

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