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COMPONENTS OF FEMALE SEX PHEROMONE OF COCOA POD BORER MOTH, *Conopomorpha cramerella*

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Abstract—The cocoa pod borer, *Conopomorpha cramerella* (Snellen) (Lepidoptera: Gracillariidae), is the most serious pest of cocoa in Southeast Asia. Analyses of ovipositor washings and entrained volatiles from virgin female moths by gas chromatography (GC) linked to electroantennography (EAG), and comparison of EAG responses from the male moth to synthetic compounds indicated the presence of the *E,Z,Z* and *E,E,Z* isomers of 4,6,10-hexadecatrienyl acetate and the corresponding alcohols, and of hexadecyl alcohol. Amounts of pheromone produced were less than 0.1 ng/female, and no peaks for the unsaturated components were observed on GC analysis. Extensive field testing of synthetic mixtures in Sabah, East Malaysia, showed that traps baited with a polyethylene vial impregnated with 1.2 mg of a mixture of the above five components in 40:60:4:6:10 ratio caught more male *C. cramerella* moths than traps baited with a virgin female moth.

Key Words—Cocoa pod borer; *Conopomorpha cramerella*; *Acrocercops cramerella*; Lepidoptera; Gracillariidae; sex pheromone; sex attractant; 4,6,10-hexadecatrienyl acetate; 4,6,10-hexadecatrien-1-ol.

INTRODUCTION

The cocoa pod borer, *Conopomorpha cramerella* (Snellen) [previously *Acrocercops cramerella* (Snellen); (Bradley, 1985)] (Lepidoptera: Gracillariidae), is the most serious pest of cocoa in Southeast Asia (Mumford, 1984). The larvae bore into immature cocoa pods and disrupt the development of the beans. This may cause production of smaller beans, difficulties in extraction of beans from

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the pods and yellowing of immature pods leading to premature harvesting. Losses can be in excess of 50% of the crop. The biology and life cycle of this pest have been summarized by Wessel (1983) and Lim et al. (1982), and control has been attempted by cultural and chemical means as described by Mumford (1984).

In 1981, Day showed that caged virgin female *C. cramerella* moths attracted male moths to sticky traps hung in cocoa trees in Sabah, East Malaysia (Mumford, 1984). Since control methods currently available for this pest are not always effective and are labor intensive, the possibility of control using a synthetic pheromone was attractive. Furthermore, the pest–crop system appeared to be suitable for control methods based on pheromones since it is a relatively low density pest with limited dispersal ability in a stable environment. Beevor et al. (1984) have reported preliminary studies to determine the best design, position, and color of pheromone traps for *C. cramerella*. This paper describes the identification and synthesis of components of the female sex pheromone and trials to determine the most attractive blends of these components in a field trapping system.

METHODS AND MATERIALS

Insect Material. Pupae were collected in Sabah, East Malaysia, and sent by air to London, still in their membranous cocoons attached to cocoa leaves. They were maintained in individual containers in an environmental cabinet on a reversed 12-hr–12-hr light–dark cycle with temperature alternating between 27°C and 22°C and relative humidity 90%. Adult moths were sexed on emergence and maintained under the same conditions.

Pheromone Collection. Ovipositor washings in carbon disulfide, dichloromethane, or heptane were prepared from virgin female moths, usually 8–9 hr into the first dark period after emergence. Female moths had been observed to be most attractive to male moths at this time in the field (R.K. Day and J.D. Mumford, unpublished observations). In attempts to improve pheromone yields, numerous variations of this procedure were tried. Thus, ovipositor washings were made from older female moths, after shorter or longer periods into the scotophase, and under natural conditions of lighting, humidity and temperature in Sabah. Whole body washes of female moths were made, as were solvent extracts of excised pheromone glands.

Volatiles were collected from individual virgin female moths on filters containing 5 mg of activated charcoal (Grob and Zurcher, 1976; Nesbitt et al., 1979; Tumlinson et al., 1982). Females were typically entrained for 10 hr into the dark period. In further attempts to improve yields of pheromone, the female moths were entrained in the presence of cocoa leaves, or placed on cocoa leaves prior to making ovipositor washings.

Electroantennography (EAG). Male *C. cramerella* moths were immobilized in an atmosphere of carbon dioxide, mounted on a plasticine block, and held in position with a strip of expanded polystyrene. The antennae were secured along the surface of the plasticine with small wire staples. The tip of one antenna was removed with a razor blade and the cut end inserted into the end of a glass microelectrode from which the tip had been broken. The other antenna was reduced to 4–5 proximal segments and inserted into a similar glass microelectrode as reference. The microelectrodes contained saline solution (Roelofs and Comeau, 1971), with 0.2% agar added to reduce leakage of saline, and were connected by means of chloridized silver wires to a Grass P16 microelectrode DC preamplifier linked to a Tetronix 5110 oscilloscope and Siemens Minograf 34T recorder.

Gas chromatographic analyses with simultaneous recording of EAG responses to the column effluent (GC-EAG) were carried out essentially as described by Moorhouse et al. (1969). The antennae were maintained under a constant stream of humidified air (400 ml/min), and the column effluent collected in the reservoir was blown over the recording antenna with nitrogen (400 ml/min) for 3 sec at 17-sec intervals.

To record EAG response profiles to synthetic hexadecenyl acetate isomers, a solution of the test compound in heptane was syringed onto the inner walls of a disposable Pasteur pipet and the solvent removed with a stream of nitrogen (500 ml/min for 10 sec). The pipet was positioned with its tip 0.5 cm above the center of the recording antenna and a 3-sec pulse of nitrogen (500 ml/min) blown through the pipet. An interval of 3 min under a constant flow of humidified air (2.5 liters/min) was allowed between successive stimuli.

Gas Chromatography (GC). Analyses by GC, with and without simultaneous recording of EAG responses to the column effluent, were carried out with the following columns and conditions: glass columns (1.8 m × 2 mm ID) with nitrogen carrier gas at 25 ml/min, packed with: (A) 5% SE30 and 0.5% Carbowax 20 M on Chromosorb W HP, temperature programmed from 100°C to 220°C at 4°C/min; and (B) 1.5% Carbowax 20 M on 100–120 mesh Chromosorb G AW DMCS, temperature programmed from 100°C to 210°C at 4°C/min; fused silica capillary columns (25 m × 0.32 mm ID, Chrompack) with Grob splitless injection and helium carrier gas at 0.4 kg/cm^2, coated with: (C) CP Sil 5CB (chemically bonded methyl silicone), oven temperature held at 70°C for 2 min, then programmed to 150°C at 34°C/min and at 0.4°C/min to 165°C; and (D) CP Wax 57CB (chemically bonded, cross-linked polyethylene glycol), oven temperature held at 70°C for 2 min, then programmed to 150°C at 31°C/min and at 1°C/min to 185°C.

Flame ionization detectors were used throughout, and in linked GC-EAG analyses the column effluent was split 1 : 1 to the GC detector and the EAG preparation by means of a glass-lined T piece (SGE). With capillary columns, carrier gas makeup (5 ml/min) was introduced just before the T piece.

Synthetic Chemicals. The monounsaturated 16-carbon acetates were prepared by standard acetylenic or Wittig routes and were purified by liquid chromatography on silica gel impregnated with 20% silver nitrate to give products containing less than 0.1% of the opposite geometrical isomer.

The (*Z*)-10 isomers of 4,6,10-hexadecatrienyl acetate and alcohol were prepared as outlined in Figures 1–5, using the appropriate sequences of acetylenic and Wittig reactions. The corresponding (*E*)-10 isomers were obtained from (*E*)-4-decen-1-ol which was derived from intermediate (IV) in Figure 1 by reduction with sodium in liquid ammonia and diethyl ether followed by deprotection, in 96% yield.

The route described for the *E,Z,Z* isomer is preparatively useful, and the yields quoted are for reactions giving 5 g of final product. Isomerization of the conjugated double bonds was easily effected by brief exposure to sunlight of a 0.1 M solution in hexane containing a catalytic amount of iodine (Klug et al., 1982). The isolated double bond was unaffected by this treatment, and the product contained the *E,E,Z*, *Z,E,Z*, *E,Z,Z* and *Z,Z,Z* isomers in 67 : 15 : 15 : 3 ratio. The pure *E,E,Z* isomer could be isolated by liquid chromatography on silica gel impregnated with 20% silver nitrate. In separate experiments, this was shown to provide good separation of the (*Z*)-10 and (*E*)-10 isomers, but separation of the isomers differing only in geometry of the conjugated double bonds was poor and only about 10% of the available *E,E,Z* isomer could be isolated pure from the isomerized mixture.

Treatment of the isomerized mixture with liquid sulfur dioxide for 5 days gave a good yield (approximately 70% of theoretical) of the cyclic sulfone (XIII in Figure 3) derived from the *E,E,Z* isomer, which could be isolated by flash

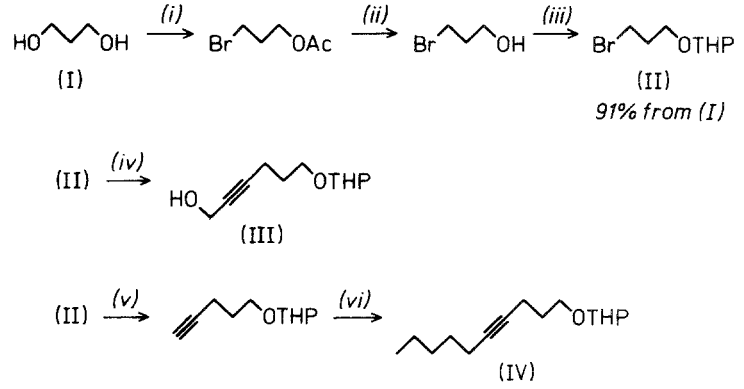

FIG. 1. Preparation of precursors II, III, and IV. (THP = 2-tetrahydropyranyl). Reagents: (i) HBr in acetic acid; (ii) K_2CO_3/MeOH; (iii) dihydropyran/ether/0°C; (iv) propargyl alcohol/$LiNH_2$/liq. NH_3/THF; (v) acetylene/$LiNH_2$/liq. NH_3/THF; (vi) $LiNH_2$/liq. NH_3/THF/$C_5H_{11}Br$.

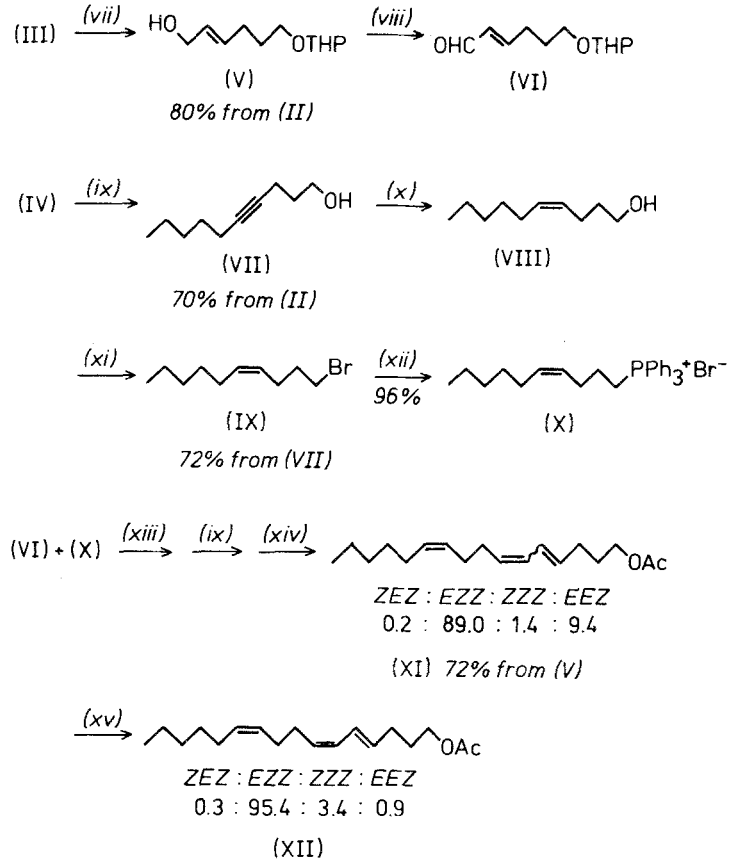

FIG. 2. Synthesis of (*E*, *Z*, *Z*)-4, 6, 10-hexadecatrienyl acetate (XII). Reagents: (vii) $LiAlH_4$/THF; (viii) pyridinium chlorochromate/sodium acetate/ CH_2Cl_2; (ix) *p*-toluenesulfonic acid/MeOH; (x) H_2/Lindlar catalyst/quinoline/EtOH; (xi) $CBr_4/Ph_3P/CH_2Cl_2$; (xii) Ph_3P/CH_3CN/reflux 18 hr; (xiii) $KOBu^t$/THF/−30°C; (xiv) Ac_2O/pyridine; (xv) tetracyanoethylene/THF.

chromatography on silica gel with 25% ethyl acetate in petroleum spirit. Injection of the sulfone onto a GC column with the injector at 200°C gave quantitatively a single peak corresponding to the *E*,*E*,*Z* isomer, but this conversion could not be carried out on a preparatively useful scale. Heating the sulfone in solvent or short-path distillation under reduced pressure gave black tars.

The products were analyzed by GC using the fused silica capillary columns described above or a column coated with OV-275 (Chrompack; 25 m × 0.22 mm ID; oven temperature held at 70°C for 2 min, then programmed at 20°C/min to 160°C; helium carrier gas at 0.8 kg/cm^2) which provided excellent sep-

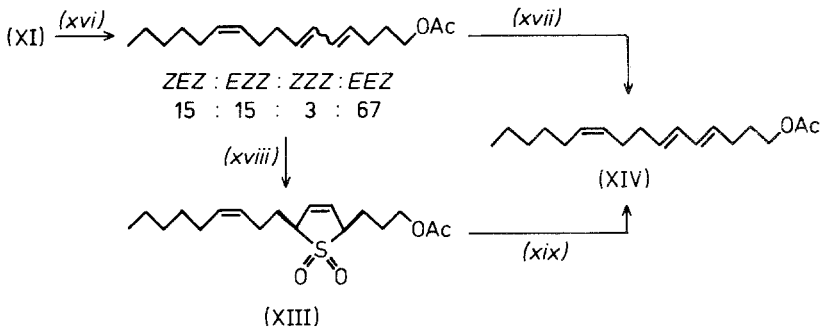

FIG. 3. Preparation of (*E*, *E*, *Z*)-4, 6, 10-hexadecatrienyl acetate (XIV). Reagents: (xvi) 0.1 M hexane solution/catalytic I_2/sunlight/15 min; (xvii) liquid chromatography/20% $AgNO_3$ on 230–400 mesh silica gel/10% ethyl acetate in petroleum spirit; (xviii) liquid SO_2/5 days; (xix) GC injector at 200°C.

arations of the isomeric acetates but which could not be used for analysis of the alcohols.

The products were characterized by the reasonably unambiguous methods of preparation used, by their GC retention characteristics, by the relative proportions observed in equilibrium mixtures of isomers, and by spectroscopic methods.

The mass spectra of all the acetate isomers were essentially identical and showed a diagnostic base peak at *m/e* 107. This presumably corresponds to the fragment $(-CH_2-CH=CH-CH=CH-CH_2-CH=CH_2)^+$ formed from the molecular ion by loss of acetic acid and cleavage to give the allylic ion stabilized by the 4,6-conjugated diene system. In the [^{1}H]NMR spectra at 270 MHz, signals due to the olefinic protons could be assigned unambiguously on a first-order basis. The [^{13}C]NMR spectra of the acetates all showed the expected 18 signals with six in the olefinic region, but these could not be assigned unambiguously.

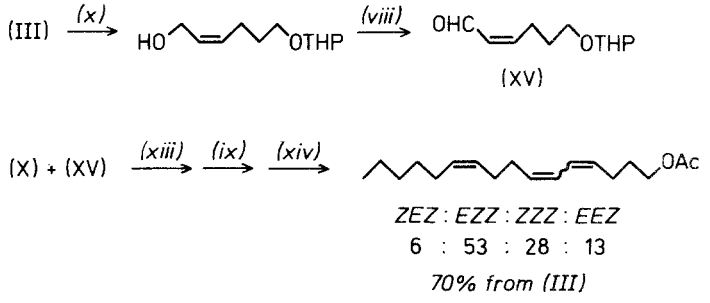

FIG. 4. Attempted preparation of (*Z*, *Z*, *Z*)-4, 6, 10-hexadecatrienyl acetate.

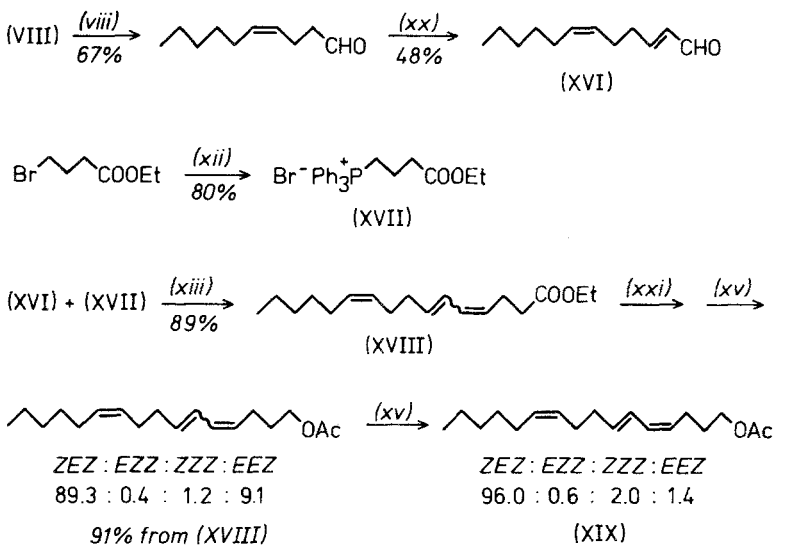

FIG. 5. Synthesis of (*Z*, *E*, *Z*)-4, 6, 10-hexadecatrienyl acetate (XIX). Reagents: (xx) $Ph_3P{=}CHCHO$/toluene/100°C/12 hr; (xxi) $LiAlH_4$/ether.

Representative spectra are as follows.

(E,Z,Z)-*4,6,10-Hexadecatrienyl Acetate.* EI mass spectrum at 70 eV [*m/e* (relative abundance)]: 278(6.0), 218(5.5), 190(4.2), 177(2.6), 163(2.6), 161(3.9), 150(3.2), 148(3.3), 147(6.3), 134(3.0), 133(5.8), 120(5.5), 119(4.2), 108(13.3), 107(100), 93(10.4), 91(24.0), 81(11.7), 80(17.5), 79(79.2), 69(10.4), 67(15.6), 55(15.5), 45(13.9), 44(11.0), 43(11.7), 42(44.2), 41(31.2). [^{1}H]NMR spectrum (270 MHz; $CDCl_3$): inter alia, δ 5.25–5.45 (3H, m: H-7, H-10, H-11); 5.64 (1H, dt, J = 17.0 and 7.5: H-4); 5.96 (1H, t, J = 11.0 and 11.0: H-6); 6.33 (1H, dd, J = 17.0 and 11.0: H-5). [^{13}C]NMR spectrum (67.9 MHz; $CDCl_3$): δ 14.2, 21.1, 22.6, 27.3, 27.4, 27.9, 28.4, 29.3, 29.5, 31.6, 64.0, 126.5, 128.7, 128.8, 130.0, 130.7, 133.0, 171.2.

(E,E,Z)-*4,6,10-Hexadecatrienyl Acetate.* EI mass spectrum at 70 eV [*m/e* (relative abundance)]: 278(4.3), 218(5.5), 161(3.7), 147(3.1), 133(7.0), 120(5.5), 119(4.7), 108(16.4), 107(100), 91(21.9), 80(13.7), 79(72.9), 69(14.9), 67(12.5), 55(18.8), 45(14.0), 43(37.5), 41(29.7). [^{1}H]NMR spectrum (270 MHz; $CDCl_3$): inter alia, δ 5.25–5.45 (2H, m: H-10, H-11); 5.45–5.65 (2H, m: H-4, H-7); 5.99 (1H, t, J = 14.5) and 6.04 (1H, t, J = 14.5) (H-5 and H-6). [^{13}C]NMR spectrum (67.9 MHz; $CDCl_3$): δ 14.2, 21.1, 22.6, 27.1, 27.3, 28.4, 29.0, 29.4, 31.6, 32.8, 64.0, 128.8, 130.4, 130.6, 130.7, 131.3, 132.5, 171.2.

Field Tests. Field testing was carried out in cocoa plantations in Sabah, East Malaysia, during 1983–1984. During initial studies, Beevor et al. (1984)

showed that many of the commonly used trap designs, e.g., sticky delta, water and funnel traps, are unsuitable for *C. cramerella*. It was also found that trap positioning was important and that traps just above the cocoa canopy caught most moths.

The most effective trap design used in this work consisted of two horizontal sheets of plastic Correx (30 × 30 cm; Corruplast Plc, Gloucester, England) held 5 cm apart by wire spacers, with another Correx sheet coated with polybutene sticker clipped to the upper surface of the lower sheet. Traps were suspended on strings from the branches of *Gliricidia* shade trees and were adjusted to a height approximately 1 m above the top of the cocoa canopy, approximately 8 m above ground level.

Pheromone dispensers were closed polyethylene vials (35 × 8 × 1.5 mm thick) impregnated with the synthetic compounds and an equal weight of 2,6-di-*tert*-butyl-4-methylphenol (BHT) as antioxidant. The dispensers were mounted at the center of the trap through a hole in the upper sheet. In virgin female-baited traps, a single virgin female moth was housed in a small plastic mesh container in place of the pheromone dispenser. Female moths were used on the second night after emergence and were renewed each night.

Typically 10–12 replicates of each experiment were carried out simultaneously over 4–5 days depending upon whether 4–5 treatments were being compared. Traps were positioned approximately 50 m apart in a circular arrangement within a replicate, and replicates were at least 100 m apart. Moth catches were recorded each day when traps were moved on one position within a replicate.

The data were analyzed using a Generalized Linear Interactive Modeling (GLIM) statistical package (Baker and Nelder, 1978). This allows one to fit a series of models, as in a stepwise regression, so that the statistical significance of treatment effects may be measured while controlling for differences in overall trap catch from night to night or plot to plot. The models used had a Poisson distribution and a "log link" (Baker and Nelder, 1978), so that the ratios between trap catches were measured, and these ratios were tested for differences between treatments significant at the 5% level by the *t* test.

RESULTS

Yields of pheromone from virgin female *C. cramerella* were very low, less than 0.1 ng/female, and these could not be improved by varying conditions for solvent extraction or entrainment of volatiles, as described above. No consistent GC peaks corresponding to the unsaturated pheromone components were ever observed, and the identification of these was carried out on the basis of GC retention data derived from linked GC-EAG analyses, EAG responses to synthetic compounds, and extensive testing of synthetic compounds as attractants in the field.

TABLE 1. RETENTION DATA[a] FOR PHEROMONE COMPONENTS AND SYNTHETIC COMPOUNDS ON PACKED GC COLUMNS

	Column	
	SE 30 (A)	Carbowax 20 M (B)
Response 1	15.0	16.7
Response 2	16.05[b]	17.6[b]
Response 3		18.4
16:OH	15.0	16.7
*Z,E*9,11-16:Ac	16.3	17.7
*E,E*9,11-16:Ac	16.6	18.0
*Z,E*9,11-16:OH	15.3	18.5
*E,E*9,11-16:OH	15.5	18.8

[a] Equivalent chain lengths relative to the retention times of *n*-alkyl acetates.
[b] Major response.

Analyses of ovipositor washings and entrained female volatiles by GC-EAG using packed GC columns gave up to three responses (Table 1). The responses were broader than expected, suggesting that they were due to more than one compound. The first response on both the nonpolar (A) and polar (B) column occurred at the retention time of 1-hexadecanol (16:OH), and a small GC peak was observed. Subsequent analyses on fused silica capillary GC columns (C and D) also showed a small peak cochromatographing with 16:OH.

The retention times for the other responses were similar to those for 16-carbon acetates with two conjugated double bonds, and the corresponding alcohols. Retention data for the isomers of 9,11-hexadecadienyl acetate (9,11-16:Ac) and the corresponding alcohols (9,11-16:OH) is given in Table 1.

The EAG responses to a range of saturated, straight-chain acetates, alcohols, and aldehydes were recorded, and the most significant responses were elicited by hexadecyl acetate (16:Ac) and 16:OH (Table 2).

TABLE 2. EAG RESPONSES OF MALE *C. cramerella* MOTH TO SATURATED COMPOUNDS

	EAG response (mV),[a] functional group		
Alkyl chain length	Acetate	Alcohol	Aldehyde
14	0.8	1.6	0
16	2.8	2.2	1.0
18	1.2	0	0.4

[a] Using linked GC-EAG as delivery system with 30 ng injected, 15 ng to the antenna.

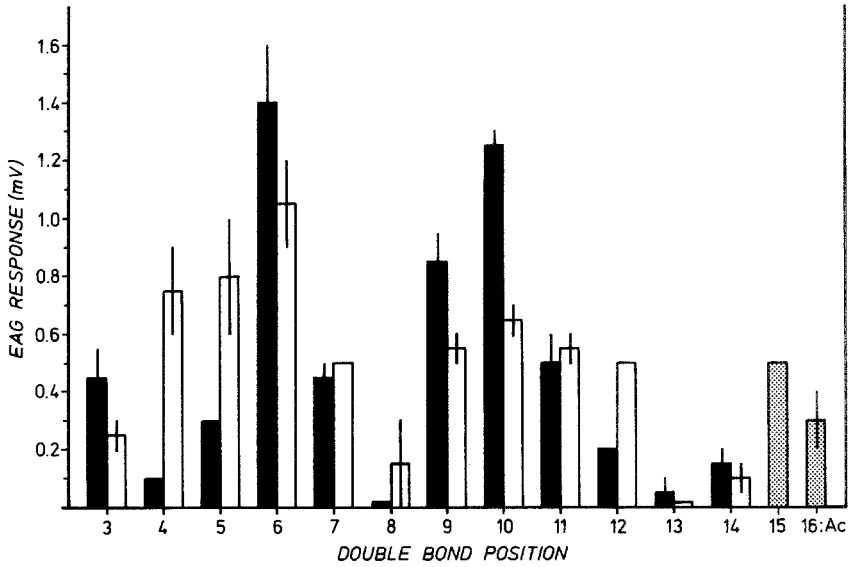

FIG. 6. EAG responses of male *C. cramerella* moth to monounsaturated 16-carbon acetates and hexadecyl acetate (16:Ac); ■ *Z* isomers; □ *E* isomers. Results are the means of duplicate recordings from the same moth to 4 ng at source, and the bars indicate the spread of the results.

The EAG responses to a range of monounsaturated, 16-carbon acetates were then recorded, and the largest responses were caused by the (*E*)-4, (*E*)-5, (*Z*)-6, (*E*)-6, (*Z*)-9, and (*Z*)-10 isomers (Figure 6). These results suggested that the pheromone might include at least one triunsaturated, 16-carbon acetate, possibly the (*E*,*Z*,*Z*)-4,6,10 and/or (*E*,*E*,*Z*)-4,6,10 isomer. The high EAG responses to the monounsaturated compounds with the double bonds between the two double bonds of a conjugated diene pheromone component—here the (*E*)-5 isomer in particular—have been observed with other insects having conjugated dienes as pheromone components (Hall et al., 1980, and references therein).

The isomers of 4,6,10-hexadecatrienyl acetate (4,6,10–16:Ac) and alcohol (4,6,10–16:OH) were synthesized, and several of these, particularly the *E*,*Z*,*Z* isomer, were found to be extremely potent olfactory stimulants for the male *C. cramerella* moth (Table 3).

Analyses of the natural pheromone by GC-EAG using fused silica capillary columns provided further evidence that the pheromone consisted of isomers of 4,6,10–16:Ac and 4,6,10–16:OH, although the results were less clear-cut than they would have been if GC peaks had been recorded. Up to five different responses were recorded during analyses of both ovipositor washings and female volatiles on the nonpolar (C) and polar (D) columns (Figure 7), and these

TABLE 3. EAG RESPONSES[a] OF MALE *C. cramerella* MOTH TO ISOMERS OF 4,6,10-HEXADECATRIENYL ACETATE

Configuration of 4,6 bonds	EAG response (mV), configuration of 10 bond	
	Z	*E*
Z,E	0.50	0.32
E,Z	1.01	0.57
Z,E	0.19	0.0
E,E	0.44	0.0

[a] Using linked GC-EAG as delivery system with 1.0 ng injected, 0.5 ng to the antenna.

were designated I–V on the basis of their order of elution from the polar column (D). Response I was only recorded in a few samples. It was not resolved from response II on the nonpolar column (C), but the retention time on the polar column (D) fitted with that of *E,Z,E*4,6,10–16:Ac. Response II was consistently the largest in all the samples analyzed, and the retention times fitted only those of *E,Z,Z*4,6,10–16:Ac on both columns. Response III occurred at retention times which matched those of *E,E,Z*4,6,10–16:Ac unambiguously on both columns. Responses IV and V occurred at the retention times of isomers of 4,6,10–16:OH. The retention times of response IV matched only those of *E,Z,Z*4,6,10–16:OH on both columns, and those of response V fitted those of *E,E,Z*4,6,10–16:OH unambiguously on both columns.

The above analytical results indicated that the pheromone of *C. cramerella* consisted of at least four components, and the available evidence suggested that these were *E,Z,Z*4,6,10–16:Ac, *E,E,Z*4,6,10–16:Ac, *E,Z,Z*4,6,10–16:OH and *E,E,Z*4,6,10–16:OH. Other possible components were 16:OH and *E,Z,E*4,6,10–16:Ac, but the results provided little information on the relative proportions of any of these components.

Field tests. In initial field tests, it was assumed that the major component of the pheromone was *E,Z,Z*4,6,10–16:Ac which was responsible for the most significant EAG response in linked GC-EAG analyses. It was also assumed that the isomeric ratio of the alcohol components was the same as that of the acetates. During 1982–1983, polyethylene vials impregnated with 1 mg *E,Z,Z*4,6,10–16:Ac + 0.1 mg *E,E,Z*4,6,10–16:Ac + 0.1 mg *E,Z,Z*4,6,10–16:OH + 0.01 mg *E,E,Z*4,6,10–16:OH + 0.1 mg 16:OH were shown to attract male *C. cramerella* moths to traps at least as effectively as virgin female moths, although catches were uniformly low.

In 1984, experiments were carried out to optimize the proportions of the above five components and to test the effect of adding *E,Z,E*4,6,10–16:Ac.

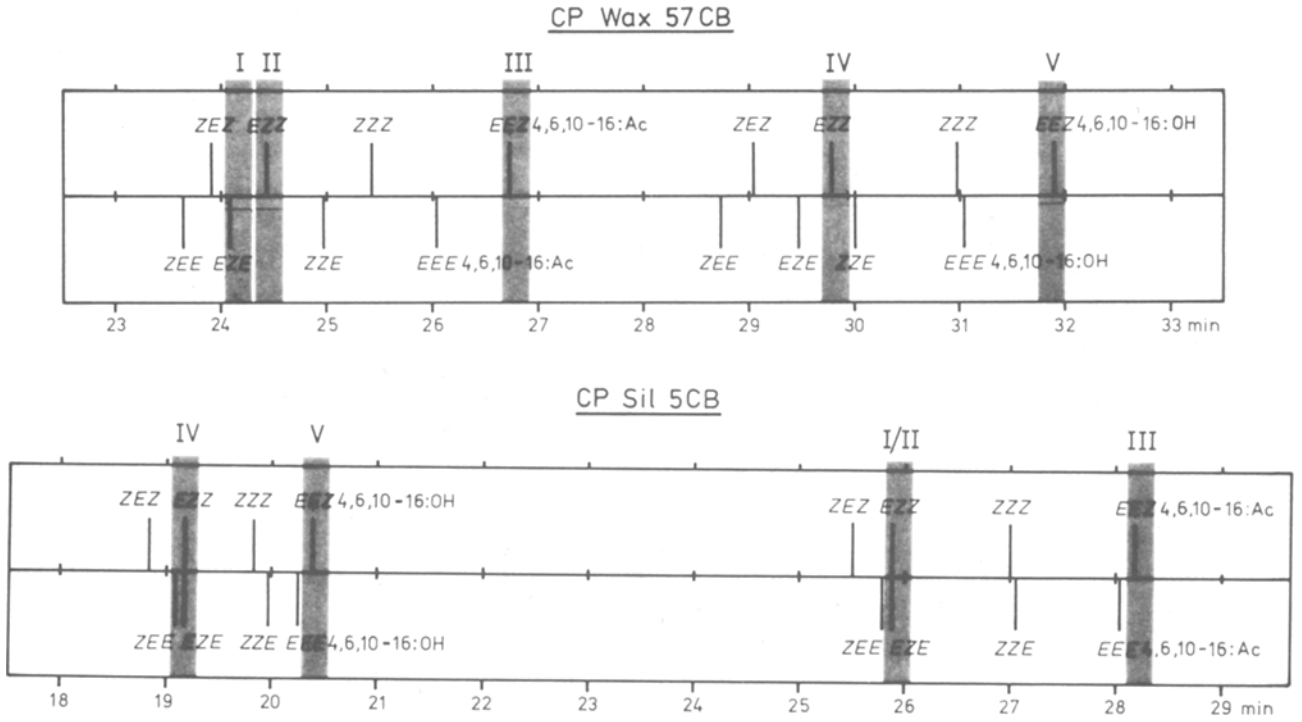

FIG. 7. GC retention data for synthetic isomers of 4, 6, 10–16:Ac and 4, 6, 10–16:OH and for EAG responses recorded during GC-EAG analyses of the natural pheromone on fused silica capillary columns. ■ Band width covered by observed maximum EAG responses numbered according to their order of elution from the polar column, as in the text. Columns coated with CP Wax 57CB (column D: 16:Ac at 16.78 min. 18:Ac at 24.52 min, 20:Ac at 34.78 min) and CP Sil 5CB (column C: 14:Ac at 15.98 min, 16:Ac at 26.39 min, 18:Ac at 40.03 min).

The most important finding was that the attractiveness of the five-component mixture tested in 1982–1983 was greatly increased by using approximately equal amounts of the *E,Z,Z* and *E,E,Z* isomers of the acetate and alcohol. A series of experiments showed that the optimum ratio fell within the range 50:50 to 30:70 *E,Z,Z–E,E,Z*, and the results of two typical experiments are shown in Tables 4 and 5. In subsequent experiments, a 40:60 ratio of *E,Z,Z* and *E,E,Z* isomers was used.

Omitting the unsaturated alcohol components markedly reduced attractiveness (Table 5), and, keeping the isomeric ratio of the alcohols the same as that of the acetates, 10% of the alcohol components relative to the acetates was found to be the optimum proportion (Table 6). Varying the proportion of each alcohol component independently showed that 10% of each, relative to the corresponding acetate, was indeed the optimum proportion (Tables 7 and 8).

Addition of *E,Z,E*4,6,10–16:Ac and *E,Z,E*4,6,10–16:OH, also in 10:1 ratio, to the five-component blend caused a progressive decrease in attractiveness with increasing amounts (Table 9).

In all the above tests, the mixtures contained 10% 16:OH relative to the total amount of *E,Z,Z*4,6,10–16:Ac and *E,E,Z*4,6,10–16:Ac. Varying the amount of 16:OH had little effect on catches except at very high levels when catches were reduced (Table 10).

From the above results, the most attractive mixture was deduced to be a 40:60:4:6:10 mixture of *E,Z,Z*4,6,10–16:Ac, *E,E,Z*4,6,10–16:Ac, *E,Z,Z*4,6,10–16:OH, *E,E,Z*4,6,10–16:OH, and 16:OH. With the polyethylene vial dispensers, those impregnated with this mixture containing 1 mg of the two acetates were shown to be more attractive than those impregnated with lesser amounts (Table 11), and very significantly more attractive than a virgin female moth (Table 12).

DISCUSSION

A synthetic mixture has been defined which catches more male *C. cramerella* moths than a virgin female moth when used to bait traps in the field. This consists of a 40:60 mixture of *E,Z,Z*4,6,10–16:Ac and *E,E,Z*4,6,10–16:Ac with 10% of the corresponding alcohols in the same proportions, dispensed from a polyethylene vial. In the experiments reported, 10% of 16:OH was also added to this mixture, but this is probably not necessary. It is not possible to say that this mixture is identical with that produced by a virgin female moth because only extremely small amounts of the natural pheromone were available for analysis, but the laboratory and field results taken together indicate that the structural assignments and general proportions are correct. However, there may be other components not identified. In particular, the EAG response recorded in some samples and attributed to *E,Z,E* 4,6,10–16:Ac may

TABLE 4. CATCHES OF MALE *C. cramerella* MOTHS IN TRAPS BAITED WITH DIFFERENT RATIOS OF *E,Z,Z* AND *E,E,Z* ISOMERS OF 4,6,10-16:Ac AND 4,6,10-16:OH

Pheromone components (μg)					Total moth catch	Moth catch/ trap/night[a]
*E,Z,Z*4,6,10-16:Ac	*E,E,Z*4,6,10-16:Ac	*E,Z,Z*4,6,10-16:OH	*E,E,Z*4,6,10-16:OH	16:OH		
10	90	1	9	10	68	0.57 c
30	70	3	7	10	124	1.03 a
40	60	4	6	10	133	1.11 a
50	50	5	5	10	154	1.28 a
70	30	7	3	10	101	0.84 b

[a]Means followed by the same letter are not significantly different (*t* test; $P = 0.05$).

TABLE 5. CATCHES OF MALE *C. cramerella* MOTHS IN TRAPS BAITED WITH DIFFERENT RATIOS OF *E,Z,Z* AND *E,E,Z* ISOMERS OF 4,6,10–16:Ac AND 4,6,10–16:OH

Pheromone components (μg)					Total moth catch	Moth catch/ trap/night[a]
*E,Z,Z*4,6,10–16:Ac	*E,E,Z*4,6,10–16:Ac	*E,Z,Z*4,6,10–16:OH	*E,E,Z*4,6,10–16:OH	16:OH		
160	40	16	4	20	83	3.46 b
120	80	12	8	20	122	5.05 a
80	120	8	12	20	133	5.63 a
40	160	4	16	20	54	2.25 c
160	40			20	31	1.92 d
120	80			20	15	0.63 e
80	120			20	9	0.38 e
40	160			20	10	0.42 e

[a]Means followed by the same letter are not significantly different (*t* test; $P = 0.05$).

TABLE 6. CATCHES OF MALE *C. cramerella* MOTHS IN TRAPS BAITED WITH *E,Z,Z*4,6,10-16:Ac AND *E,E,Z*4,6,10-16:Ac IN 40:60 RATIO COMBINED WITH DIFFERENT AMOUNTS OF *E,Z,Z*4,6,10-16:OH AND *E,E,Z*4,6,10-16:OH ALSO IN 40:60 RATIO

Pheromone components (μg)					Total moth catch	Moth catch/ trap/night[a]
*E,Z,Z*4,6,10-16:Ac	*E,E,Z*4,6,10-16:Ac	*E,Z,Z*4,6,10-16:OH	*E,E,Z*4,6,10-16:OH	16:OH		
40	60	0	0	10	4	0.07 d
40	60	2	3	10	77	1.28 b
40	60	4	6	10	179	2.98 a
40	60	8	12	10	69	1.15 b
40	60	20	30	10	19	0.38 c

[a]Means followed by the same letter are not significantly different (t test; $P = 0.05$).

TABLE 7. CATCHES OF MALE *C. cramerella* MOTHS IN TRAPS BAITED WITH *E,Z,Z*4,6,10-16:Ac, *E,E,Z*4,6,10-16:Ac, AND *E,Z,Z*4,6,10-16:OH IN 40:60:4 RATIO COMBINED WITH DIFFERENT AMOUNTS OF *E,E,Z*4,6,10-16:OH

Pheromone components (μg)					Total moth catch	Moth catch/ trap/night[a]
*E,Z,Z*4,6,10-16:Ac	*E,E,Z*4,6,10-16:Ac	*E,Z,Z*4,6,10-16:OH	*E,E,Z*4,6,10-16:OH	16:OH		
40	60	4	0	10	244	2.44 bc
40	60	4	3	10	278	2.78 bc
40	60	4	6	10	343	3.43 a
40	60	4	12	10	289	2.88 b
40	60	4	30	10	222	2.22 c

[a]Means followed by the same letter are not significantly different (*t* test; $P = 0.05$).

TABLE 8. CATCHES OF MALE *C. cramerella* MOTHS IN TRAPS BAITED WITH *E,Z,Z*4,6,10–16:Ac, *E,E,Z*4,6,10–16:Ac AND *E,E,Z*4,6,10–16:OH IN 40:60:6 RATIO COMBINED WITH DIFFERENT AMOUNTS OF *E,Z,Z*4,6,10–16:OH

Pheromone components (μg)					Total moth catch	Moth/catch/ trap/night[a]
*E,Z,Z*4,6,10–16:Ac	*E,E,Z*4,6,10–16:Ac	*E,Z,Z*4,6,10–16:OH	*E,E,Z*4,6,10–16:OH	16:OH		
40	60	0	6	10	103	1.03 d
40	60	2	6	10	308	3.08 b
40	60	4	6	10	475	4.75 a
40	60	8	6	10	188	1.88 c
40	60	20	6	10	59	0.59 e

[a] Means followed by the same letter are not significantly different (*t* test; $P = 0.05$).

TABLE 9. CATCHES OF MALE *C. cramerella* MOTHS IN TRAPS BAITED WITH *E,Z,Z*4,6,10-16:Ac, *E,E,Z*4,6,10-16:Ac, *E,Z,Z*4,6,10-16:OH, *E,E,Z*4,6,10-16:OH, AND 16:OH IN 40:60:4:6:10 RATIO COMBINED WITH DIFFERENT AMOUNTS OF *E,Z,E*4,6,10-16:Ac AND *E,Z,E*4,6,10-16:OH IN 10:1 RATIO

Pheromone components (μg)							Total moth catch	Moth catch/ trap/night[a]
*E,Z,Z*4,6,10-16:Ac	*E,E,Z*4,6,10-16:Ac	*E,Z,Z*4,6,10-16:OH	*E,E,Z*4,6,10-16:OH	16:OH	*E,Z,E*4,6,10-16:Ac	*E,Z,E*4,6,10-16:OH		
40	60	4	6	10	0	0	98	1.96 a
40	60	4	6	10	10	1	81	1.62 ab
40	60	4	6	10	20	2	65	1.30 bc
40	60	4	6	10	50	5	43	0.86 d
40	60	4	6	10	100	10	49	0.98 cd

[a] Means followed by the same letter are not significantly different (t test; $P = 0.05$).

TABLE 10. CATCHES OF MALE *C. cramerella* MOTHS IN TRAPS BAITED WITH *E,Z,Z*4,6,10-16:Ac, *E,E,Z*4,6,10-16:Ac, *E,Z,Z*4,6,10-16:OH, AND *E,E,Z*4,6,10-16:OH, IN 40:60:4:6 RATIO COMBINED WITH DIFFERENT AMOUNTS OF 16:OH

Pheromone components (μg)					Total moth catch	Moth catch/ trap/night[a]
*E,Z,Z*4,6,10-16:Ac	*E,E,Z*4,6,10-16:Ac	*E,Z,Z*4,6,10-16:OH	*E,E,Z*4,6,10-16:OH	16:OH		
Experiment 1						
40	60	4	6	0	441	4.41 a
40	60	4	6	10	402	4.02 a
Experiment 2						
40	60	4	6	10	194	2.43 a
40	60	4	6	100	230	2.88 a
40	60	4	6	1000	200	2.50 a
40	60	4	6	10000	126	1.56 b

[a] Means followed by the same letter within experiments are not significantly different (*t* test; $P = 0.05$).

TABLE 11. CATCHES OF MALE *C. cramerella* MOTHS IN TRAPS BAITED WITH DIFFERENT AMOUNTS OF *E,Z,Z*4,6,10-16:Ac, *E,E,Z*4,6,10-16:Ac, *E,Z,Z*4,6,10-16:OH, *E,E,Z*4,6,10-16:OH, AND 16:OH IN 40:60:4:6:10 RATIO

Total pheromone loading (μg)	Total moth catch	Moth catch/ trap/night[a]
Experiment 1		
1200	161	2.68 a
120	127	2.12 b
Experiment 2		
120	149	2.48 a
12	101	1.68 b

[a] Means followed by the same letter within experiments are significantly different (t test; $P = 0.05$).

TABLE 12. COMPARISON OF CATCHES OF MALE *C. cramerella* MOTHS IN TRAPS BAITED WITH SYNTHETIC PHEROMONE AND VIRGIN FEMALE MOTH

Attractive source	Total moth catch	Moth catch/ trap/night[a]
Synthetic pheromone[b] (1.2 mg)	373	12.93 a
Virgin female moth	29	0.97 b
Unbaited	11	0.37 c

[a] Means followed by the same letter are not significantly different (t test; $P = 0.05$).
[b] *E,Z,Z*4,6,10-16:Ac + *E,E,Z*4,6,10-16:Ac + *E,Z,Z*4,6,10-16:OH + *E,E,Z*4,6,10-16:OH + 16:OH in 40:60:4:6:10 ratio.

be due to some other compound which is an essential component of the pheromone.

This is the first recorded identification of a female sex pheromone in the family Gracillariidae, although attractants for several species in this family have been found by random screening (Roelofs et al., 1977; Ando et al. 1977, 1981; Voerman and Herrebout, 1978; Booij and Voerman, 1984). These attractants include a range of unsaturated acetates, alcohols, and aldehydes, but there is a noticeable predeliction for compounds with the double bond at the 10-position.

Only one other triunsaturated acetate or alcohol has been identified as a pheromone component of a lepidopterous species: (*E,Z,Z*)-4,7,10-tridecatrienyl acetate is one of the components of the pheromone of the potato tuberworm moth, *Phthorimaea operculella* (Lepidoptera: Gelechiidae) (Persoons et al., 1976; Yamaoka et al., 1976).

Use of pheromone-baited traps for control of *C. cramerella* by mass trapping is under investigation in a 200-hectare trial in Sabah.

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SENSORY AND BEHAVIORAL EFFECTS OF GOSSYPLURE ALCOHOL ON SEX PHEROMONE RESPONSE OF MALE PINK BOLLWORM MOTHS, *Pectinophora gossypiella*

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Abstract—(*Z,Z*)- and (*Z,E*)-7,11-hexadecadienol, reported to be pheromone precursors, interfere with the normal sequence of behavioral response of male *Pectinophora gossypiella* to sex pheromone. The magnitude of the interference can be diminished with higher release rates of the sex pheromone. (*Z,Z*)-7,11-Hexadecadienol is more effective than its *Z,E* isomer in eliciting the reduction in the behavioral response. Electroantennographic evidence suggests that each alcohol may be interfering more with receptor sites for the conformationally similar pheromone acetate than with receptor sites for the other pheromone isomer. Defining behavioral and physiological effects of pheromone analogs such as the alcohols of gossyplure may help to determine their potential for behavioral manipulations.

Key Words—Lepidoptera, Gelechiidae, *Pectinophora gossypiella*, pink bollworm, (*Z,Z*)-7,11-hexadecadienyl acetate, (*Z,E*)-7,11-hexadecadienyl acetate, (*Z,Z*)-7,11-hexadecadienol, (*Z,E*)-7,11-hexadecadienol, pheromone analog, behavior, electroantennogram.

INTRODUCTION

The pheromonally mediated behavioral responses of many species of moths have been found to be adversely affected by compounds that are related in structure to the pheromone components (Beevor and Campion, 1979; Cardé, 1976;

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Roelofs and Cardé, 1977). Some of these compounds have been synthesized and tested in part because of their structural similarity to the naturally occurring sex pheromone (e.g., Beevor and Campion, 1979). Other chemicals have been identified from extracts of sex pheromone glands and subsequently were found to interfere with the normal sequence of male responses to sex pheromone (e.g., Cardé et al., 1973). These latter compounds may not be emitted by the female and often are likely candidates to be pheromone precursors.

The sex pheromone of the pink bollworm, *Pectinophora gossypiella* (Saunders), has been identified as a 1:1 blend of (*Z*,*Z*)-7,11-hexadecadienyl acetate [(*Z*,*Z*)-7,11-16:Ac] and (*Z*,*E*)-7,11-hexadecadienyl acetate, [(*Z*,*E*)-7,11-16:Ac] (Hummel et al., 1973; this blend was called gossyplure). When Bierl et al. (1974) confirmed the identity of this sex pheromone, they also determined that (*Z*,*Z*)-7,11-hexadecadienol [(*Z*,*Z*)-7,11-16:OH] and (*Z*,*E*)-7,11-hexadecadienol [(*Z*,*E*)-7,11-16:OH] were present in gland extracts of female moths and they assumed that these alcohols were precursors of the sex pheromone. Zhu et al. (1983) detected the same two alcohols only in mated female *P. gossypiella*. In addition they determined that ca. a 1:1 blend of these alcohols (gossyplure OH) reduced captures of male *P. gossypiella* in traps in the field. We felt that it was important to gain a better understanding of the mode of action of this communicative interference at the sensory and behavioral levels in order to determine the potential for mating disruption using these compounds.

METHODS AND MATERIALS

Insects. P. gossypiella larvae were reared in half-gallon cartons containing shredded wheat-germ diet modified from the diet described by Adkisson et al. (1960). Pupae were segregated by sex, and male moths were aged daily and held in 25 × 25 × 30-cm screen cages. Larvae and adults were maintained at ca. 28°C on a 14:10 light–dark cycle (lights on at noon). Adult males had access to an 8% sucrose solution. Individual 2- to 4-day-old male moths were transferred to cylindrical screen cages (3.5 × 3.3 cm diam) during the photophase and were tested during the seventh through ninth hour of the next scotophase.

Chemicals. (*Z*,*Z*)-7,11-16:Ac and (*Z*,*E*)-7,11-16:Ac were purchased from Pest-Select (Buckeye, Arizona), and we determined that they were greater than 93% free of other sex pheromone-like volatiles by a GC analysis using a 3-m column packed with 4.2 g of 10% Silar 10C (N_2 flow rate 30 ml/min; temperature 180°C; OD 4 mm). (*Z*,*Z*)-7,11-16:Ac contained as much as 4% (*Z*,*E*)-7,11-16:Ac and no detectable (*Z*,*Z*)- or (*Z*,*E*)-7,11-16:OH. (*Z*,*E*)-7,11-16:Ac contained as much as 3% (*Z*,*Z*)-7,11-16:Ac and no detectable (*Z*,*Z*)- or (*Z*,*E*)-7,11-16:OH. (*Z*)-7-16:Ac was obtained from Farchan (Wil-

loughby, Ohio), and was greater than 96% free of other sex pheromone-like volatiles. (*Z,Z*)-7,11-16:OH, (*Z,E*)-7,11-16:OH, and (*Z*)-7-16:OH were derived from the acetates by base hydrolysis (as in Bjostad et al., 1984). (*Z,E*)-7,11-16:OH contained as much as 3% (*Z,Z*)-7,11-16:OH and no detectable 7,11-16:Ac. (*Z,Z*)-7,11-16:OH used in behavioral tests contained as much as 4% (*Z,E*)-7,11-16:OH and 5% (*Z,Z*)-7,11-16:Ac. The presence of up to 5% (*Z,Z*)-7,11-16:Ac in the solution of (*Z,Z*)-7,11-16:OH did not pose a problem for behavioral tests since this alcohol was always incorporated into a blend of (*Z,Z*)-7,11-16:Ac and (*Z,E*)-7,11-16:Ac. The blend ratio of the pheromone would only be shifted by at most 1.2%. However, in the electroantennogram (EAG) study, it was important that the alcohols contained no acetates. The alcohols were rederived from the acetates, and this time we could detect no evidence of acetate contaminants in our alcohol solutions. Solutions of the compounds to be used in behavioral and EAG tests were made in hexane.

Behavioral Tests. Male *P. gossypiella* in individual screen cages were transferred to the room housing the flight tunnel at least 0.5 hr prior to the beginning of observations, in order to acclimate the moths to wind-tunnel conditions (0.3 lux from incandescent white lights; supplemental incandescent red lighting; temperature ca. 26°C; wind speed 0.5 m/sec). The wind tunnel was similar to that described by Miller and Roelofs (1978). Cotton dental rolls (1 × 1 cm diam) were loaded with 10-μl hexane solutions containing the indicated amount of compounds about 10 min before the start of a test. Treatments within an experimental series were drawn in random order for each day's test. At the beginning of a trial, a cotton wick treated with the test compounds was placed at the center of a piece of sheet metal (15 × 15 cm) situated 15 cm above the tunnel floor on a sheet-metal platform that was 50 cm from the upwind end of the tunnel and equidistant from the walls of the tunnel. Each male was released 1.4 m directly downwind of the source by placing a cage, open end up, on a metal platform. Cages and platforms were washed with acetone daily after use. The behavioral responses of males were noted in the following categories: wing fanning in the release cage (WF); taking flight (F); upwind flight in the pheromone plume (UFW); making contact with the source (SC); and the time from introduction of the moth into the flight tunnel to initiation of flight. Ryan's (1960) multiple-comparison test for proportions was used to evaluate differences between probabilities of displaying a behavior, and Duncan's multiple-range test following an analysis of variance was used to separate mean latencies.

Electroantennogram. Aspects of our EAG studies followed procedures previously described by Baker and Roelofs (1976), Payne and Dickens (1976) and Roelofs (1977). A 2- to 4-day-old male was captured and its head was removed. The base of the head was placed into saline solution (9.0 g/liter NaCl, 0.2 g/liter KCl, 1.0 g/liter BES (buffer), 4.0 g/liter sucrose, 0.2 g/liter $CaCl_2$) on a wedge of clay that was partially submerged. One antenna was teased up and the terminal two or three segments were removed. A capillary input elec-

trode (containing saline solution and silver chloride-coated silver wire) was moved into position so that it contacted the cut end of the antenna. A chloridized silver wire submerged in the saline solution served as the ground electrode. The EAG response was preamplified with a gain of 100 and frequency range of DC 5 kHz. The signal was displayed on a Tektronic 5113 storage oscilloscope with 1 vertical division equal to 1 mV and 1 horizontal division equal to 1 sec. The EAG responses were recorded to the nearest 0.1 mV.

The first electroantennogram study involved exposing the antenna of a male *P. gossypiella* to one of three adapting stimuli: (1) solvent blank (10 μl of hexane); (2) 10 μg of (*Z*,*Z*)-7,11-16:Ac in 10 μl hexane; and (3) 10 μg (*Z*,*E*)-7,11-16:Ac in 10 μl hexane. Adapting stimuli were loaded onto a 1 × 2-cm piece of fluted filter paper (Z-fold) and, following solvent evaporation, were placed into the proximal end of elbow-shaped glass cartridges (each arm of an elbow was 4 cm long and 1 cm ID). Each cartridge was connected to a laboratory airstream flowing at 2 liters/min after passing through an activated charcoal filter. Test stimuli were the following: (1) solvent blank (10 μl of hexane); (2) 10 μg of (*Z*,*Z*)-7,11-16:Ac in 10 μl of hexane; and (3) 10μg (*Z*,*E*)-7,11-16:Ac in 10 μl of hexane, each on a 1 × 2-cm fluted filter paper. The filter paper was inserted following solvent evaporation into the lumen of a disposable Pasteur-type glass pipet (14.5 cm long). During the first 3 min of exposure to each adapting stimulus, a test stimulus was puffed into the airstream using 2 ml of air forced from a 10-ml glass syringe connected by a rubber septum to the test cartridge. Adapting stimuli and test stimuli were presented in random order within each block of treatments. Each antenna was exposed to one adapting stimulus, and all of the test stimuli. In order to examine antennal recovery following removal of adapting stimuli, test stimuli were again puffed onto the antennae after 2 min in clean air.

The second EAG experiment was designed to examine the effect of the alcohols of gossyplure on the response to (*Z*,*Z*)-7,11-16:Ac and (*Z*,*E*)-7,11-16:Ac. In this experiment the adapting stimuli were 30 μg of the alcohols loaded in 30 μl hexane onto separate pieces of fluted filter paper. The solvent blank adapting stimulus was 30 μl of hexane. Test stimuli were the same as in the previous experiment and were puffed the same way into the adapting airstream as above.

RESULTS

Effects of Gossyplure Alcohol on Behavioral Responses to Gossyplure. As the total quantity of a 1:1 blend of (*Z*,*Z*)-7,11-16:OH and (*Z*,*E*)-7,11-16:OH (this blend will henceforth be referred to as gossyplure OH) was increased, there were several effects on the behavioral responses of males to 1000 ng of gossyplure (Table 1). The addition of 100 ng of gossyplure OH resulted in a sig-

TABLE 1. EFFECTS OF VARIOUS DOSES OF GOSSYPLURE OH ON BEHAVIORAL RESPONSES OF MALE *Pectinophora gossypiella* TO 1000 ng OF GOSSYPLURE (INITIAL SAMPLE SIZE FOR EACH TREATMENT WAS 100 MALES)

Treatment		Conditional probability of transition[a]					Mean latency of taking flight[b] (sec + SD)
Gossyplure (ng)	Gossyplure OH (ng)	S to WF	S to F	F to UWF	UWF to SC	S to SC[c]	
1000	0	0.69a	0.99a	0.47a	1.00a	0.47a	3.5 ± 2.3c
1000	10	0.53a	0.95ab	0.34ab	0.97a	0.31b	3.8 ± 3.2c
1000	100	0.17b	0.88b	0.19b	0.76b	0.13c	5.9 ± 5.2b
1000	1000	0.01c	0.29c	0.00c		0.00d	10.0 ± 8.5a

[a] Proportions in the same column are significantly different if they do not share a letter in common ($P < 0.05$; Ryan's multiple-comparison test for proportions. S = stationary; WF = wing fanning; F = flight; UWF = upwind flight in pheromone plume; SC = source contact.

[b] Time from introduction of the moth into the wind tunnel to initiation of flight. Mean latencies in the same column are significantly different if they do not share a letter in common ($P < 0.05$; analysis of variance followed by Duncan's multiple-range test).

[c] Probability of completing the entire behavioral sequence from stationary to contacting the source of pheromone.

nificant decrease in the probability that males would initiate wing fanning (S to WF) and flight (S to F). In addition, this quantity of gossyplure OH resulted in a significant decrease in the conditional probabilities of the transition from flight to upwind flight (F to UWF) and upwind flight to source contact (UWF to SC). A significant decrease in the overall behavioral sequence was noted even when only 10 ng of the alcohols were added to 1000 ng of gossyplure. When equal amounts of gossyplure and gossyplure OH were present on the cotton wick, no males completed the sequence of behaviors involved in location of a pheromone source. In addition to these effects on the behavioral transitions of mate location, the gossyplure OH affected the mean latency of taking flight; a significant increase in the latency was found at the 100-ng dose.

A constant amount of gossyplure OH (100 ng) added to wicks loaded with various quantities of gossyplure (1, 10, and 100 μg) resulted in a significant reduction in males landing at the source (SC) only at the lowest ratio of gossyplure to gossyplure OH (10:1) (Table 2). When the ratio was 100:1, the percentage of males contacting the pheromone source was reduced slightly, but not significantly.

Effect of (Z,Z)- *and* (Z,E)-*7,11-16:OH on Behavioral Responses to Gossyplure.* Gossyplure OH (100 ng) and one of its component isomers, (*Z,Z*)-7,11-16:OH (100 ng), were similarly effective in reducing the percentage of landing at the pheromone source in the wind tunnel. In contrast, (*Z*)-7-16:OH and (*Z,E*)-7,11-16:OH had no effect on this response at the same amount (Table 3). However, in another experiment, increasing the amount of (*Z,E*)-7,11-16:OH to 1000 ng resulted in a significant decrease in the percentage of males reaching the source (reduced from 46.7% to 13.3%; $N = 30$; $P < 0.05$). No males contacted the source when 1000 ng of (Z,Z)-7,11-16:OH was present (0%, not significantly different from 13.3%).

TABLE 2. EFFECT OF 100 ng OF GOSSYPLURE OH ON PERCENTAGE OF MALE *Pectinophora gossypiella* CONTACTING PHEROMONE SOURCE[a]

Gossyplure (μg)	Gossyplure OH (ng)	S to SC (%)[b]	Reduction (%)
1	0	58.8a	
1	100	35.0b	40.5
10	0	68.8a	
10	100	58.8a	14.5
100	0	76.3a	
100	100	75.0a	1.7

[a] 80 males/treatment. Percentages in the same column are significantly different if they do not share a letter in common [$P < 0.05$; Ryan's (1960) multiple-comparison test].

[b] Percentage of males that completed the entire behavioral sequence from stationary to contacting the source of pheromone.

TABLE 3. EFFECT OF (Z)-7-16:OH, (Z,E)-7,11-16:OH, (Z,Z)-7,11-16:OH, AND GOSSYPLURE OH ON PERCENTAGE OF MALE *Pectinophora gossypiella* CONTACTING A SOURCE OF PHEROMONE[a]

Gossyplure (ng)	16-Carbon alcohols	S to SC (%)[b]	Reduction (%)
1000		42.4a	
1000	(Z)-7-16:OH (100 ng)	41.2a	2.8
1000	(Z,E)-7,11-16:OH (100 ng)	43.5a	−2.6
1000	(Z,Z)-7,11-16:OH (100 ng)	21.2b	50.0
1000	Gossyplure OH (100 ng)	22.4b	47.2

[a] 85 males/treatment. Percentages in the same column are significantly different if they do not share a letter in common [$P < 0.05$; Ryan's (1960) multiple-comparison test].
[b] Percentage of males that completed the entire behavioral sequence from stationary to contacting the source of pheromone.

Effect of (Z,Z)-*7,11-16:OH and* (Z,E)-*7,11-16:OH on Behavioral Response to Blend Ratios of Pheromone.* The addition of 10 ng of (Z,E)-7,11-16:OH to a 30:70 blend of (Z,Z)-7,11-16:Ac and (Z,E)-7,11-16:Ac (1000 ng) resulted in a significant increase in the number of males contacting the pheromone source (Figure 1). The same quantity of (Z,E)-7,11-16:OH resulted in a significant decrease in the response to an 80:20 blend of (Z,Z)-7,11-16:Ac and (Z,E)-7,11-16:Ac and had no effect on the response to a 60:40 blend ratio. (Z,Z)-7,11-16:OH (at a 10-ng dose) did not significantly affect the response to any of the blend ratios of (Z,Z)-7,11-16:Ac and (Z,E)-7,11-16:Ac. The percentage of males contacting the pheromone source was significantly attenuated by 100 ng of (Z,Z)-7,11-16:OH at the 60:40 and 80:20 blend ratios of pheromone (Figure 1).

Electroantennogram. The adapting stimuli (Z,Z)-7,11-16:Ac or (Z,E)-7,11-16:Ac reduced the EAG amplitudes to puffs of either of these same isomers (Figure 2A). However, (Z,Z)-7,11-16:Ac diminished response to (Z,Z)-7,11-16:Ac more than it diminished the response to (Z,E)-7,11-16:Ac, as illustrated by the significant differences in the ratio of antennal response (Z,Z:Z,E). Similarly, the (Z,E)-7,11-16:Ac adapting stimulus diminished EAG responses to superimposed puffs of (Z,E)-7,11-16:Ac more than it reduced responses to puffs of (Z,Z)-7,11-16:Ac. This bias in the ratio of response was still significant 2 min after the adapting stimulus was turned off (Figure 2A).

When (Z,Z)-7,11-16:OH or (Z,E)-7,11-16:OH were used as adapting stimuli, the response to the acetate isomers was significantly diminished (Figure 2B). Again there seemed to be some specificity to this adaptation as evidenced by significant differences between the ratio of response to the acetates during

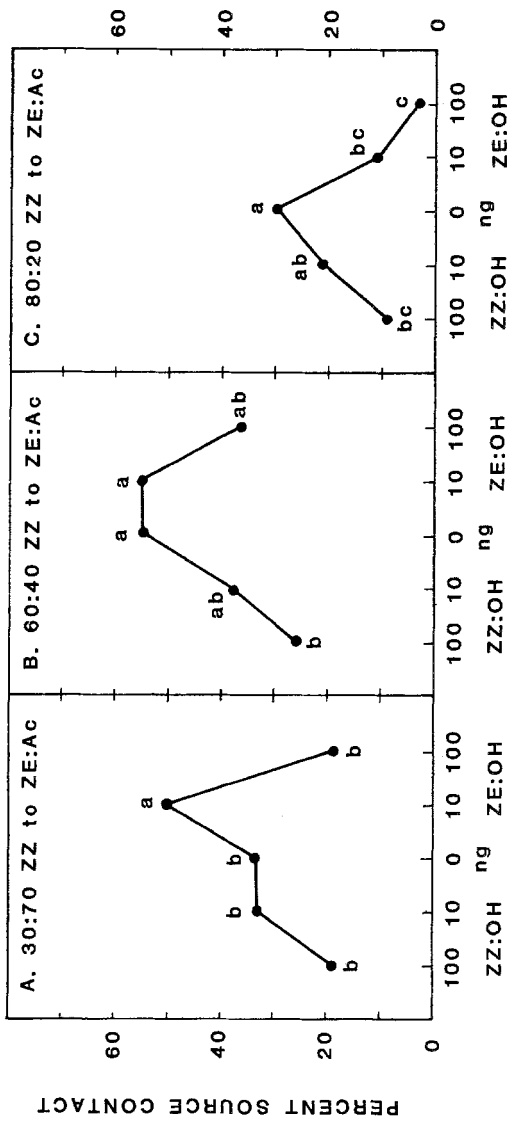

FIG. 1. Percentages of male *Pectinophora gossypiella* contacting a source (cotton dental wick) of three different blends of (*Z*,*Z*)-7,11–16:Ac (*Z*,*Z*:Ac) and (*Z*,*E*)-7,11–16:Ac (*Z*,*E*:Ac) (total 1 μg) when either (*Z*,*Z*)-7,11–16:OH (*Z*,*Z*:OH) or (*Z*,*E*)-7,11–16:OH (*Z*,*E*:OH) is added to the source. Percentages are significantly different if they do not share a letter in common [Ryan's (1960) multiple-comparison test].

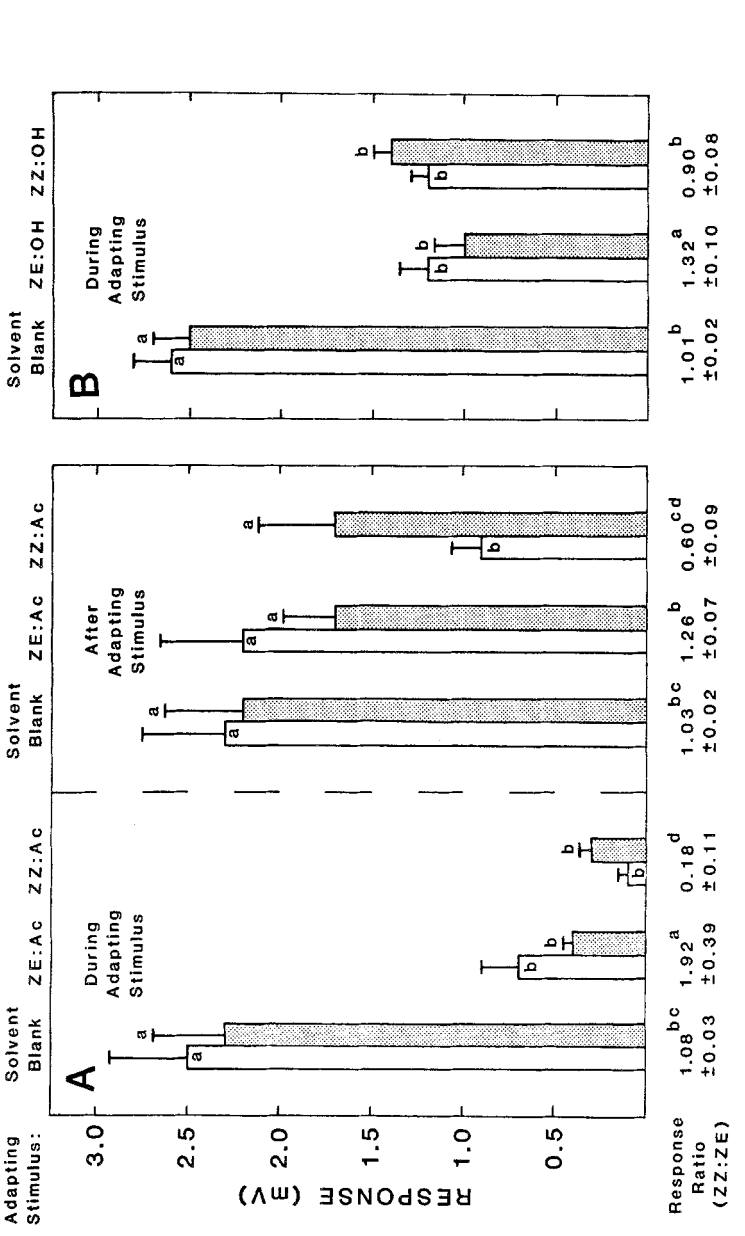

FIG. 2 (A) The electroantennogram responses of male *Pectinophora gossypiella* to (*Z*,*Z*)-7,11–16:Ac (*Z*,*Z*:Ac), and (*Z*,*E*)-7,11–16:Ac (*Z*,*E*:Ac) puffed concurrently (left) or after (right) a continuous adapting airstream containing either solvent blank, *Z*,*E*:Ac, or *Z*,*Z*:Ac. (B) Electroantennogram responses to *Z*,*Z*:Ac, and *Z*,*E*:Ac puffed concurrently with a continuous adapting airstream containing either solvent blank, (*Z*,*E*)-7,11–16:OH (*Z*,*E*:OH) or (*Z*,*Z*)-7,11–16:OH (*Z*,*Z*:OH). Vertical lines above bars indicate standard errors. Mean response ratios are shown ±SE. Mean EAG responses to the same puffed stimulus and mean response ratios within an experiment are significantly different if they do not share a letter in common ($P \leq 0.05$; Duncan's new multiple range test following ANOVA).

the (*Z*,*Z*)-7,11-16:OH adapting stimulus versus the (*Z*,*E*)-7,11-16:OH adapting stimulus.

DISCUSSION

Gossyplure OH effectively interferes with the normal sequence of behavioral responses observed when male *P. gossypiella* are stimulated by sex pheromone (Table 1). The negative effects are significant at each step in the sequence including the transition of upwind flight to source contact. This contrasts with reduced responses to off-ratios of the two acetate isomers, which most severely diminish the transition from flight to upwind flight without reducing the probability of the upwind flight to source contact transition (Linn and Roelofs, unpublished data). The cumulative effects of gossyplure OH on the behavioral responses at all stages account for the observations of Zhu et al (1983), who found that this alcohol blend interferes with the effectiveness of pheromone-baited traps. They observed that 2.5 μg of these alcohols in 30 μg of gossyplure resulted in a 97.6% reduction in trap catch of male *P. gossypiella* (0.5 μg of gossyplure OH reduced trap catch by 81.0%).

In other insects, "inhibitors" have been documented to have effects at specific stages in the behavioral sequence. For instance, (*Z*)-7-dodecenol, which decreases trap catch dramatically, does not eliminate upwind flight to pheromone in *Trichoplusia ni* (McLaughlin et al., 1974). In the gypsy moth, *Lymantria dispar*, the putative pheromone precursor found in the pheromone glands (2-methyl-*cis*-7-octadecene) actually stimulates "searching" behavior by males when it is dispersed in the environment, but reduces pheromone trap catch when volatilizing from a point source releasing disparlure (Cardé et al., 1973, 1975). Clearly the type of behavioral effect elicited by so-called inhibitors varies from species to species and from one compound to another.

The absolute amount of gossyplure OH is not as critical as its amount relative to gossyplure in determining the potential for behavioral interference. A large amount of gossyplure can overcome the negative effect of gossyplure OH. In addition, (*Z*,*Z*)-7,11-16:OH (*Z*,*Z*:OH) was more effective than (*Z*,*E*)-7,11-16:OH (*Z*,*Z*:OH) in interfering with the ability of male moths to locate a pheromone source in the wind tunnel, suggesting that the antagonistic effect of gossyplure OH may be largely due to *Z*,*Z*:OH. To explain these two findings, we propose that there are independent receptors for the two pheromone isomers, (*Z*,*Z*)-7,11-16:Ac (*Z*,*Z*:Ac) and (*Z*,*E*)-7,11-16:Ac (*Z*,*E*:Ac). Second, we suggest that *Z*,*Z*:OH and *Z*,*E*:OH interact more with receptor sites for their corresponding acetate isomers than with sites for the "opposite" isomers. Third, the interaction of alcohol molecules with acetate receptor sites interferes with the ability of those sites to produce optimal responses in the presence of the acetate isomers.

Our data provide much support for these hypotheses. If they are correct, then there should be a suboptimal blend of acetate isomers that is actually *improved* by the addition of a low dose of one of the alcohol isomers, because it interferes with the activity of the corresponding acetate. This increased response to an off-ratio should be eliminated at higher doses of that alcohol isomer. We observed precisely this pattern in response to a 30:70 blend of Z,Z and Z,E:Ac. The addition of 10 ng of Z,E:OH improved the overall response to this blend, and the improvement was eliminated at a higher dose of the alcohol. [The natural blend of Z,Z:Z,E:Ac is 61:39 according to Haynes et al. (1984), and Linn and Roelofs (unpublished data) have found the peak response of males is centered about a 60:40 and 65:35 blend ratio of Z,Z to Z,E:Ac.] Furthermore, as we expected, the addition of 10 ng of Z,E:OH to an 80:20 blend of Z,Z and Z,E:Ac resulted in a significant reduction in the response. However, we did not find a dose of Z,Z:OH that improved the response to 80:20 blend, so our hypotheses were only partially supported by the behavioral data.

The EAG results were consistent with the existence of functionally different receptor sites for each acetate isomer. In our experiments, during adaptation, the EAG response was greater in response to a puff of the acetate isomer that was not used as the adapting stimulus. Tang et al. (1980) have also postulated the presence of independent receptor sites based on an EAG study. Since both alcohols have a detrimental effect on the EAG response to both acetates, it is possible that their observed negative effect on behavior is a general, not a specific one. However, behavioral results (Figure 1) indicate that there is some independent activity of the two alcohol isomers. This independence is also seen at the receptor site level in the small but significant differences in the ratio of acetate response amplitude during adaptation with alcohols. Thus it appears plausible that the negative behavioral effects of the gossyplure alcohols are caused by an interaction of the alcohols with the acetate receptor sites, thereby reducing optimal binding with the acetates.

There are many reports of pheromone-related compounds that can interfere with the behavioral responses of male moths to sex pheromone (see references in Beevor and Campion, 1979; Cardé, 1976; Roelofs and Cardé, 1977). Unfortunately, most of the time we must infer the types of behavioral effects that these pheromone analogs elicit, since usually their effectiveness has been documented only by measuring a reduction in the number of males caught in traps. Compounds that reduce attraction are often closely related in structure to a pheromone component, suggesting that they may be interacting (suboptimally) with the same receptor sites as the pheromone component (Arn et al., 1974; Cardé et al., 1973; Roelofs and Comeau, 1971). In contrast, some evidence suggests there may be independent receptor sites for some pheromone components and "inhibitors" (McLaughlin et al., 1974; Miller et al. 1977; O'Connell et al., 1983; Schneider et al., 1974, 1977). The putative presence of independent receptors for the pheromone and "inhibitors" suggests a functional rationale for

the behavioral inhibition, such as detection of pheromone components of other species (and thus reproductive isolation could be ensured, as suggested by Roelofs and Cardé, 1977) or elimination of behavioral responses to mated females. The latter hypothesis was tested and was not supported by Cardé et al. (1973) for disparlure's olefin precursor which interrupts the response of male gypsy moths to pheromone traps.

Our behavioral and EAG studies suggest that gossyplure OH has some potential as a behavioral disruptant, but this potential is limited relative to the synthetic pheromone. Bartell (1982) suggested several mechanisms which could explain communication disruption using synthetic pheromone, including: (1) adaptation or habituation; (2) "false trail following;" (3) inability of insects to distinguish pheromone plumes from an odor background; and (4) an "imbalance in the pattern of sensory input." Bartell (1982) included an additional category which broadly covered communication disruption with nonpheromone compounds. Clearly this latter category includes compounds that could operate through one or more of the four mechanisms mentioned above. In *P. gossypiella*, gossyplure OH reduces both activation and long-range orientation to sources of pheromone, and thus may have an advantage over some "inhibitors" of other species that only affect close-range orientation [e.g., (*Z*)-7-dodecenol for *T. ni*]. However, communication disruption with gossyplure OH may be limited because sensory adaptation to *Z,Z* or *Z,E*:OH was not as severe as adaptation to *Z,Z* or *Z,E*:Ac, suggesting that one of the mechanisms hypothesized to work in mating disruption (Bartell, 1982) may not be as effective with the alcohols as with the acetates. In addition, gossyplure OH could not serve as a "false trail," and thus another hypothetical mode of action of communication disruption would be limited.

Presumably, one could use just *Z,Z*:OH and stimulate an "imbalance in the pattern of sensory input" when male *P. gossypiella* perceived the pheromone blend released by a female and this alcohol simultaneously. However, because of the greater impact of the acetates on the EAG response, it would seem that using a single acetate to disrupt mating would be more effective in this respect if one's objective is this sensory imbalance. Flint and Merkle (1983, 1984) have already documented the potential for this "sensory imbalance" using just *Z,Z*:Ac to disrupt mating. In addition to the aforementioned limitation, gossyplure OH could not be used in "attracticide" or "bioirritant" approaches, since the "attracticide" formulation relies on responders contacting the odor source (which includes insecticide), and the "bioirritant" approach relies on increased movements of the target insect in fields sprayed with the volatile chemical and an insecticide, thus presumably increasing the probability that a male will pick up a lethal dose.

We are left with two major questions that can only be addressed through field tests: (1) Does the effectiveness of gossyplure OH in interfering with ori-

entation to a point source extend to the very different situation of mating disruption? (2) How effective is mating disruption with gossyplure OH (or only a single alcohol isomer) relative to mating disruption with gossyplure (or a single acetate isomer)? The practicality and biological potential for mating disruption with gossyplure OH rests on economic considerations and the answers to these questions.

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BEHAVIORAL RESPONSES OF MALE AND FEMALE MEXICAN FRUIT FLIES, *Anastrepha ludens*, TO MALE-PRODUCED CHEMICALS IN LABORATORY EXPERIMENTS

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Abstract—The behavioral responses of male and female Mexican fruit flies elicited by male abdominal extracts were measured in laboratory cages where pheromone was applied to the undersides of some leaves on a treated tree but to none of the leaves on a control tree. After arrival to the treated tree, females came directly to pheromone sources. Females on the treated tree visited leaves and fought other females at higher rates than on the control tree. Females stayed on treated leaves and trees longer than on control leaves and trees. In separate experiments, the number of males on pheromone-treated trees and leaves was higher than on controls, but other behavior was unchanged. The results indicate that the pheromone stimulates a complex of behavior involved in the mating ecology of the species.

Key Words—Sex pheromones, Mexican fruit flies, Diptera, Tephritidae, *Anastrepha ludens*.

INTRODUCTION

Male-produced sex pheromones are known in several species of tephritid fruit flies including *Ceratitis capitata* (Ohinata et al., 1977), *Dacus tryoni* (Fletcher, 1968), *D. dorsalis* and *D. cucurbitae* (Ohinata et al., 1982), *D. oleae* (De Marzo et al., 1978), *Rhagoletis pomonella* (Prokopy, 1975), *R. cerasi* (Katsoyannos, 1982), *Anastrepha suspensa* (Nation, 1972) and *A. ludens* (Esponda-Gaxiola, 1977). Most studies have focused on attraction of sexually active females to a pheromone source. A few studies have also demonstrated that male-produced chemicals attract conspecific males in the field (Fletcher, 1968; Per-

domo et al., 1976; Ohinata et al., 1977). Other than attraction, little has been published on the behavior of either males or females elicited by the pheromones of their species. The purpose of this paper is to investigate behavior of both male and female Mexican fruit flies (*Anastrepha ludens* Loew) elicited by the male-produced pheromone in laboratory bioassays.

METHODS AND MATERIALS

General. All flies were from a laboratory culture maintained for ca. 60 generations with no wild fly introductions. The original stock came from mango (*Mangifera indica* L.) fruit collected near Mexico City, Mexico.

All experiments were conducted in the laboratory. Temperature varied between 20 and 30°C and relative humidity between 50 and 70%. Photoperiod was shifted so that lights came on at 0230 hr and went off at 1630 hr CST or CDT depending on the time of year.

Pheromone extract was prepared by grinding abdomens of sexually mature, virgin males in hexane and allowing them to soak overnight. The extract was filtered through glass wool and concentrated to one male equivalent (ME) per 5 μl of solvent under a stream of nitrogen. Extracts were stored at 0°C.

Experiments. The experiments were intended to investigate the following aspects of behavior: attraction to the vicinity of the pheromone, leaf visitation rate in the vicinity of pheromone, searching strategy near pheromone sources, attraction directly to pheromone point sources, searching strategy after arrival to point sources, and agonistic behavior and sexual displays by males near and at pheromone sources. Attraction to the vicinity of the pheromone was measured by the number of arrivals to the trees. Leaf visitation rate in the vicinity of pheromone was investigated by analysis of the number of flights (walking from leaf to leaf was negligible) originating and ending within the trees, per female on the trees. The number of females was estimated from the number observed during counts. The null hypothesis was that the number of leaves visited per female on each tree should be the same if the pheromone did not stimulate leaf visitation. Searching strategy near pheromone sources was evaluated by analysis of the time spent by individual females on the treated (T) and control (C) trees. Attraction directly to pheromone point sources was determined from the number of arrivals to T and C leaves on the T tree. Searching strategy after arrival to point sources was investigated by analysis of the time spent by females on T and C leaves. Tendencies for agonistic behavior and male displays (calling) were determined from the number of fights and calling displays, respectively, which occurred in various locations, per fly present.

Bioassays were conducted in wood-framed cages (0.7 × 1.6 × 1.0 m) with aluminum screening and a Plexiglas door (0.7 × 0.9 m) with two round service openings (15 cm diam). The cages contained a 2-cm layer of sand and

2 potted sour orange (*Citrus aurantium* L.) host trees, ca. 1 m tall. Filter paper squares (1.5 × 1.5 cm) were attached to the undersides of 20 leaves of each tree with double stick cellophane tape. Pheromone extract was applied to 10 T papers on the T tree (T = pheromone treated). The other papers on both the T and C trees were treated with hexane only and served as controls (C). Undersides of leaves were chosen as treatment sites since male sexual displays (calling) and mating frequently occurs there in *A. ludens* (Robacker and Hart 1985).

For each replication, 50 sexually mature, virgin females (experiment 1) or males (experiment 2) were released into a cage containing water but not food between 0900 and 1100 hr on the test day. *A. ludens* is not sexually active at this time (Robacker and Hart 1985). Flies were between the ages of 7 and 20 days posteclosion. Tests began at 1400 hr when *A. ludens* is sexually active. At the beginning of each test, the 10 T leaves were treated with 1.0 ME of abdominal extract in 5 μl of hexane and the 30 C leaves with 5 μl of hexane. One observer then recorded the number of flies to arrive on the T tree during a 2-min period, followed by 2-min observations of arrivals to the C tree, the T tree again, and the C tree again. The number of arrivals on T and C leaves was also recorded at this time. Next, the number of flights originating and ending in each tree was recorded for 2 min per tree. At this point, the numbers of flies on the T and C leaves and on the T and C trees were counted. Finally, arrivals to trees and leaves were monitored for another 2 min. per tree.

Agonistic interactions (physical fighting only) were recorded whenever observed throughout the experiment. During experiment 2, the number of sexually displaying (abdomen puffing, rectal pouch eversion, rapid wing vibration; Robacker and Hart, 1985) males was also recorded during the fly-location counts. In both experiments, a second observer recorded the amount of time individual flies spent on various locations during the experiment. For this purpose, a fly was considered to be on a tree until it flew away from the tree and landed somewhere else. A fly was considered to be on a leaf until it arrived at another location. Thus, flights which ended where they began were not departures from the original location.

As part of the same replication, the positions of the two trees were switched and the papers on the leaves were treated again with 1.0 ME of abdominal extract or hexane. The same series of observations was recorded again. After each test the trees were cleaned with soap and water. The two trees alternated as T and C trees and the positions of the T and C leaves were rerandomized for each replication. Twenty replications of experiment 1 and 30 of experiment 2 were conducted. Each replication took about 1 hr to conduct.

Statistical Analyses. Responses of flies on T vs. C trees were compared using paired t tests. Responses on T leaves, C leaves on T trees, and C leaves on C trees were compared with t tests using the error mean square obtained from a randomized complete block analysis of variance. Tendencies for certain

behaviors to vary either with the number of flies present at various locations or with other behaviors was measured by regression.

RESULTS AND DISCUSSION

Experiment 1. Responses of Females. The average number of arrivals to T trees was not significantly greater ($P = 0.14$) than to C trees (Table 1). Therefore, no conclusions concerning attraction to the vicinity of the pheromone source from distances of at least 0.3 m, the distance from the cage walls to the T tree canopy, can be inferred from the experiment. The variable attraction response may have been due to lack of consistent air currents in the cage which could have enabled orientation to the T tree.

The total number of within-tree flights on the T tree was significantly greater ($P < 0.01$) than on the C tree (Table 1). The number of within-tree flights per female on the T trees was also significantly greater ($P < 0.01$) than the corresponding values for C trees. Regressions of the number of within-tree flights per female on the number of females on trees were not significant (C tree: $b = 0.035$, $P = 0.5$; T tree: $b = 0.24$, $P = 0.21$), thus discounting the possibility that within-tree activity per female was related to the number of females. Regression of within-tree flights on the number of agonistic interactions was also not significant for the C trees ($b = 1.9$, $P = 0.5$), but was significant for the T tree ($b = 2.3$, $P = 0.05$). These results demonstrate that females visited leaves (made "within-tree" flights) at higher rates in the vicinity of the pheromone, but they do not show whether the increase in activity was caused directly by pheromone or indirectly through agonistic encounters with other females on the T trees.

Another question investigated was whether females modified their searching strategy so as to remain for longer periods near the pheromone source. The amount of time spent searching (flying, walking, and sitting) T trees was significantly greater ($P = 0.01$) than C trees (Table 1). Combining this result with the effects on leaf visitation rate, we conclude that females not only searched more leaves per unit time but also spent more time searching on the T trees. These pheromone-induced modifications in searching should cause higher encounter rates with males.

The number of arrivals to T leaves was significantly greater ($P = 0.001$) than to C leaves (Table 1), demonstrating that females are probably attracted directly onto male territories after arrival to the vicinity (<0.3 m) of the pheromone source. This confirms and expands the finding of Esponda-Gaxiola (1977), who showed that male abdominal extract acts as an attractant to sexually active females.

Females spent significantly greater ($P = 0.001$) amounts of time on T than on C leaves of the T trees (Table 1). Thus, searching strategy after arrival to

TABLE 1. RESPONSES OF VIRGIN FEMALE MEXICAN FRUIT FLIES TO MALE ABDOMINAL EXTRACT APPLIED TO UNDERSIDES OF LEAVES ON CITRUS TREES IN INDOOR CAGES[a]

Measurement	T tree	C tree	T tree		C tree
			T leaves	C leaves	C leaves
Arrivals[b]	6.2x	3.8x	1.0x	0.46y	0.21z
Number present[c]	2.3x	1.3y	0.13x	0.088x	0.042y
Within-tree flights[d]	15.7x	6.0y			
Within-tree flights/number present on tree[c,d]	5.6x	3.4y			
Time spent (min)[e]	3.7x	2.7y	2.8x	1.4y	1.9xy
Arrivals/number on tree[b,c]			0.25x	0.098y	0.10y
Agonistic interactions[f]	2.5x	0.25y	0.25x	0.024y	0.012y
Agonistic interactions/ number present on tree or leaf[c,f]	0.28x	0.088y	0.64x	0.076y	0.062y

[a] Data entries within the same row, corresponding to comparisons between trees or among leaves, followed by the same letter are not significantly different at the 5% level. T = pheromone treated; C = untreated.
[b] Number of females to arrive, per 12 min of observation, per tree or leaf.
[c] Number of females present, per count, per tree or leaf.
[d] Number of flights originating and ending in tree, per 4 min of observation, per tree.
[e] Per observation.
[f] Number of agonistic interactions, per tree or leaf.

pheromone point sources was also modified relative to strategy on untreated leaves. It was not possible to determine from our data whether locomotor arrest or a modified searching strategy in which females engaged in short, round-trip flights was responsible for the increase in time spent on T leaves since both behaviors were regarded as time spent on the leaf. However, the conclusion that females modified their searching strategy so as to remain near the pheromone point source is justified. This translates into a tendency for females to remain on male territories if pheromone is released by the male.

At this point it is appropriate to return to the subject of leaf-visitation rates. The number of arrivals to C leaves was almost identical on the T and C trees, per female on each tree (Table 1). This result suggests similar searching rates on the two trees, thus contradicting the previous conclusion that the pheromone stimulated activity on the T tree. However, the number of females actually available to respond to C leaves on the T tree was probably less than the number observed on the tree since females spent so much time on or near T leaves as a result of modified searching strategy. If the number of females available to respond to C leaves could be determined, the number of arrivals to C leaves on the T tree per available female would probably be greater than the arrival rate to leaves on the C tree.

Much more agonistic behavior occurred on T vs. C trees and T vs. C leaves of the T tree both before and after the appropriate conversions to the number of agonistic interactions per female present on the trees or leaves ($P < 0.01$). Regression of the number of agonistic interactions per female on either the number of females present on trees (C tree: $b = 0.04$, $P = 0.07$; T tree: $b = 0.08$, $P = 0.05$) or the number of females on leaves of the T trees (C leaves: $b = 0.05$, $P = 0.10$; T leaves: $b = 0.16$, $P = 0.08$) showed that, with more replications, significant positive relationships may exist. However, in each case, the regression coefficient is too small to account for the dramatic differences in the number of agonistic interactions on T vs. C treatments. This discounts the possibility that the greater number of females on T trees and leaves was primarily responsible for the increase in agonistic behavior. Rather, the increase in agonistic behavior was probably caused by the presence of pheromone which altered the tendency of individual females to fight.

Experiment 2. Responses of Males. Behavior at pheromone-treated vs. untreated trees and leaves was not quantitatively different, with a few exceptions. The numbers of males on the T trees during counts was significantly higher ($P < 0.05$) than on the C trees (Table 2). Also, the number of males on T leaves was significantly greater ($P = 0.01$) than on C leaves of the C tree. These results suggest that pheromone either attracted or arrested flight behavior of males. However, there were no significant differences in arrivals to T vs. C trees and leaves or in the time spent at T vs. C trees and leaves. The contradictory nature of these results compels us to accept the null hypothesis that male distribution was not affected by pheromone.

TABLE 2. RESPONSES OF VIRGIN MALE MEXICAN FRUIT FLIES TO MALE ABDOMINAL EXTRACT APPLIED TO UNDERSIDES OF LEAVES ON CITRUS TREES IN INDOOR CAGES[a]

Measurement	T tree	C tree	T tree		C tree
			T leaves	C leaves	C leaves
Arrivals[b]	5.9x	6.1x	0.72x	0.61x	0.72x
Number present[c]	4.9x	3.9y	0.15x	0.12xy	0.10y
Within-tree flights[d]	11.4x	11.8x			
Within-tree flights/number present on tree[c,d]	2.4x	2.9x			
Time spent (min)[e]	2.6x	2.7x	2.7x	2.5x	2.6x
Arrivals/number on tree[b,c]			0.12x	0.095x	0.15x
Sexually displaying males[f]	4.3x	3.9x	0.15x	0.14xy	0.11y
Sexually displaying males/number on tree or leaf[c,f]	0.90x	0.97x	0.96x	1.0x	0.97x
Agonistic interactions[g]	3.8x	2.8x	0.23x	0.15xy	0.14y
Agonistic interactions/number present on tree or leaf[c,g]	0.78x	0.72x	1.4x	1.1x	1.3x

[a] Data entries within the same row, corresponding to comparisons between trees or among leaves, followed by the same letter are not significantly different at the 5% level. T = pheromone treated; C = untreated.
[b] Number of males to arrive, per 12 min of observation, per tree or leaf.
[c] Number of males present, per count, per tree or leaf.
[d] Number of flights originating and ending in tree, per 4 min of observation, per tree.
[e] Per observation.
[f] Number of males calling, per count, per tree or leaf.
[g] Number of agonistic interactions, per tree or leaf.

More sexual displays and agonistic interactions were also observed on T leaves (T tree) than on C leaves of the C tree ($P = 0.05$) (Table 2). Dividing by the number of males on T and C leaves showed that these results reflect the greater number of males on T leaves rather than any stimulatory effect of the pheromone on sexual displays or agonistic behavior.

Compared to female responses in Table 1, male responses to pheromone treatments were weak or absent for most behaviors examined. To date, no published reports have demonstrated that tephritid males respond in laboratory bioassays specifically to pheromone from conspecific males, even though conspecific females do respond in laboratory bioassays (Nation, 1977). Males of at least three species apparently are attracted to male-produced pheromones in field assays (Fletcher, 1968; Perdomo et al., 1976; Ohinata et al., 1977). Also, the lek system used by most tropical Tephritidae requires that males have an aggregating mechanism, and possible roles of pheromones have been proposed (Perdomo et al., 1976; Prokopy, 1980; Burk and Calkins, 1983). It seems likely that experimental designs are at fault for the lack of success at proving responses of males to pheromone in the lab. One possible problem is that males themselves act as treatments wherever they produce pheromone in the bioassay chamber. These unwanted pheromone sources may then compete with the experimental treatments and also convert controls to treatments. Field tests avoid this problem since males are less likely to produce pheromones at sites where control traps are installed. Better designed laboratory and field experiments are needed to elucidate the roles of male-produced pheromones in the mating ecology of both male and female tephritid fruit flies.

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FLAVONOID PIGMENTS AND WING COLOR IN *Melanargia galathea*

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Abstract—*Melanargia galathea* L. from four different populations were classified into three categories, white, cream, and yellow, according to the background color of their wings. The flavonoid content of the wing and body tissues of butterflies in each of these categories was spectrophotometrically determined. A direct relationship was found between the wing background color and the flavonoid content of the wings; the greater the flavonoid content, the yellower the wings. Butterflies from the yellow category contained an average of 19.6 μg flavone/mg wing tissue; those from the cream, an average of 14.1 μg/mg tissue; and those from the white an average of 8.3 μg/mg wing tissue. Flavonoids represent on average about 1.9% by weight of the yellow wings; 1.4% of the cream wings; and about 0.8% by weight of the white wings. The most frequently encountered wing background color in each of the populations examined was cream; far fewer individuals had white or yellow wings. Flavonoids were not evenly distributed in the wings of *M. galathea*; greater quantities occur on the underside of the hindwings and front margins of the forewings, while the ocelli centers of the underside of the hindwings, and the inner margins on the upperside of the hindwings lack flavonoids. Furthermore, flavonoids were found in the white scales of *M. galathea* but not in the black. The flavonoid content of the body of *M. galathea* was found to be much lower than that of the wings. When expressed as a percentage of that in the wings, the flavonoid content of the body of both sexes was relatively constant; however, the flavonoid content of the female body represents on average about 59% of that in the wings, compared to about 20% in the male. Both the gross flavonoid content and the flavone concentration per milligram of tissue were found to be greater in female than male butterflies. Since the concentration of flavone per cm^2 of wing tissue was the same in both sexes, the greater flavonoid content of female than male *M. galathea* has been attributed to the deposition of relatively large quantities in the reproductive tissues of the female. The possible role(s) of flavonoid pigments in wing coloration, in protecting vulnerable tissues from damaging ultraviolet radiation, in mate selection and/or sex recognition, in insect de-

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fense against vertebrate predators, and as antimicrobial agents in *M. galathea* has been discussed.

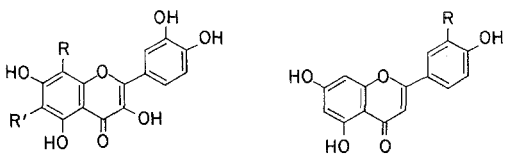
Key Words—*Melanargia galathea*, Lepidoptera, Satyridae, marbled white butterfly, flavonoids, wing pigmentation, flavonoid localization, scales, reproductive tissues, sex differences, ultraviolet radiation protection, Oudeman's phenomenon, sex recognition/mate selection, chemical defense, insect-plant interactions.

INTRODUCTION

Flavonoid pigments are known to contribute to flower color in most angiosperms. The yellow flavonols quercetagetin, gossypetin (Figure 1), and their derivatives, for instance, are responsible for providing color in the primrose, cotton flowers, and in various composites, while derivatives of the flavones luteolin and apigenin are common pigments of white, ivory, or cream flowers (Scott-Moncrieff, 1936; Harborne, 1967, 1977).

Ford (1941) suggested that flavonoids perform a similar role in butterflies, providing wing pigmentation. Although not common, flavonoids are widespread within the lepidoptera, occurring in 36 of 327 butterfly genera, 11.5%, (six of 11 families) examined, and in 10 of 192 moth genera, 5% (four of 13 families) examined (Ford, 1941; 1944). The contribution made by flavonoids to butterfly coloration is particularly noticeable in the forms of *Eurytides lysithous* Hbn. (syn: *Graphium lysithous* Hbn.), *Eurytides ariarathes* Esp. (syn: *Graphium ariarthes* Esp.), and *Atrophaneura* (syn: *Polydorus*) species, with yellow or cream markings, but they are absent from the forms with white markings (Ford, 1941).

In the marbled white butterfly (*Melanargia galathea* L.), the white coloring is structural; however, the background color of the wings is not a pure white, and varies from a creamy white to a distinct yellow. It has been shown that qualitative differences in the flavonoids sequestered by *Melanargia* from its larval food plants are not responsible for this color variation; all the individuals examined from six *Melanargia* species have the same flavonoid pattern



gossypetin, R=OH, R'=H
quercetagetin, R=H, R'=OH

apigenin, R=H
luteolin, R=OH

FIG. 1. Some flavonoid pigments of yellow and white flowers.

(Wilson, 1985a). However, it is possible that quantitative differences in their flavonoid contents are responsible.

In this paper the concentrations of flavonoids in the wings of individual *M. galathea* butterflies have been measured and the relationship between flavonoid content and wing color determined. The distribution of flavonoids within the wing and body tissues has also been examined, and the possible role(s) of flavonoid pigments in *M. galathea* is discussed.

METHODS AND MATERIALS

M. galathea was collected by the author from sites at North Moreton and Aston Tirrold, in Oxfordshire; Wayland Smithy, in Berkshire; and Barbury Castle, in Wiltshire, during July and August 1982. Only small numbers of female butterflies were collected from each site to minimize damage to population numbers in subsequent years. Butterflies were killed in the field using ethyl acetate vapor.

M. galathea Color Classification. Four of the author's collegues (two males and two females) independently classified male *M. galathea* collected from each population into three categories, white, cream, and yellow, according to the wing background color. Female *M. galathea* were not color classified; instead the flavonoid contents of their wing and body tissues were measured to determine if the quantities present were similar to those in the males.

Determination of Flavonoid Content. Flavones, which represent the class of flavonoid present in *M. galathea* exhibit two major absorption peaks in the region 200–400 nm, referred to as bands I and II. Band II of the flavones characteristically occurs between 240 and 280 nm, and band I between 304 and 350 nm.

Flavonoid extracts of *M. galathea* exhibit peaks at 272 nm and 347 nm. Because the absorbance of flavonoids in band I is less affected by impurities, the absorbance of extracts at 347 nm was selected as a measure of the flavonoid content; the higher the absorbance, the greater the concentration of flavonoids.

Ten male *M. galathea* were randomly selected from each of the white, cream, and yellow color categories of all four populations. The wings and body of each butterfly were separated, weighed, and then ground up using a pestle and mortar in a small volume (1–2 ml) of 70% ethanol. Each extract was then filtered into a 50-ml round-bottomed flask using Whatman No. 1 filter paper, concentrated to dryness in vacuo on a rotary evaporator, and the absorbance at 347 nm in 10 ml of 100% methanol determined on a Perkin-Elmer 551S spectrophotometer.

Ultraviolet Absorbance of M. galathea Wings. The presence of flavonoids in *M. galathea* causes the wings to exhibit absorption properties very similar to those of pure flavonoids. In long-wave ultraviolet light, the areas of wing which

are white in visible light appear as dark absorbing regions; the black areas of wing, though, remain black. The intensity of the absorbance in ultraviolet light is proportional to the concentration of UV-absorbing flavonoid in the butterflies' wings; the greater the absorbance, the higher the concentration.

In long-wave ultraviolet light, the appearance of the wings of two yellow category males, one white male, and one uncategorized female, from the North Moreton population, was examined. Flavonoids were removed from wings on the right-hand side of one of the yellow category males by first dipping them into 95% ethanol to "wet" them, and then immersing and gently agitating the wings in 70% ethanol at room temperature for 5 min. The immersion process was repeated several times using fresh 70% ethanol, and the wings were then dried using a hair-dryer. Unfortunately, handling this butterfly to remove flavonoids from the right-hand side damaged some of the scales on the left hand side. Ultraviolet photography, using a UV filter (wavelengths 320–366 nm) was used to record differences in the UV absorbance of these butterflies.

Determination of Distribution of Flavonoids in Wings. Ten male and ten female *M. galathea* were randomly selected from the butterflies collected. The wings of each butterfly were removed and their areas measured. Each wing was ground up using a pestle and mortar in a small volume of 70% ethanol, the extract filtered into a 50-ml round-bottomed flask using Whatman No. 1 filter paper, and concentrated to dryness in vacuo on a rotary evaporator.

To the residues of the wing extracts of male and female *M. galathea*, 2 ml and 2.5 ml, respectively of 100% methanol were added, and the absorbance at 347 nm determined.

Determination of Flavonoid Content of Black and White Scales. Areas of wing composed predominantly of black or white scales were cut from the wings of a number of butterflies. Obtaining samples of black or white scales free from contamination by each other was virtually impossible due to the nature of the black and white patterning; areas of black scales on one surface of the wings often coincide with white on the other.

Derived from predominantly black or white areas of wing, 1.8 mg of each of the black and white scales, respectively, were ground up using a pestle and mortar in a small volume of 70% ethanol, the extracts filtered using Whatman No. 1 filter paper, concentrated to dryness in vacuo on a rotary evaporator, and their absorbance at 347 nm in 2 ml of 100% methanol determined.

RESULTS

M. galathea males from all four populations were readily classified into the three color categories, white, cream, and yellow, by all four colleagues. The numbers of butterflies placed in each category are given in Table 1. More male butterflies from each population were placed in the cream category than

TABLE 1. NUMBERS OF MALE *M. Galathea* FROM FOUR POPULATIONS WITH WHITE, CREAM, AND YELLOW WING BACKGROUND COLORS

Population	No. of males in color category[a]			Total No. of males
	White	Cream	Yellow	
North Moreton	24 (24)	52 (53)	22 (23)	98
Wayland Smithy	22 (28)	41 (52)	16 (20)	79
Barbury Castle	15 (28)	27 (51)	11 (21)	53
Aston Tirrold Downs	12 (21)	30 (54)	14 (25)	56

[a] Percentages of butterflies from the four populations in each color category are given in parentheses.

in either the white or yellow. Cream therefore appears to be the most common wing background color in the wild.

In Table 2 the absorbance at 347 nm of extracts of the body and wings of male *M. galathea* in the three color categories from all four populations are given. From these data it can be seen that the wing absorbance value is directly related to the wing background color; the yellower the wings the higher the absorbance. Despite variation in the flavonoid contents of butterflies, the range of variation within each population is similar. The absorbance values range from 0.23 to 0.99 for white category wings; from 1.03 to 1.47 for cream wings; and from 1.49 to 1.94 for yellow category wings.

The mean absorbance and mean weight of the wing and body tissues of male butterflies in each color category from all four populations has been used to calculate the mean flavone concentrations (μg flavone/mg tissue) (Table 3). Flavone concentrations have been calculated on the basis that a solution of tricin containing 30 μg/ml methanol has an absorbance of 1.45 at 347 nm. Butterflies from the yellow category contain an average of 19.6 μg flavone/mg of wing tissue; those from the cream, an average of 14.1 μg/mg; and those from the white an average of 8.3 μg flavone/mg wing tissue. Flavonoids represent approximately 1.9% by weight of the yellow wings, 1.4% of the cream wings, and 0.8% by weight of the white wings.

The flavonoid content of the body of male *M. galathea* is much lower than that of the wings. From the absorbance values which are directly related to the concentration of flavone present, it can be seen that there is approximately five times as much flavone in the wings than body of *M. galathea*. In addition, when the body absorbance values are expressed as a percentage of the wing absorbance values, it appears that the flavonoid content of the body represents a relatively constant proportion, on average 20%, of that in the wings (Table 3). The wing and body absorbance values have been used to calculate the values above because, although calculations based on the absorbance of the tricin stan-

TABLE 2. ABSORBANCE VALUES AT 347 nm OF MALE *M. galathea* WITH WHITE, CREAM, AND YELLOW WING BACKGROUND COLORS

	Absorbance values (347 nm) of male *M. galathea*								
	White			Cream			Yellow		
Population	Wings	Body	%[a]	Wings	Body	%[a]	Wings	Body	%[a]
North Moreton	0.85	0.16	19	1.45	0.30	21	1.49	0.29	20
	0.57	0.12	21	1.08	0.12	11	1.89	0.37	20
	0.24	0.13	54	1.20	0.15	13	1.57	0.31	20
	0.41	0.12	29	1.03	0.14	14	1.75	0.33	19
	0.89	0.20	22	1.39	0.22	16	1.62	0.31	19
	0.35	0.14	40	1.17	0.13	11	1.55	0.31	20
	0.66	0.11	17	1.43	0.25	17	1.94	0.35	18
	0.73	0.16	22	1.10	0.11	10	1.50	0.30	20
	0.96	0.15	16	1.13	0.13	12	1.82	0.32	18
	0.69	0.15	22	1.24	0.17	14	1.87	0.34	18
Wayland Smithy	0.35	0.14	40	1.27	0.21	17	1.71	0.27	16
	0.71	0.18	25	1.45	0.30	21	1.80	0.32	18
	0.93	0.21	23	1.23	0.21	17	1.61	0.34	21
	0.52	0.15	29	1.10	0.12	11	1.55	0.30	19
	0.23	0.12	52	1.06	0.13	12	1.92	0.37	19
	0.99	0.20	20	1.40	0.31	22	1.49	0.30	20
	0.68	0.18	26	1.20	0.20	17	1.64	0.32	20
	0.55	0.16	29	1.33	0.22	17	1.53	0.30	20
	0.89	0.21	24	1.13	0.18	16	1.69	0.31	18
	0.88	0.20	23	1.39	0.26	19	1.58	0.33	21

Barbury Castle	0.95	0.19	20	1.35	0.27	20	1.58	0.32	20
	0.99	0.19	19	1.16	0.26	22	1.82	0.35	19
	0.57	0.16	28	1.39	0.28	20	1.51	0.31	21
	0.95	0.18	19	1.07	0.20	19	1.89	0.36	19
	0.76	0.17	22	1.20	0.26	22	1.51	0.31	21
	0.80	0.17	21	1.13	0.25	22	1.53	0.31	20
	0.97	0.18	19	1.10	0.21	19	1.91	0.36	19
	0.80	0.17	21	1.10	0.20	18	1.50	0.30	20
	0.75	0.17	23	1.30	0.27	21	1.52	0.32	21
	0.82	0.18	22	1.47	0.29	20	1.49	0.30	20
Aston Tirrold Downs	0.58	0.13	22	1.45	0.20	14	1.61	0.32	20
	0.92	0.15	16	1.13	0.15	13	1.68	0.31	18
	0.47	0.13	28	1.31	0.20	15	1.88	0.34	18
	0.25	0.12	48	1.40	0.19	14	1.55	0.31	20
	0.53	0.14	26	1.10	0.14	13	1.90	0.36	19
	0.88	0.16	18	1.23	0.16	13	1.66	0.31	19
	0.67	0.12	18	1.03	0.16	16	1.52	0.30	20
	0.95	0.14	15	1.12	0.12	11	1.79	0.31	17
	0.61	0.14	23	1.05	0.12	11	1.58	0.32	20
	0.77	0.15	19	1.21	0.11	09	1.76	0.33	19
Mean	0.70	0.16	25	1.23	0.19	16	1.67	0.32	19
Standard deviation	0.22	0.03	9	0.14	0.06	4	0.15	0.02	1
Range	0.23–0.99	0.11–0.21	15–54	1.03–1.47	0.11–0.31	9–22	1.49–1.94	0.27–0.37	16–21

[a] The flavonoid content of the body is expressed as a percentage of that in the wings.

TABLE 3. MEAN FLAVONE CONCENTRATIONS[a] IN MALE *M. galathea* WITH WHITE, CREAM, AND YELLOW WING BACKGROUND COLORS

	Wings					Body			Whole butterfly		
Color category	Mean absorbance value	Mean weight (mg)	Concentration of flavone (μg flavone/mg tissue)	% by weight of wings composed of flavone	Mean absorbance value	Mean weight (mg)	Concentration of flavone (μg flavone/mg tissue)	% by weight of body composed of flavone	Mean weight (mg)	Mean flavone content (μg)	% by weight of butterfly composed of flavone
White	0.70	17.51	8.3	0.83	0.15	56.04	0.55	0.06	73.55	176.15	0.24
Cream	1.22	17.88	14.1	1.41	0.20	55.49	0.75	0.08	73.37	293.72	0.40
Yellow	1.67	17.65	19.6	1.96	0.32	55.82	1.19	0.12	73.47	416.36	0.57

[a] Flavone concentrations have been calculated on the basis that a solution of tricin containing 30 μg/ml methanol has an absorbance of 1.45 at 347 nm.

dard provide a fair estimation of the quantities of flavone present, tricin is only one of 18 flavonoids previously identified in *M. galathea* (Wilson, 1985a). Further calculations based on these figures would therefore increase the likelihood of error.

The flavonoid content of the wings of the female *M. galathea* examined lies within the same range as those of the males (Table 4) and, as in the males, the flavonoid content of the body is lower than that of the wings. However, when the body absorbance value is expressed as a percentage of the wing absorbance value, it can be seen that the proportion of flavonoids in the female body is greater than that in the males; on average the flavonoid content of the female body represents about 59% of that in the wings, compared to about 20% in the male.

Differences in the flavonoid concentrations of male *M. galathea* with white, cream, and yellow wing background colors revealed by spectrophotometric examination are supported by their appearance in long-wave ultraviolet light. The ultraviolet absorbance of both the upper and undersides of the wings of males in the yellow category is greater than that of males in the white category (Figures 2 and 3). Since the intensity of UV absorbance is related to the quantity of flavonoid present, the greater absorbance of the yellow than white category males indicates that the wings of the former contain more flavonoids.

Washing the wings on the right-hand side of the yellow category males with 70% ethanol to remove flavonoids altered their behavior in long-wave ultraviolet light; wings previously ultraviolet absorbing become ultraviolet reflecting, whereas the unwashed left-hand side wings remained UV absorbing (Figures 2 and 3). Unfortunately, holding the yellow category male by the left-hand side wings during the washing procedure damaged some of the scales on this side. As a result, the wings on the left-hand side appear to be absorbing less than those of the unwashed yellow category male whose scales are intact. Prior to washing the wing, absorbance of both males was the same.

Judging from the appearance of the female butterfly in Figures 2 and 3, it is likely that had this butterfly been color classified it would have been placed in the white category since its absorbance in long-wave ultraviolet light is nearer to that of the white than to that of the yellow category male.

Similar concentrations of flavone per cm^2 of wing tissue were found in the forewings of each male and female butterfly, and, despite variation in the flavonoid contents of individual butterflies, the mean flavone concentration in the forewings of both sexes was approximately the same (Tables 5 and 6). Mean flavone concentrations of 26.65 and 25.89, and 26.63 and 25.62 $\mu g/cm^2$ wing tissue were found in the forewings of male and female butterflies, respectively. The hindwings of each butterfly also contain similar flavone concentrations, and again, the mean flavone concentration in the hindwings of both male and female butterflies was approximately the same. Mean flavone concentrations of 35.6 and 35.43, and 35.70 and 37.57 $\mu g/cm^2$ wing tissue were found in the hind-

TABLE 4. ABSORBANCE VALUES (AT 347 nm) OF FEMALE *M. galathea* FROM FOUR POPULATIONS

Population	Absorbance values (347 nm) of female M. galathea		
	Wings	Body	%[a]
North Moreton	1.68	0.91	54
	1.55	0.80	52
	1.10	0.66	60
	0.98	0.68	69
	1.24	0.72	58
	1.32	0.74	56
	1.46	0.78	53
Wayland Smithy	1.25	0.61	49
	1.43	0.76	53
	1.55	0.84	54
	1.23	0.70	57
	0.94	0.66	70
	0.82	0.63	77
	1.12	0.60	54
Barbury Castle	1.66	0.85	51
	1.54	0.82	53
	1.40	0.78	56
	0.73	0.54	74
	0.92	0.62	67
	1.37	0.76	55
	0.98	0.65	66
Aston Tirrold	1.42	0.80	56
	1.01	0.68	67
	1.66	0.90	54
	0.85	0.64	75
	1.67	0.83	50
	1.25	0.70	56
	0.97	0.66	68
Mean	1.25	0.73	59
Standard deviation	0.29	0.09	8
Range	0.73–1.68	0.54–0.91	49–77

[a]The flavonoid content of the body is expressed as a percentage of that in the wings.

wings of male and female *M. galathea*, respectively. The concentration of flavone in the hindwings of every *M. galathea* butterfly is higher than that in the forewings.

The ultraviolet absorbance of the underside of the hindwings and front margins of the forewings is greater than that anywhere else on the wings (Fig-

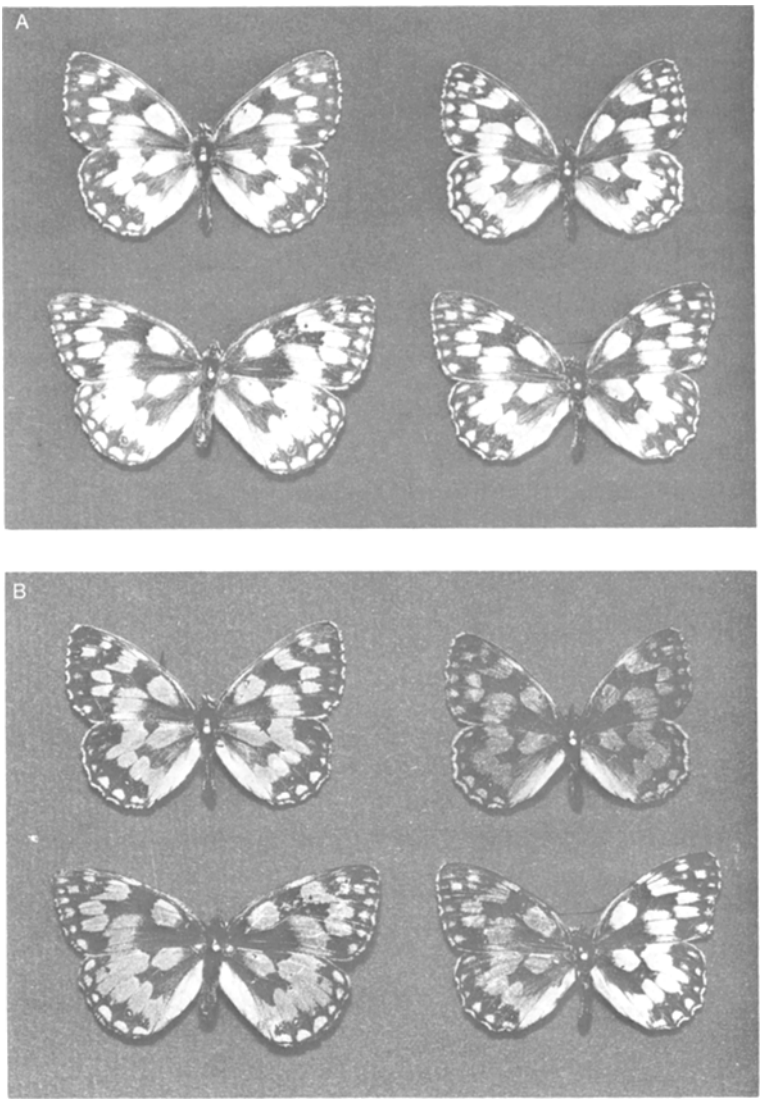


FIG. 2. (A) *M. galathea* upper sides as photographed in light visible to the human eye. Upper left, white category ♂; upper right, yellow category ♂; lower left, ♀; lower right, yellow category ♂. The right-hand side wings have been extracted with 70% ethanol. (B) The same butterflies as in (A) but as they appear in an image forming system sensitive to only ultraviolet light. Note the increased UV reflectance of the extracted wings compared to their unextracted counterparts.

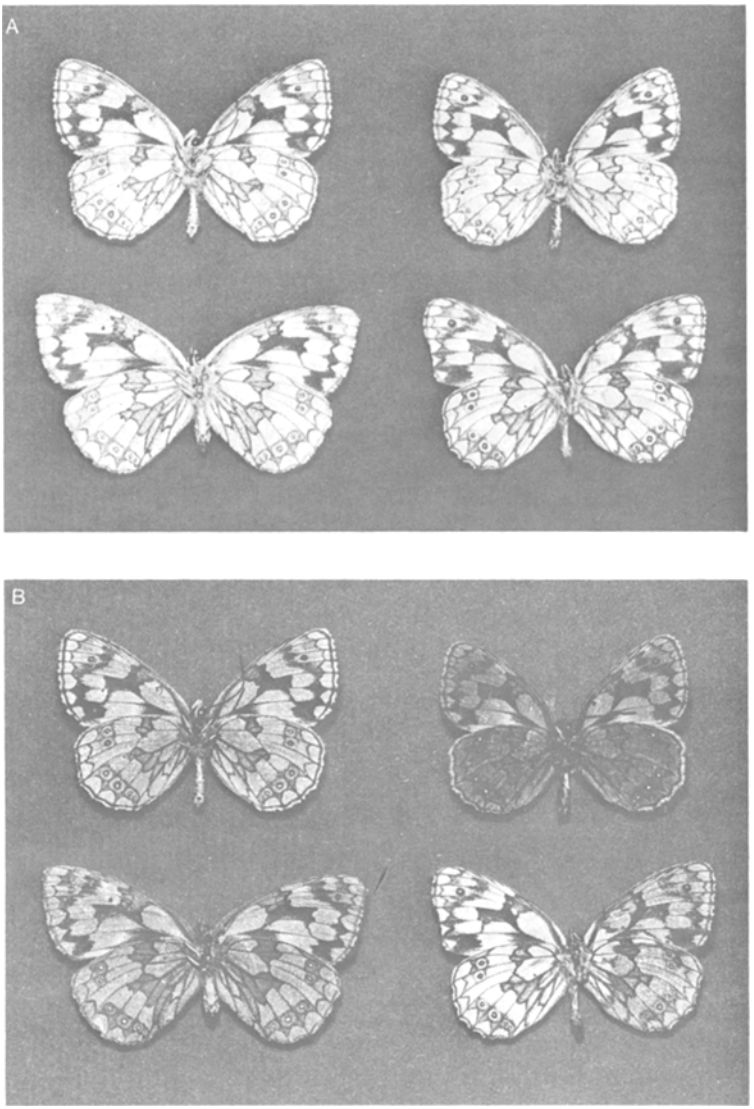


FIG. 3. (A) *M. galathea* undersides as photographed in light visible to the human eye. Upper left, white category ♂; upper right, yellow category ♂; lower left, ♀; lower right, yellow category ♂. The right-hand side wings have been extracted with 70% ethanol. (B) The same butterflies as in (A) but as they appear in an image forming system sensitive to only ultraviolet light. Note the increased UV reflectance of the extracted wings compared to their unextracted counterparts.

TABLE 5. ABSORBANCE VALUES AT 347 nm (IN 2 mls OF 100% METHANOL), WING AREA, AND CONCENTRATION OF FLAVONE ($\mu g/cm^2$ WING TISSUE) IN WINGS OF MALE *M. galathea*

	Right forewing			Left forewing			Right hindwing			Left hindwing		
Specimen No.	Absorbance value	Area (cm^2)	Flavone conc. ($\mu g/cm^2$)	Absorbance value	Area (cm^2)	Flavone conc. ($\mu g/cm^2$)	Absorbance value	Area (cm^2)	Flavone conc. ($\mu g/cm^2$)	Absorbance value	Area (cm^2)	Flavone conc. ($\mu g/cm^2$)
1	1.22	2.08	24.27	1.31	2.40	22.59	1.72	2.12	33.57	1.55	2.04	31.44
2	1.34	2.04	27.18	1.29	2.40	22.24	1.69	2.04	34.28	1.84	2.16	35.25
3	1.26	2.00	26.07	1.50	2.36	26.30	1.64	2.00	33.93	1.69	2.12	32.99
4	1.58	2.40	27.24	1.29	2.08	25.66	1.81	2.08	36.01	1.79	2.08	35.61
5	1.33	2.12	25.96	1.61	2.44	27.30	1.74	2.04	35.29	1.84	2.20	34.61
6	1.48	2.44	25.10	1.28	2.04	25.96	1.76	2.16	33.72	1.59	1.96	33.57
7	1.37	2.12	26.74	1.55	2.48	25.86	1.86	2.12	36.30	1.72	2.04	34.89
8	1.51	2.40	26.03	1.39	1.96	29.35	1.79	2.04	36.31	1.94	2.00	40.14
9	1.40	2.04	28.40	1.60	2.40	27.59	1.98	2.00	40.97	1.89	2.04	38.34
10	1.65	2.48	27.53	1.31	2.08	26.06	1.79	2.08	35.61	1.81	2.00	37.45
Mean	1.41	2.21	26.45	1.41	2.26	25.89	1.78	2.07	35.60	1.77	2.06	35.43
Standard deviation	0.14	0.19	1.22	0.14	0.19	2.14	0.09	0.05	2.17	0.13	0.08	2.61
Range	1.22–1.65	2–2.48	24.27–28.40	1.28–1.61	1.96–2.48	22.24–29.35	1.64–1.98	2.0–2.16	33.57–40.97	1.55–1.94	1.96–2.20	31.44–40.14

TABLE 6. ABSORBANCE VALUE AT 347 nm (IN 2.5 mls OF 100% METHANOL), WING AREA, AND CONCENTRATION OF FLAVONE ($\mu g/cm^2$ WING TISSUE) IN WINGS OF FEMALE *M. galathea*

	Right forewing			Left forewing			Right hindwing			Left hindwing		
Specimen No.	Absorbance value	Area (cm^2)	Flavone conc. ($\mu g/cm^2$)	Absorbance value	Area (cm^2)	Flavone conc. ($\mu g/cm^2$)	Absorbance value	Area (cm^2)	Flavone conc. ($\mu g/cm^2$)	Absorbance value	Area (cm^2)	Flavone conc. ($\mu g/cm^2$)
11	1.52	2.68	29.34	1.54	2.76	28.94	1.64	2.52	33.66	2.00	2.56	40.41
12	1.48	2.80	27.34	1.38	2.64	27.12	1.58	2.44	33.58	1.58	2.28	35.93
13	1.45	2.56	29.26	1.44	2.72	27.38	1.59	2.48	33.20	1.68	2.32	37.46
14	1.21	2.52	24.79	1.18	2.76	22.19	1.64	2.40	35.34	1.76	2.36	38.57
15	1.30	2.52	26.77	1.38	2.80	25.42	1.93	2.44	40.87	1.62	2.40	34.83
16	1.34	2.60	26.73	1.45	2.84	26.37	1.65	2.40	35.52	1.72	2.44	36.46
17	1.12	2.88	20.11	1.11	2.68	21.46	1.60	2.36	35.07	1.55	2.32	34.60
18	1.40	2.72	26.62	1.54	2.80	28.52	1.89	2.44	40.02	1.62	2.36	35.59
19	1.46	2.68	28.26	1.36	2.92	24.09	1.60	2.48	33.37	1.89	2.40	40.69
20	1.36	2.60	27.06	1.38	2.88	24.71	1.74	2.48	36.37	1.88	2.36	41.20
Mean	1.36	2.66	26.63	1.38	2.78	25.62	1.69	2.44	35.70	1.73	2.38	37.57
Standard deviation	0.13	0.12	2.66	0.14	0.09	2.53	0.13	0.05	2.72	0.15	0.08	2.50
Range	1.12–1.52	2.52–2.88	20.11–29.34	1.11–1.54	2.64–2.92	21.46–28.94	1.58–1.93	2.36–2.52	33.2–40.87	1.55–2.0	2.28–2.56	34.6–41.2

TABLE 7. ABSORBANCE VALUES (AT 347 nm) AND FLAVONE CONCENTRATIONS IN BLACK AND WHITE SCALES OF *M. galathea*

Scale color	Absorbance/1.8 mg tissue	Flavonoid conc. (μg flavone/mg tissue)
Black	0.04	0.92
White	1.75	40.23

ures 2 and 3). In view of the limited distribution of this intense UV absorption, it appears that the greater hind- than forewing flavonoid content is due to a higher concentration of flavonoids in the scales on the underside but not on the upperside of the hindwings.

Besides the areas of wing which contain relatively large quantities of flavonoids there are areas of wing which reflect UV light and therefore lack flavonoids. These occur at the inner margins of the hindwings on the upperside, and at the ocelli centers on the underside of the hindwings (Figures 2 and 3). When viewed in UV light, the absence of flavonoids from the ocelli centers produces a series of white or light reflecting spots along the outer margin of the hindwings, particularly noticeable due to the deposition of relatively large quantities of flavonoids elsewhere on the underside of the hindwings.

The flavone content of the white scales of *M. galathea* (40.23 μg flavone/mg tissue) is almost 44 times greater than that of the black (0.92 μg flavone/mg tissue) (Table 7). Although a small amount of flavone was found in the black scales, this is probably due to contamination of the extract by white scales. The black scales of *M. galathea* therefore lack flavonoids.

DISCUSSION

The results of this investigation have shown that there is a direct relationship between the flavonoid content of *M. galathea* and the wing background color; the greater the flavonoid content, the yellower the wings. Even in those butterflies with the palest wing background color, flavonoids represent on average about 0.83% by weight of the wing materials and therefore contribute "body" as well as some color to the wings. In flowers, it is usually the presence of specific flavonoids that are responsible for color differences; this is in contrast to the situation in *M. galathea* where it is different concentrations of the same combination of flavonoids that are responsible for color variation.

At first glance, the greater number of butterflies in the cream than in either the white or yellow categories of each population, and their ratios to one another (1:2:1), suggests a fairly simple Mendelian inheritance, i.e., the white and yellow winged individuals are homozygous recessive (ff) or homozygous dom-

inant (FF), respectively, for the flavone alleles, while the cream individuals are heterozygous (Ff). This, however, is not the case as the wings were only divided into three color categories for convenience; in fact, a gradation exists between them and the wings could have been divided into many more color classes, for example, very white, white, cream-white, cream, cream-yellow, yellow, very yellow. Thus, it is unlikely that a simple Mendelian inheritance is involved, and rather that wing color in *M. galathea* is polygenic and susceptible to environmental modification.

Expressing the flavonoid content of the butterfly body as a percentage of that in the wings has revealed that the proportion of flavonoids in the female body is greater than that in the male, and therefore that a sex difference exists in the distribution of flavonoids. Wilson (1985a) has shown that the eggshell, first instar pharate larvae, and first instar larvae of *M. galathea* before feeding on the larval food plants, contain flavonoids which could only have been derived from the reproductive tissues of the female. The higher concentration of flavonoids in the female than male body is therefore probably due to the localization of flavonoids in both the scales and reproductive tissues of the former. Since the flavonoids in *M. galathea* are of a dietary origin, obtained by the larval feeding habits (Wilson, 1985b), a decrease in the flavonoid content of the female is likely throughout the season concomitant with the deposition of flavonoids in the eggs.

The sequestration of flavonoids by *M. galathea* has a number of features in common with the sequestration of other secondary plant substances, particularly the cardenolides, by insects. In an earlier report (Wilson, 1985b), it has been shown that distinct and constant flavonoid patterns are produced in *M. galathea* reared on different species of grasses, defined as the *Pestuca rubra* L. flavonoid fingerprint profile, the *Lolium perenne* L. fingerprint profile, and so on. A similar phenomenon occurs when the monarch butterfly *Danaus plexippus* L. is reared on *Asclepias eriocarpa* Benth, *A. speciosa* Torr., and *A. californica* Greene; relatively constant patterns of 16–20, 20, and 18 distinct cardenolides appear in the insect, defined as the *A. eriocarpa*, *A. speciosa*, and *A. californica* cardenolide fingerprint profiles respectively (Brower et al., 1982, 1984a, b).

The flavonoid content of female *M. galathea*, which is the larger sex, when expressed as either the mean concentration per milligram of tissue, or as the total concentration per butterfly, is greater than that of the males; the mean flavone concentration per cm^2 of wing tissue is approximately the same in both sexes. In *D. plexippus* the mean cardenolide content (μg/mg tissue) of the females is also greater than that of the males; however, the males of this species are heavier than the females so that the total cardenolide content of the two sexes is approximately the same (Brower et al., 1982). More flavonoids and cardenolides, respectively, are present in the wings than body of both *M. galathea* and *D. plexippus*; approximately twice as much cardenolide occurs in the

wings than body (thorax and abdomen) of *D. plexippus* (Brower and Glazier, 1975) compared to about five times as much flavone in the wings than body of *M. galathea*. In *D. plexippus* it appears that it is not only the quantity of cardenolides in the respective tissues that differs but also their emetic potency (Brower and Glazier, 1975). While the concentration of cardenolides is greatest in the wings, intermediate in the abdomen, and lowest in the thorax, the most emetic cardenolides are found in the abdomen, followed by the wings and then the thorax, in both sexes. As yet it remains to be determined whether the different concentrations of flavonoids in the various tissues of *M. galathea* confer different properties to them.

On average flavonoids represent between 0.2 and 0.56% of the dry weight of *M. galathea*. These values are very close to those reported for *D. plexippus* in which cardenolides on average represent between 0.14 and 0.61% of the dry weight (Brower et al., 1982). The similarity in these figures suggests that both cardenolides and flavonoids are sequestered to the same extent by *D. plexippus* and *M. galathea*, respectively.

The deposition of flavonoids in relatively large quantities on the underside of the hindwings and front margins of the forewings, but their absence from the ocelli centers on the underside of the hindwings and the inner margins of the hindwings on the upperside, suggests that the distribution of these pigments within the wings is under very fine control and that they have a specific role in *M. galathea*. As the insect visual system possesses both UV and visible light receptors, it is likely that both areas of wing can be detected by *M. galathea*. Hence, it is possible that these UV reflecting areas of wing may be used by *M. galathea* in mate selection and/or sex recognition, particularly if used in conjunction with wing movements. Further support for the participation of flavonoids in a role involving the insect's visual system is provided by Ford's (1941) observation that flavonoids are only found in day-flying forms of lepidoptera.

Compared to the rest of the wings, the areas of relatively high flavonoid concentrations, namely the underside of the hindwings and front margins of the forewings, are a much darker yellow color. This may be seen in connection with Oudeman's phenomenon in which the areas of wing on display when a butterfly is in its resting position are adjusted to the color of its environment. When *M. galathea* is resting, it is the dark yellow areas of wing that are displayed; the butterfly's color therefore resembles that of the dry grasses typically found in its habitat during July and August, thus concealing it from potential predators.

The deposition of flavonoids in the reproductive tissues of *M. galathea*, particularly those of the female, mirrors the deposition of carotenoids in the reproductive organs of animals (Goodwin, 1950), and suggests their well-marked physiological function. Flavonoid pigments in *M. galathea* may function by protecting vulnerable tissues from damaging ultraviolet radiation; such a role has been suggested for flavonoids in plants (Harborne et al., 1975). Be-

sides protecting the gametes, the localization of flavonoids in the reproductive tissues will permit their transfer to the developmental materials, and so protect the developing pharate larva after leaving the insect's body.

Variation in the cardenolide content of butterflies in *D. plexippus* populations produces the well-documented palatability spectrum (Brower et al., 1969, Brower and Moffitt, 1974); variation in the flavonoid content of *M. galathea* produces a wing background color spectrum. Although flavonoids are not normally noted for their toxicity to birds and mammals, it is possible that they may be responsible for the unpalatability of *M. galathea*. Pocock (1911) reported that *M. galathea* was distinctly unpalatable although eaten by a number of birds, while Lane (1957) found that a male Shama (*Kittacincla malabarica* Gm.) would not eat *M. galathea*. Hence, the wing background color spectrum observed in *M. galathea* may be the result of a palatability spectrum involving the flavonoids. Unfortunately, at this stage, *M. galathea*'s unpalatability cannot be attributed any more to the presence of flavonoids in the insect than to any of the other substances present. However, it does seem that the striking black and white coloration of this butterfly is aposematic.

In view of the meager immunological system of insects, and the considerable bacteriocidal and antimycetic activities of flavonoids (Havsteen, 1983), it is possible that if flavonoids are involved in a protective role, their prime targets are alien microbes and invertebrate parasites rather than vertebrate predators, thus performing a role similar to that suggested for aristolochic acids in *Battus archidamas* Bsdv. (Urzúa et al., 1983) and cardenolides in *Danaus chrysippus* L. (Smith, 1978).

Despite the lack of information concerning the role of flavonoids in insects, there are a number of similarities between the roles suggested for them in this paper and those suggested in the literature for other secondary plant substances in insects. Both carotenoids (Kayser, 1982) and flavonoids appear to be involved in insect pigmentation; while both pyrrolizidine alkaloids (Edgar et al., 1974; Edgar and Culvenor, 1974) and flavonoids may be used in courtship displays. Common to the aristolochic acids (Urzúa et al., 1983), cardenolides (Smith, 1978), and flavonoids are antibiotic properties. Finally, it is possible that the cardenolides (Brower and Brower, 1964; Brower et al., 1982; Roeske et al., 1976), pyrrolizidine alkaloids (Rothschild and Aplin, 1971), aristolochic acids (Urzúa et al., 1983; Rothschild et al., 1972), mustard oils (Aplin et al., 1975), and flavonoids are involved in insect defense against vertebrate predators. Unfortunately, these roles must remain as mere speculation until further investigations elucidate the precise role(s) of flavonoid pigments in *M. galathea* and other insects.

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K. Wilson and Messrs. D. and S. Wilson for assistance collecting *M. galathea*, and finally my supervisor Professor J. B. Harborne for introducing me to the flavonoids.

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2,6-DICHLOROPHENOL IN THE TICK *Rhipicephalus appendiculatus* NEUMANN
A Reappraisal

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Abstract—Certain olfactory cells of male *Rhipicephalus appendiculatus* Neumann are known to be stimulated by 2,6-dichlorophenol. *R. appendiculatus* has been reexamined for the presence of 2,6-dichlorophenol, and it has been detected in both male and female adults. Unfed females contain 12 ng/tick, declining to 2 ng/tick after six days of feeding, while unfed males contain 2 ng/tick, falling to 0 after feeding. Larval tick extracts which are also known to stimulate 2,6-dichlorophenol-sensitive cells were examined and found to contain this phenol, whereas *R. appendiculatus* eggs did not contain this compound.

Key Words—*Rhipicephalus appendiculatus*, Acari, Ixodidae, Tick, 2,6-dichlorophenol, female, larvae, eggs.

INTRODUCTION

According to Berger (1983), the presence of 2,6-dichlorophenol (2,6-DCP) (I) has been reported in nine tick species since its first discovery in the lone star tick *Amblyomma americanum* (L.) (Berger et al., 1971; Berger, 1972), where the principal role assigned to it was that of a sex pheromone. A report by Silverstein et al., (1983) on additional species containing this compound brings the number to 12.

Other phenols such as phenol (II) itself, *p*-cresol (III), and salicylaldehyde (IV) have also been proposed as sex pheromones in ticks (Wood et al., 1975) (Scheme 1).

Rhipicephalus appendiculatus Neumann, the vector of East Coast fever (ECF) was reported by Wood et al. (1975) to contain phenol (II), *p*-cresol (III),

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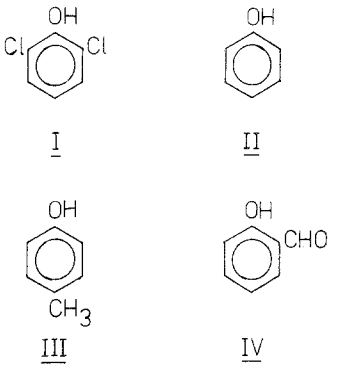


SCHEME 1

and salicylaldehyde (IV). These investigators did not find evidence of 2,6-DCP, but did, however, report that authentic 2,6-DCP attracted male *R. appendiculatus* in their T-tube bioassay. Waladde (1982) reported electrophysiological studies on *R. appendiculatus* and *Amblyomma variegatum* (Fabricius). Certain cells of particular olfactory sensillae were found to respond to low doses of 2,6-DCP (ca. 0.04 ng), while other compounds (such as II–IV) required much larger amounts. Responses of the same sensillae to tick odors and hexane washings were similar to those of 2,6-DCP. This led us to reassess *R. appendiculatus* for the presence of 2,6-DCP through analysis with capillary gas chromatography with electron capture detection (ECD), gas chromatography–mass spectrometry, and UV spectroscopy.

METHODS AND MATERIALS

Rhipicephalus appendiculatus in various stages of development were obtained from the colony maintained at the Kenya Veterinary Research Organisation, Muguga, Kenya, and maintained and fed on rabbits as described by Bailey (1960).

Extracts of male and female ticks were made by using sufficient redistilled hexane to cover the ticks and extracting for 24–48 hr at 4°C, before removing the material which was then reextracted with a minimum of hexane. The combined washings were dried briefly over sodium sulfate, then reduced in volume under a stream of nitrogen to a convenient volume depending on the original number of ticks extracted (in the range 100 μl to 2 ml). Both unfed and fed male and female ticks were extracted. Fed ticks were extracted after six days of feeding.

Each extract of adult ticks was separated by extraction with 1 N NaOH into a neutrals and bases fraction (NB) and, after acidification and reextraction of the aqueous extract with hexane, an acids and phenols fraction (AP). The acids and phenols fraction was further separated by extraction with 5% aqueous

sodium bicarbonate into a phenols fraction (P) and, after acidification and reextraction, an acids fraction (A).

An extract of 44.46 g *R. appendiculatus* larvae was obtained using hexane for 24 hr at 4°C, then removing the ticks and evaporating the solvent to a volume of 2 ml. A portion of this was further concentrated by a factor of ca. 4 for analysis.

An extract of *R. appendiculatus* eggs was obtained by thorough extraction of 10 g of eggs (produced by 75 females) with hexane at 4°C. Following removal of the eggs, the extract was briefly dried over sodium sulfate and reduced in volume to 200 μl for analysis.

The electrophysiological tests were carried out as described previously by Waladde (1982). Briefly, adult male ticks were immobilized on their ventral surface and one of the forelegs oriented to expose the desired sensillum (md 3) and the pedal nerve severed to eliminate motor activity. The tip of the sensillum was removed using metal knives on micromanipulators. Glass microelectrodes filled with tick saline and mounted on Ag–AgCl wires were used. The indifferent electrode was inserted into the tick body, while the tip of the recording electrode was brought into contact with the distal open end of the sensillum. The electrodes were connected to a Grass PI 6 preamplifier. Signals were displayed on a Tektronix oscilloscope and stored on magnetic tape using a stereo tape recorder (Recal 4DS). Olfactory stimuli were presented in an air stream of 100 ml/min passed through a glass cartridge containing a filter paper impregnated with the test sample, for a period of 1.3 sec. Clean air was passed over the preparation at all other times through a similar cartridge. Both cartridges were positioned with the outlet at 45 mm from the preparation. The air stream was switched between cartridges using an electrically operated two-way valve.

Capillary column gas chromatography (CGC) was performed on a Packard model 438 chromatograph (Packard-Becker B.V., 2624 AV Delft, The Netherlands) equipped with a Packard model 902 electron capture detector (ECD) employing a 10 mCi ^{63}Ni foil as the beta source. Capillary columns employed were either 50-m × 0.22-mm ID fused silica CP Sil 5 (OV-101 type) WCOT column operated at 170 or 180°C isothermally, or 50-mm × 0.22-mm ID fused silica CP Wax 51 (Carbowax 20 M type) WCOT column operated at 220°C isothermally (Chrompack, P.O. Box 3, 4330 AA Middleburg, The Netherlands). Injections were performed in the split mode with a split ratio of 10:1 or by splitless injection. Nitrogen, the carrier and scavenge gas for the detector, was passed through an indicating oxygen trap (Chrompack) as well as through molecular sieve water traps.

Packed column gas chromatography (GC) was performed on a Packard model 428 chromatograph (Packard-Becker B.V.) equipped with a Packard model 902 electron capture detector (ECD) employing a 10 mCi ^{63}Ni foil as the beta source. An all-glass column (3-m; 2-mm ID, 6-mm OD) packed with 5% OV-17 coated on Chromosorb W-HP, 80–100 mesh (Johns-Manville). The

column was operated at 150°C isothermally with the injector at 210°C and the detector at 300°C.

Preparative gas chromatography was carried out using an all-glass splitter as described by Baker et al. (1976), modified in shape to fit the Packard model 428 configuration. Preparative separation of *R. appendiculatus* female extract was performed on the 5% OV-17 column adapted to fit the preparative splitter device. Preparative GC was performed on the phenols fraction (P) from an extract of 2880 unfed female ticks. Three preparative fractions were obtained: PF1, PF2, and PF3.

GC peak area integration was achieved by the use of a Packard model 603 computing integrater.

Identification of 2,6-DCP from adult females ticks was confirmed by gas chromatography–mass spectrometry (GC-MS) on a Finnigan 1015D GC-MS, fitted with a Teclab I microcomputer (Tecmar Inc., Cleveland, Ohio) acting as a data system. The ion source was operated at 70 eV energy, with mass scanning in the range m/z 160–170. Identification of 2,6-DCP in larval tick extract was performed by GC-MS on a VG 12-250 quadrupole instrument at VG Analytical, Manchester, U.K. Ultraviolet spectra were recorded on a Perkin-Elmer model 402 UV-Visible spectrophotometer.

RESULTS

Acid–base extraction of male and female *R. appendiculatus* extracts indicated that electrophysiological responses were produced primarily by components in the phenol-containing fraction (P). Figure 1 shows single-cell responses to the acids and phenols fraction (AP) and neutrals and bases fraction (NB) from both fed and unfed female extracts, while Figure 2 indicates the responses from the separated phenols fraction (P) and acids fraction (A) from unfed female extracts. The response to 2,6-DCP is shown in each case for comparison. GC analysis of the extracts revealed the presence of a component with the retention time 2,6-DCP in females, fed and unfed, and in unfed males. None was detected in the fed males. Figures 3A and 3B show typical chromatograms of the phenols fraction (P) from (A) unfed females and (B) unfed males. Coinjection studies by GC with authentic 2,6-DCP showed that component 1 (Figure 3) was 2,6-DCP [coincidence occurred on all columns: OV-17 (packed column), and CP Sil 5 and CP Wax 51 capillary columns]. The largest amounts appeared in unfed females, while fed females and unfed males contained similar amounts as shown in Table 1.

Preparative gas chromatography on a 5% OV-17 packed column was used to isolate components 1 and 3 (Figure 3A) from the phenols fraction (P) of an extract of 2880 unfed adult females. Three fractions were obtained; PF1 con-

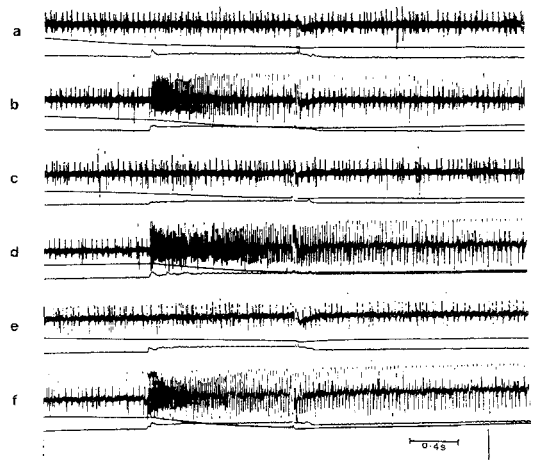


FIG. 1. Responses of a 2,6-diclorophenol-sensitive cell in sensillum md3 of a male *R. appendiculatus* to extracts of *R. appendiculatus* females. Below the AC trace is the DC trace followed by the stimulus marker trace; duration of the stimulus was 1.3 sec. (A) Control stimulation with air (no effect upon spontaneous activity). (B) Acids and phenols fraction (AP) from extract of fed females. (C) Neutrals and bases fraction (NB) from extract of fed females. (D) Acids and phenols fraction (AP) from extract of unfed females. (E) Neutrals and bases fraction (NB) from extract of unfed females. (F) 0.4 ng 2,6-dichlorophenol.

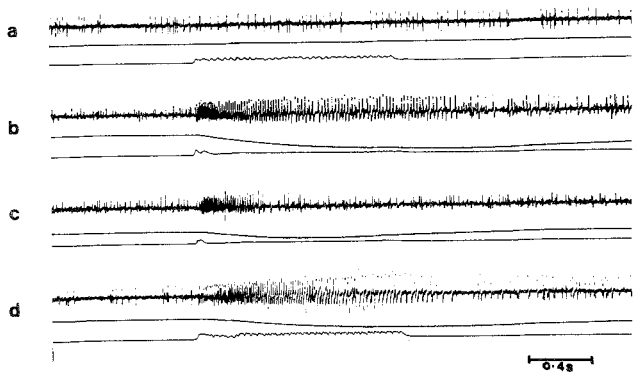
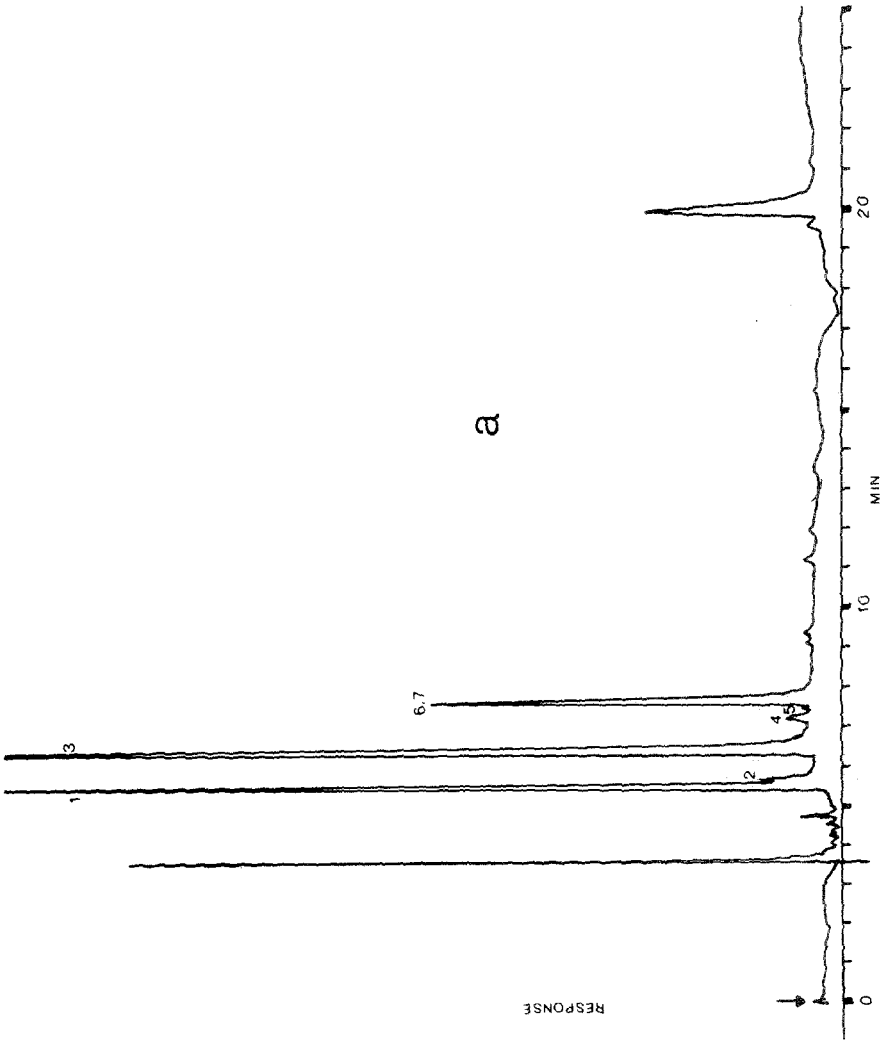


FIG. 2. Responses of a 2,6-diclorophenol-sensitive cell in sensillum md3 of a male *R. appendiculatus* to extracts of *R. appendiculatus* females. (A) Control stimulation with air (no effect upon spontaneous activity). (B) Phenols fraction (P) from extract of unfed females. (C) Acids fraction (A) from extract of unfed females. (D) 0.4 ng 2,6-dichlorophenol.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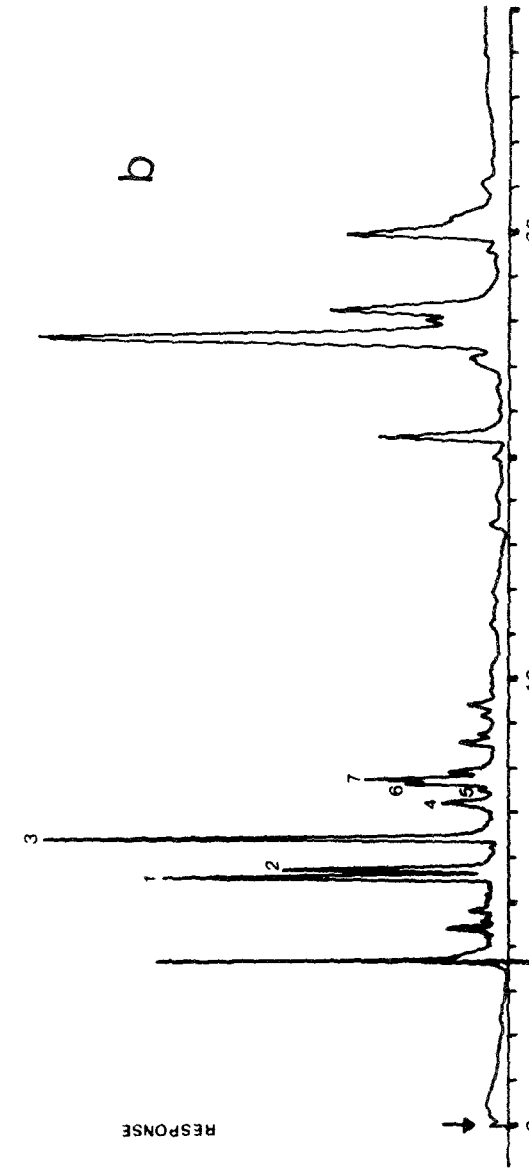

FIG. 3. Gas chromatograms of phenolic fractions of unfed *R. appendiculatus* adults. Capillary GC on 50-m CP Sil 5 fused silica column, 0.22 mm ID, 170°C isothermal, injection split ratio 10:1. (A) Unfed female *R. appendiculatus*; (B) unfed male *R. appendiculatus*.

Table 1. Estimated Quantities[a] of 2,6-DCP per Tick in *Rhipicephalus appendiculatus*

	Determination (ng/tick ± SD)				
	1	2	3	4	Mean
Unfed female	11.3 ± 1.6 $N = 9$	12.5 ± 2.2 $N = 9$	12.2 ± 1.8 $N = 9$	11.8 ± 1.5 $N = 9$	11.9
6-day fed female	2.1 ± 0.4 $N = 9$	1.9 ± 0.4 $N = 9$	2.1 ± 0.3 $N = 9$	2.3 ± 0.3 $N = 9$	2.1
Unfed male	2.2 ± 0.3 $N = 5$	1.9 ± 0.3 $N = 5$	1.8 ± 0.5 $N = 5$		2.0

[a] Quantities estimated by peak area comparisons with 2,6-DCP solutions of known concentration. The means of four separate determinations are given (three for unfed male).

tained component 1, PF2 contained component 3, while PF3 contained all other components. Components 1 and 3 were in high purity as estimated by reanalysis.

Ultraviolet spectra were recorded for fractions PF1 and PF2. PF1 displayed a spectrum similar to that of an authentic sample of 2,6-DCP with maxima occurring at λ_{max} 268, 276, and 284 nm. The relative intensities of the peaks were slightly different from those of the standard. This was shown to be due to a contribution in the UV spectrum from GC column bleed during preparative fractionation (OV-17 is a 50% phenyl silicone). Figure 4 shows the UV spectrum of component 1 and the effect of column bleed.

To confirm the identity of component 1, a mass spectrum was obtained. The spectrum was acquired only in the region of the molecular ion cluster *m/z* 160–170 in order to provide greater sensitivity. The isotope ions at *m/z* 162, 164, and 166 were found in the ratio of ca. 8.9:6.6:1.0, which approximates to the theoretical value for a compound containing two chlorine atoms (9:6:1).

The structure of component 3 (Figure 3A) remains unknown. The UV spectrum revealed little of its nature.

Fractions PF1, PF2, and PF3 were assayed electrophysiologically by the tip recording technique described by Waladde (1982). Both fractions PF1 and PF2 produced strong responses similar to those produced by 2,6-DCP, while fraction PF3 produced little stimulation.

From these data, we concluded that 2,6-dichlorophenol is indeed present in *R. appendiculatus* males and females, but predominantly in unfed females.

Waladde (1982) also reported that hexane extracts from larvae of *R. appendiculatus* stimulated the same 2,6-DCP-sensitive cells in the male, whereas extracts from eggs did not produce stimulation. A larval extract was examined by GC and GC-MS for the presence of this compound. Fig. 5 shows a GC (EC detection) of whole larval extract. The chromatogram showed a peak with the

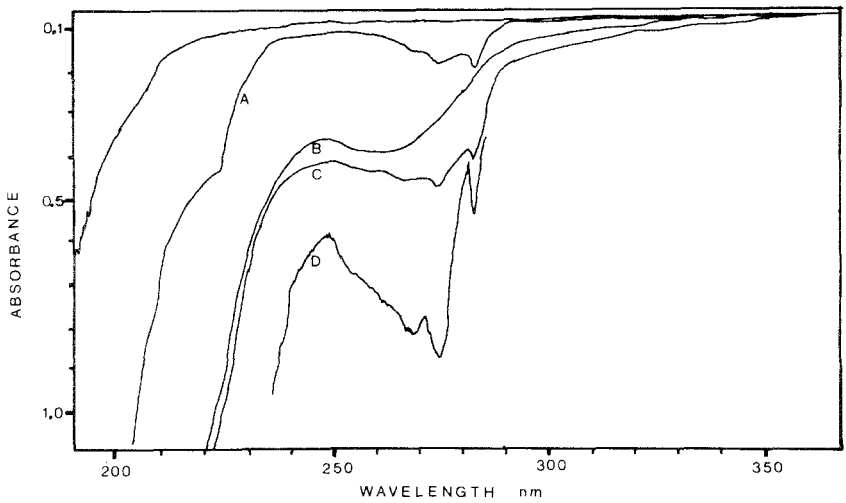

FIG. 4. Ultraviolet spectra of 2,6-dichlorophenol (authentic and isolated) in hexane solution from 190 nm to 360 nm. (A) Authentic 2,6-dichlorophenol; λ_{max}: 268, 276, and 284 nm. (B) Background spectrum due to OV-17 preparative GC column bleed. (C) 2,6-Dichlorophenol isolated from extracts of unfed female *R. appendiculatus*, λ_{max}: 268, 276, and 284 nm. (D) Trace C with magnification factor of five.

correct retention time for 2,6-DCP. GC-MS analysis of this extract showed that this component had a mass spectrum (Figure 6) matching that of authentic 2,6-DCP. Extracts of eggs were also examined by GC and 2,6-DCP was not detected. We therefore concluded that larval ticks also possess the compound.

DISCUSSION

The ubiquitous 2,6-dichlorophenol has been found in *R. appendiculatus*, contrary to the original report on this species (Wood et al., 1975). Both adult males and females contain the compound, with largest amounts occurring in unfed females adults. The levels of 12 ng/unfed females are comparable to quantities found in other species.

The onset of feeding in the ticks appears to stimulate the release of 2,6-DCP into the environment as evidenced by the reduced amounts found in fed ticks. This is in keeping with observations made by Waladde (1982) that, while tick washes from unfed and partially fed females were equally stimulatory, vapors emanating from live unfed females were not as stimulating as those from live partially fed virgin females.

Other investigators have noted variations in 2,6-DCP content with feeding. For example, in *Dermacentor variablis* (Say), feeding appears to cause release

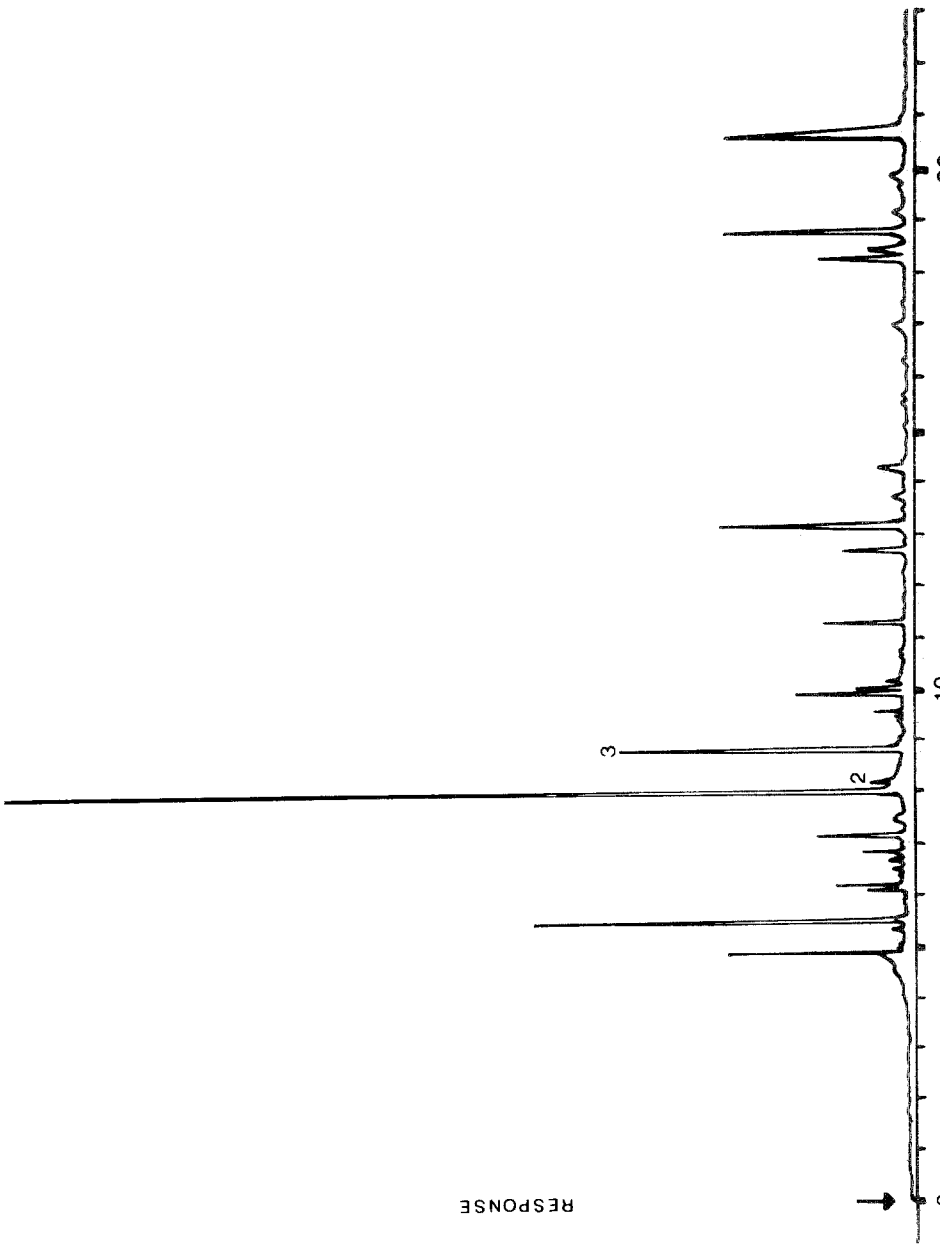

FIG. 5. Gas chromatogram of extract of *R. appendiculatus* larvae. Capillary GC on 50-m CP Sil 5 fused silica column, 0.22 mm ID, splitless injection at 30°C (injector 200°C) with isothermal analysis at 180°C.

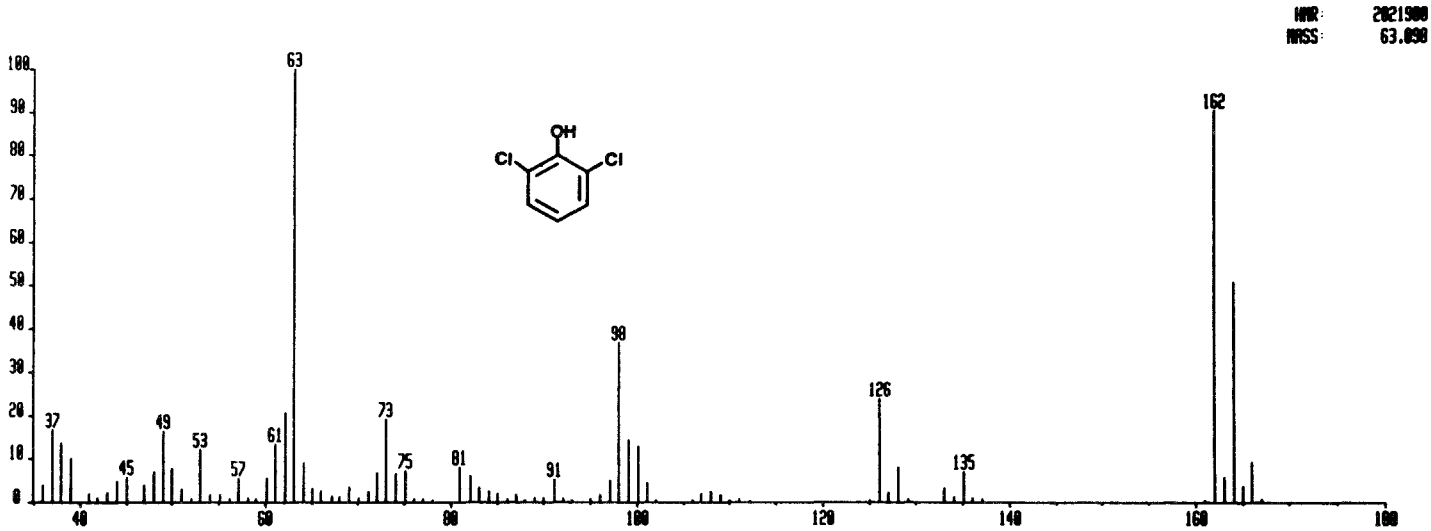

FIG. 6. Mass spectrum of 2,6-dichlorophenol from larval tick extract.

of the compound (Sonenshine et al., 1982). In other cases, feeding does not appear to cause a decrease in 2,6-DCP content. In *Amblyomma americanum* and *A. maculatum* (Koch) females, 2,6-DCP content increases from almost nothing in newly emerged adults to a maximum after 16 days. Subsequent feeding does not significantly increase or decrease the 2,6-DCP content (Kellum and Berger, 1977). These investigators suggested that it would be dangerous to assume that whole-body analysis gives a true indication of the dynamic production of 2,6-DCP. It is rather a measure of the ability of the animal to synthesize and store the compound. In the case of *R. appendiculatus*, it seems that the newly emerged adults already have a significant reserve of the compound. While feeding stimulates the release of 2,6-DCP in the adult, it is not known if further synthesis takes place, as suggested by the general production and storage scheme proposed by Sonenshine et al. (1979).

Waladde (1982) clearly showed that larval washes of *R. appendiculatus* contained a compound or compounds that stimulated 2,6-DCP-sensitive cells in the male, whereas vapors from egg washes did not stimulate these cells. Our analyses of egg and larval extracts have shown that eggs do not contain 2,6-DCP, whereas larvae do contain the compound. We therefore presume that larvae are capable of producing the compound, although we have not yet estimated the quantities they contain. Analyses are currently being performed to quantitate 2,6-DCP in larvae and to detect and quantitate 2,6-DCP in nymphae (preliminary evidence suggests that it is present in nymphae).

The precise role of 2,6-DCP in *R. appendiculatus* is unclear, but it may well act as a sex pheromone, as in other species. Females attach and feed at one site until they are fully engorged, but males achieve sexual maturity after feeding for a few days and later detach to seek receptive females. This male activity coincides with the copious release of 2,6-DCP by the females. It is possible that copulatory behavior is evoked when a male encounters a female emitting a high concentration of 2,6-DCP. Wood et al. (1975) clearly demonstrated that 2,6-DCP attracts male ticks in a T-tube bioassay, although they did not detect it in *R. appendiculatus* females. Waladde's results (1982) suggested that the cells of sexually immature males were more responsive to 2,6-DCP than those of sexually mature males, the opposite of the situation for *R. americanum* as reported by Haggart and Davis (1981).

A multicomponent system seems a likely possiblity here in that phenol and *p*-cresol, which increases with feeding in *R. appendiculatus*, also attracted males in a T-tube bioassay (Wood et al., 1975). The sensitivity of the tick olfactory cells to phenols is evidently variable. In the case of the latter two phenols the cells highly responsive to 2, 6-DCP in males require much higher doses of phenol and *p*-cresol to give responses (Waladde, 1982). It is clear that the role of phenolic compounds in *R. appendiculatus* is poorly understood and behavioral studies are planned to define the role played by 2,6-DCP and other phenols

in this species. Further chemical studies are ongoing to structurally elucidate other compounds which stimulate *R. appendiculatus* olfactory cells, and confirm the presence and quantitate 2,6-DCP in larvae and nymphs.

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INHIBITORY ACTION OF (4*S*,6*S*,7*R*)-ISOMER TO PHEROMONAL ACTIVITY OF SERRICORNIN, (4*S*,6*S*,7*S*)-7-HYDROXY-4,6-DIMETHYL-3-NONANONE

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Abstract—By adding the effects of a series of the stereoisomers to the pheromonal activity of serricornin, (4*S*,6*S*,7*S*)-7-hydroxy-4,6-dimethyl-3-nonanone, the sex pheromone of the cigarette beetle *Lasioderma serricorne* (F.) was investigated. The experiments using synthetic enantiomeric mixtures and optically active stereoisomers showed that the (4*S*,6*S*,7*R*)-isomer inhibited significantly the pheromonal activity of serricornin.

Key Words—Sex pheromone, cigarette beetle, *Lasioderma serricorne* (F.), *Coleoptera*, *Anobiidae*, serricornin, 7-hydroxy-4,6-dimethyl-3-nonanone, inhibitory action of diastereoisomer.

INTRODUCTION

Serricornin, (4*S*,6*S*,7*S*)-7-hydroxy-4,6-dimethyl-3-nonanone, is the sex pheromone of the cigarette beetle *Lasioderma serricorne* (F.), which is a worldwide pest for cured tobacco leaves and various dried foodstuffs (Coffelt and Burkholder, 1972). As the serricornin molecule has three chiral centers at C-4, C-6, and C-7, there are eight possible stereoisomers shown in Figure 1, in which the isomers in the upper row, such as (4*S*,6*S*,7*S*), and the corresponding ones in the lower row, such as (4*R*,6*R*,7*R*), are enantiomerically paired, respectively.

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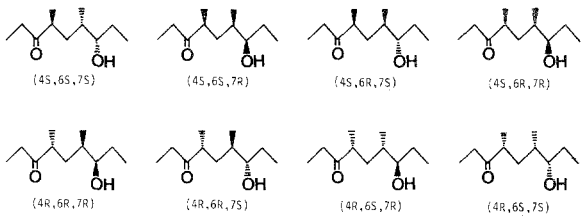

FIG. 1. Eight possible structures of serricornin and diastereoisomers in acyclic forms.

Up to now six of them have been synthesized as such or as the O-acetyl derivatives (Chuman et al., 1981; Mori et al., 1982a,b; Mori and Watanabe, 1985).

Our serial studies on the relationship between the stereochemistry of the serricornin molecule and biological activity revealed that the (4*S*,6*S*,7*S*)-stereoisomer, which is the natural form of the sex pheromone (Mori et al., 1982a,b), is at least 10^3 times more active than any of the other diastereoisomers (Chuman et al., 1982a,b; Mochizuki et al., 1984). In this paper we report the additive effect of these stereoisomers in enantiomeric mixtures and of the optically active stereoisomers on the pheromonal activity of serricornin.

The enantiomeric mixtures used are designated herein as SSS, SSR, SRS, and SRR which mean mixtures of (4*S*,6*S*,7*S*) and (4*R*,6*R*,7*R*) isomers, (4*S*,6*S*,7*R*) and (4*R*,6*R*,7*S*) isomers, (4*S*,6*R*,7*S*) and (4*R*,6*S*,7*R*) isomers, and (4*S*,6*R*,7*R*) and (4*R*,6*S*,7*S*) isomers, respectively.

METHODS AND MATERIALS

Rearing the Insects. The beetles were reared on corn flour supplemented with 8% dried brewer's yeast (EBIOS®, Asahi Breweries Ltd., Tokyo) at 28°C and 60% relative humidity. For the following pheromonal bioassay, virgin males were sexed during the pupal stage by their external characteristics and reared separatedly from the atmosphere of female adults under the same condition.

Pheromonal Bioassay. The method of pheromonal bioassay is essentially the same as described previously by Chuman et al. (1982a) and Mochizuki et al. (1984). At 9–10 AM of the day for bioassay, ten healthy virgin males 7–10 days after adult emergence were put on an arena of filter paper tightly fitted on the floor of a Petri dish (60-mm ID, 15-mm height). Meanwhile almost all of these insects came to rest on the arena. At 2–3 PM of the same day, the cover was opened and a small screen made of rectangular filter paper (5-mm height, 25-mm length, folded in W-letter shape), which was previously impregnated with the test substance, was put on the center of the arena, the dish was covered again immediately (Figure 2). Impregnation of the test substance on the screen was performed just before the bioassay by adding 2 μl of *n*-hexane solution, pipetted from an appropriately diluted solution prepared from stock solution (5

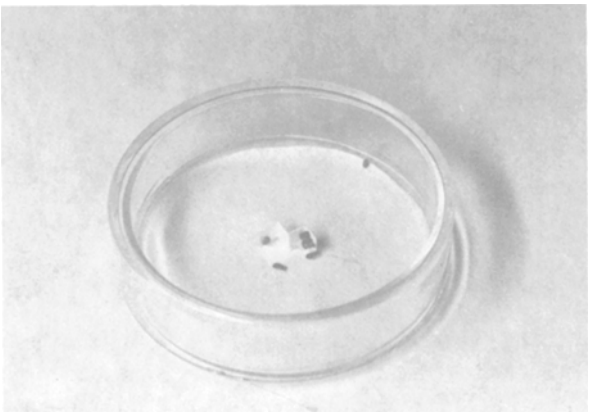

FIG. 2. Pheromonal bioassay using a Petri dish. Many virgin males are crowding on the folded paper screen (center), which has been previously impregnated with a pheromonally active substance. Some of them are attempting to mate with each other. See Methods and Materials for the preparation.

mg/ml), and evaporating the solvent by allowing the preparation to stand for ca. 10 sec.

If the test substance was active as the sex pheromone, the males became alert, raised their antennae, and quickly walked in a zig-zag fashion on the arena seeking the pheromone source. Then they climbed and dropped from the screen and, after a while, attempted to mate with each other. Observation was maintained for 10 min after setting the screen on the arena. Evaluation of the pheromonal activity of each test substance was performed with respect to the following three parameters: (1) attractiveness (accumulated number of times that males climbed the screen), (2) sex stimulation (accumulated number of attempted matings), and (3) maximum aggregation number (maximum number of males staying simultaneously on the screen). Each male was used only once during bioassays.

Enantiomeric Mixtures. Four enantiomeric mixtures SSS, SSR, SRS, and SRR were obtained with purities of more than 99.6% by chromatographic separation of the conventionally synthesized serricornin, a mixture of diastereomers (Ono et al., 1980) with or without C-4 epimerization (Mori et al., 1984a).

(4S,6S,7S)-7-Hydroxy-4,6-dimethyl-3-nonanone (Serricornin). Optically active serricornin was synthesized by the method of Mori and Watanabe (1985). This compound exists as a mixture of the cyclic hemiacetal and acyclic forms (Mori et al., 1984b) in a molar ratio of 2.5 : 1 in benzene solution.

(4S,6S,7R) Isomer. The (4*S*,6*S*,7*R*) isomer was prepared according to the synthetic method for serricornin (Mori and Watanabe, 1985) by the coupling of

diethyl ketone with (2*R*,3*R*)-3-*t*-butyldimethylsilyloxy-2-methylpentyl iodide instead of its (2*R*,3*S*) isomer, followed by silica gel column separation. GLC analysis (cross-linked methyl silicone, fused silica capillary column, 50 m) of the corresponding acetate, $[\alpha]_D^{23}$ + 21.38° (c = 0.32, *n*-hexane), showed the optical purity to be more than 98%. The 500-MHz PMR spectrum of the underivatized sample in deuterobenzene also showed the absence of any of the other diastereoisomers.

RESULTS AND DISCUSSION

Biological Assays Using Enantiomeric Mixtures. As SSS has been proven to have an efficient activity as the sex pheromone substance to male cigarette beetles (Chuman et al., 1979), the effect of the addition of other enantiomeric mixtures, namely SSR, SRS, and SRR, was examined individually. In these systems, the level of SSS was fixed as 1×10^{-1} μg and that of added agents was varied as 0.1×10^{-1}, 0.5×10^{-1}, and 1×10^{-1} μg, respectively. The results are summarized in Figure 3, in which the activity is expressed in relative values. The absolute values for SSS itself (1×10^{-1} μg) were 70.7 (±SD 25.6, $N = 5$), 8.5 (3.9), and 7.9 (1.6) for attractiveness, sex stimulation, and responding ratio, respectively.

These data indicate that the activity of SSS is remarkably decreased in all three parameters by adding SSR even in the ratio of 1:0.1 ($t = 3.51$; $P < 0.01$, $t = 2.48$; $P < 0.05$, and $t = 3.19$; $P < 0.01$ for attractiveness, sex stimulation, and maximum aggregation, respectively). The addition of SRS or SRR caused neither inhibitory nor synergistic effect on the activity of SSS up to the ratio of 1:1. (The differences between controls and $0.1–1.0 \times 10^{-1}$ μg additions were not significant at $P > 0.10$ in all cases.) From this observation, the enantiomeric mixture SSR was considered to play an inhibitory role against the natural stereoisomer of serricornin.

Inhibition by (4S,6S,7R) Isomer. To confirm the above results and to determine the exact inhibitory component, the same bioassay was carried out by using optically active serricornin and the (4*S*,6*S*,7*R*) isomer. Here also the level of serricornin was fixed as 1×10^{-1} μg and that of the adding agent (4*S*,6*S*,7*R*) isomer was varied as 0.1×10^{-1}, 0.5×10^{-1}, and 1×10^{-1} μg. As seen in Figure 4, the result obtained in this experiment seemed to be almost identical to that obtained for SSR against SSS (Figure 3). Both parameters, attractiveness and sex stimulation, for serricornin are decreased to 20–30% of the originals by adding the same amount of (4*S*,6*S*,7*R*) isomer ($t = 7.91$; $P < 0.001$ and $t = 8.93$; $P < 0.001$, respectively, Figure 4). This result indicates that the inhibitory component against the pheromonal activity of serricornin is its (4*S*,6*S*,7*R*) isomer.

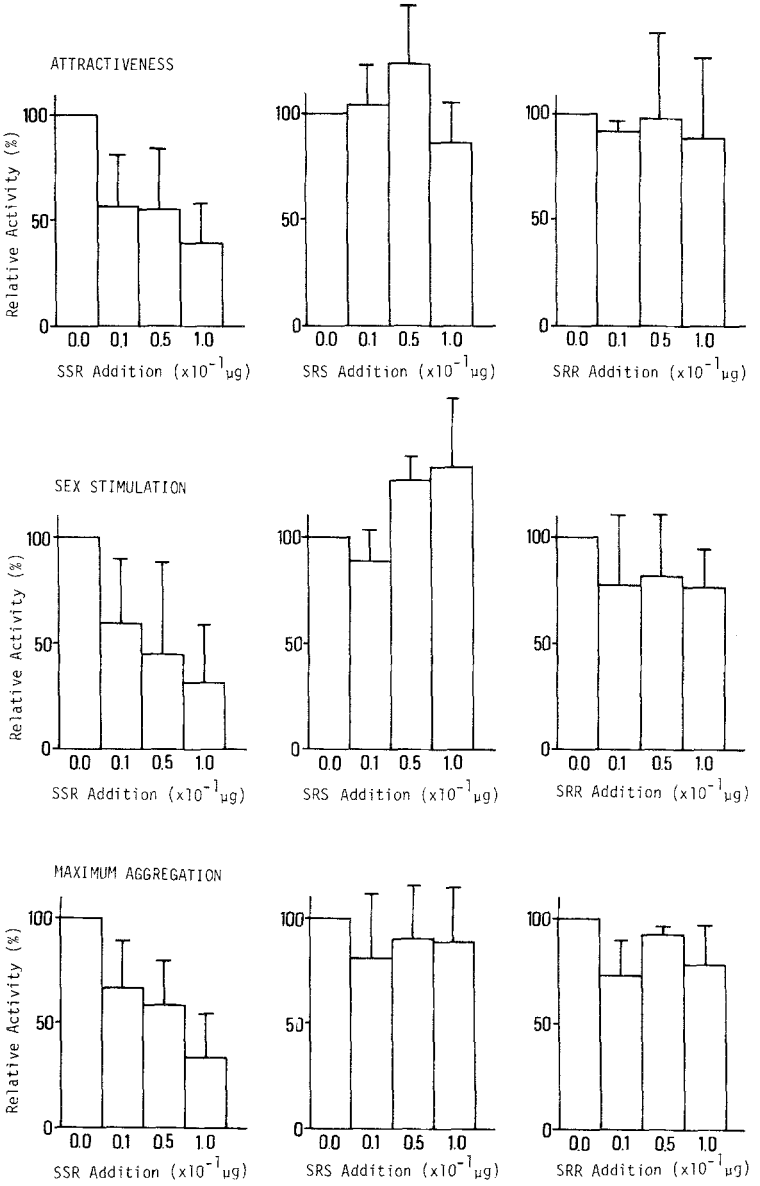

FIG. 3. The effect of enantiomeric mixture addition on the pheromonal activity of SSS. Each test was replicated five times and the standard deviations are shown by T bars.

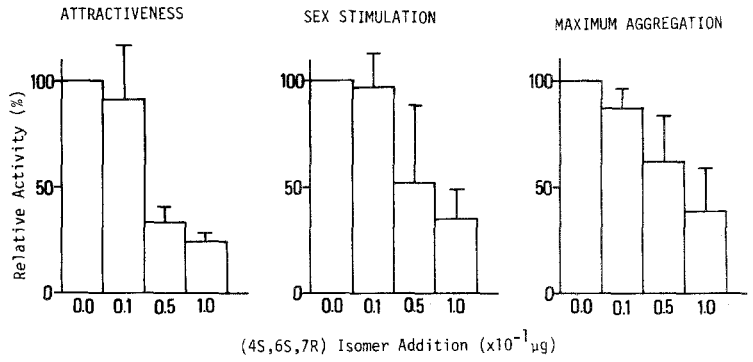

FIG. 4. The effect of (4*S*,6*S*,7*R*) isomer addition on the pheromonal activity of serricornin. See the caption of Figure 3.

These findings suggest that (4*S*,6*S*,7*R*) isomer competitively participates in the receptor for serricornin on the antennae of male cigarette beetles. In fact, recent results of EAG (electroantennography) experiments for all four enantiomeric mixtures indicated higher responses of SSS and SSR than those of SRS and SRR (Mochizuki et al., 1984).

One possibility, that the (4*R*,6*R*,7*S*) isomer, which is the enantiomer of (4*S*,6*S*,7*R*) isomer, also inhibits the pheromonal action of serricornin still remains. But this possibility is precluded because the profiles and the magnitudes of the inhibitory actions of SSR against SSS (Figure 3) and of (4*S*,6*S*,7*R*) isomer against serricornin (Figure 4) were coincident with each other.

Serricornin thus is an example of those pheromones in which a nonnatural, synthetic diastereoisomer inhibits the pheromonally active stereoisomer; similar inhibition by the enantiomers of certain sex pheromones has already been reported for gypsy moth *Porthetria dispar* (Yamada et al., 1976; Klimetzek et al., 1976), Japanese beetle *Popillia japonica* (Tumlinson et al., 1977), and southern pine engraver *Ips calligraphus* (Vité et al., 1976).

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ORIENTATION OF BOLL WEEVIL, *Anthonomus grandis* BOH. (COLEOPTERA: CURCULIONIDAE), TO PHEROMONE AND VOLATILE HOST COMPOUND IN THE LABORATORY[1]

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Abstract—Behavioral responses of male and female boll weevils to the aggregation pheromone, grandlure, and the major volatile of cotton, β-bisabolol, were investigated using a new dual-choice olfactometer. Dosage-response experiments revealed both males and females to be attracted by the aggregation pheromone at the 1.0 μg dosage. However, only males were attracted to β-bisabolol (1.0 μg). Both sexes were repelled by the highest dosage of β-bisabolol tested (10 μg). In preference experiment, males chose grandlure over β-bisabolol, while both sexes chose the combination of grandlure + β-bisabolol over β-bisabolol alone. There was some evidence for synergism between pheromone and plant odor for the females. The results correlate well with previous electrophysiological and behavioral experiments.

Key Words—Boll weevil, pheromone, host odor, olfactometer, preference, *Anthonomus grandis*, Coleoptera, Curculionidae, cotton, behavior, bioassay, orientation.

INTRODUCTION

The orientation of insects to their host plants and mates has been the subject of numerous investigations (see Bell and Cardé, 1984). In certain coleopterous insects this relationship is complex since the sex reponsible for host selection

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

often releases in its frass an aggregation pheromone which attracts members of both sexes for both feeding and mating purposes (Birch, 1984; Dickens, 1984). Thus at any one time in the field, insects must choose between suitable host plants and those that have already been colonized or fed upon by pheromone-producing conspecifics. The relationship of the boll weevil, *Anthonomus grandis* Boh. (Coleoptera: Curculionidae), to its host plant, *Gossypium hirsutum* L. (Malvales: Malvaceae), is particularly interesting since this species is narrowly oligaphagous and must find a suitable host for production of its pheromone and subsequent mating and egg deposition (Cross, 1983).

Overwintered males consistently compose 60–65% of boll weevil populations emerging in the spring, and there is some evidence that the first few weevils to emerge are males (Cross, 1983). These males may be responsible for locating a suitable host. Upon locating a suitable host, the male begins to feed and release an aggregation pheromone which attracts both sexes for feeding and mating (Tumilinson et al., 1969; Hardee et al., 1969, 1972).

Two theories have been proposed for host location by adult weevils. One theory asserts that boll weevils locate potential hosts by random flights and are not attracted to the cotton plant from distances over a few centimeters (Hunter and Pierce, 1912; Hardee et al., 1969; Cross, 1973, 1983). Another theory maintains that volatiles emanating from the cotton plant attract boll weevils from some distance (Parencia et al., 1964; Smith et al., 1965; Mistric and Mitchell, 1966; Mitchell and Taft, 1966). Both of these theories have their adherents. Those who adhere to the random flight theory cite the experiments of Hardee et al. (1969) which showed that response of overwintered and migrating boll weevils to males and plants + males greatly exceeded response to fruiting cotton plants which was similar to control (empty) traps. However, cotton plant volatiles have been shown to be attractive in laboratory bioassays (Hardee et al., 1966; Minyard et al., 1969; Thompson et al., 1970) and field tests (Keller et al., 1965; McKibben et al., 1977).

Recent electrophysiological investigations revealed male boll weevils to be as sensitive to certain plant odors, e.g., β-bisabolol (the major volatile of cotton, Thompson et al., 1971), as to components of their aggregation pheromone (Dickens, 1984). Furthermore, threshold and saturation levels determined for various odorants by these investigations also indicated the presence of at least two olfactory receptor populations: one primarily responsive to pheromone components and related compounds; the other primarily responsive to plant odors, e.g., β-bisabolol.

The purpose of this study was to investigate the behavioral effects of activating pheromone and plant odor receptor populations. The experiments were also intended to elucidate enigmatic field and laboratory data on response of the boll weevil to insect and host-plant volatiles. To accomplish these objectives, a new dual-choice olfactometer for the boll weevil was designed.

METHODS AND MATERIALS

Insects. Newly emerged *A. grandis* adults were obtained from a small laboratory colony annually infused with feral insects. Insects collected in the morning (ca. 0800 hr) were sexed and held in Petri dishes (1.0 × 10 cm; 5 insects/dish) on moist filter paper at 26°C without feeding until testing that afternoon (ca. 1400–1700 hr).

Chemical Stimuli. Odorous stimuli used in this study are listed in Table 1. The aggregation pheromone of the boll weevil was a 3:4:3 mixture of compounds I:II:III + IV. The plant attractant chosen, β-bisabolol, was previously shown to be attractive in laboratory bioassays (Minyard et al., 1969). Both the aggregation pheromone and plant attractant were diluted in nanograde pentane. Stimulus dilutions were delivered as 10-μl aliquots placed on filter paper discs (2.15 cm diameter) inserted diagonally into the test vials (Figure 1).

Bioassay Device. A dual-choice behavioral bioassay was devised for use in these experiments (Figure 1). Five insects were released from a 1 dram screw-cap vial through a hole (1 cm diameter) located 1 cm from the center of the bottom of an 8.0 × 4.0 cm crystallizing dish (lower chamber). The lower chamber was covered by Plexiglas into which two holes (1.5 cm diameter) were cut 0.5 cm from the sidewall of the lower chamber. Test vials (4 dram shell vials; 7.0 × 2.1 cm) containing odorous stimuli were positioned over the two holes leading to the lower chamber. In order that volatiles emanating from the test vials did not accumulate in the lower chamber, and to facilitate gradation of olfactory stimuli, air was continuously exhausted from the device at a rate of 1.8 liters/min from a hole (1.0 cm diameter) in the center of the lower chamber.

TABLE 1. SOURCE AND PURITY OF ODOROUS STIMULI USED IN BEHAVIORAL EXPERIMENTS

Function	Compound	Chemical purity (%)	Source of supply[a]
Aggregation pheromone	I [(±)-*cis*-2-isopropenyl-1-methylcyclobutaneethanol]	85–90	A
	II (*cis*-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexane ethanol)	85–90	A
	III + IV (50:50 mixture of *cis*- and *trans*-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexane-acetaldehyde)	85–90	A
Plant attractant	β-Bisabolol	83	B

[a] A, Frank Enterprises Inc., Columbus, Ohio; B, P.A. Hedin, USDA-ARS, Mississippi State, Mississippi.

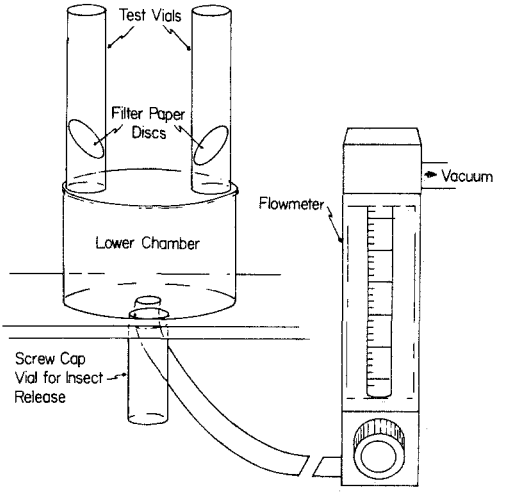

FIG. 1. Dual-choice olfactometer for the boll weevil. See text for details.

Experimental Protocol. Just prior to beginning an experiment, insects were taken from Petri dishes and placed in groups of five in screw-cap vials. Odorous compounds were then applied to filter paper discs within test vials which were subsequently put in place on the bioassay device. Positions of test stimuli were alternated for each replicate. The screw-cap vial from which the insects were to be released was then screwed to the bottom of the lower chamber. The insects were allowed to orient for 20 min in the dark. At the end of the orientation period, lights were switched on and the number of insects in each test vial was counted and recorded. For each experiment 20 replicates were performed for both males and females. A paired *t* test was used to evaluate differences between various treatments (Ostle, 1969).

RESULTS AND DISCUSSION

Response to Serial Dilutions. Responses of both male and female *A. grandis* to serial dilutions of grandlure were significantly different from reponses to the pentane control only for the 1.0 μg dosage ($P < 0.01$; Figure 2). Although responses by both sexes at 0.1 μg were slightly greater than the control and slightly less than the control at 10 μg, these differences were not significant.

The attraction of both males and females to grandlure is similar to results obtained by other investigators in field tests (Hardee et al., 1972). Furthermore, that both sexes respond to the aggregation pheromone is further supported by electrophysiological studes indicating receptors for each pheromone component in both sexes (Gutman et al., 1981; Dickens, 1984). A previous study indicated

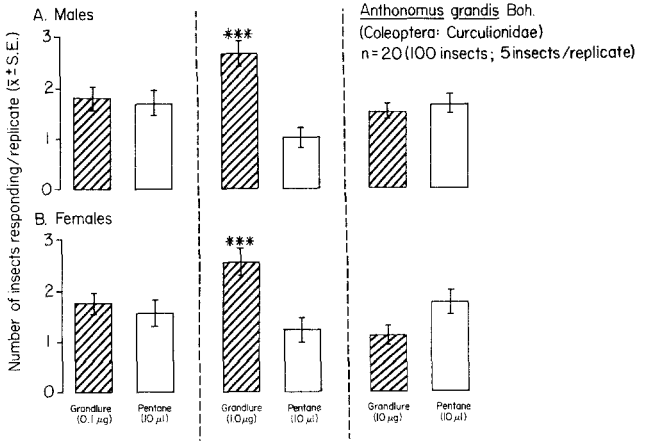

FIG. 2. Mean response of male (A) and female (B) boll weevils to serial dilutions of grandlure. Vertical bars represent standard errors. $***P < 0.01$.

that grandlure alone was unattractive to male boll weevils in laboratory bioassays, although the pheromone alone did attract males in field tests (Hedin et al., 1979). It is difficult to understand the lack of male reponse in laboratory bioassays, especially with regard to our current results. However, the lack of response of males in these earlier laboratory bioassays could be explained by the fact that: (1) males used in those studies were 4–6 days old and already producing pheromone (Gueldner and Wiygul, 1978); (2) methanol was used as a solvent in their study; and (3) a different olfactometer (Hardee et al., 1967) was used in their study.

Only the response of *A. grandis* males to the 1.0 µg dosage of β-bisabolol was significantly greater than response to the pentane control ($P < 0.05$; Figure 3). Both males and females were repelled by the 10 µg dosage of β-bisabolol ($P < 0.01$).

Male boll weevils are the first to emerge in the spring and are responsible for aggregation pheromone production. The attraction of males to β-bisabolol, the major volatile of cotton (Thompson et al., 1971), would greatly facilitate host plant location and subsequent pheromone production (Figure 3). The repulsion of both sexes by β-bisabolol at the highest dosage tested could also be significant since a high concentration of plant odor could signal previous feeding, oviposition, or other damage.

Response in Preference Experiments. Although both grandlure and β-bisabolol were attractive to males when offered versus pentane solvent (Figure 3), males preferred grandlure over β-bisabolol in preference experiments ($P < 0.01$) (Figure 4). In fact, male preference for grandlure over β-bisabolol in this experiment was similar to male reponse to grandlure vs. pentane solvent alone

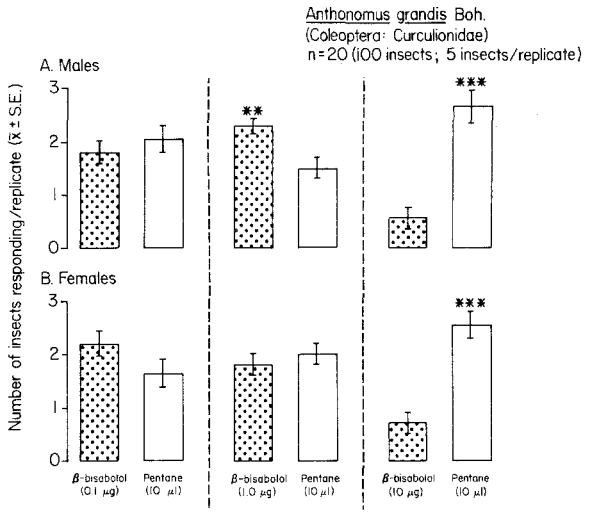

FIG. 3. Mean response of male (A) and female (B) boll weevils to serial dilutions of β-bisabolol. Vertical bars represent standard errors. $^{**}P < 0.05$; $^{***}P < 0.01$.

(Figure 2). When offered the same choice, females exhibited no significant preference for either grandlure or β-bisabolol (Figure 4).

In order to determine possible synergism between the pheromone and plant odor, both sexes were offered the choice between pheromone + plant odor vs. pheromone or plant odor alone (Figure 4). In each instance both sexes preferred the stimulus containing the most grandlure. Additionally, there was some evidence for synergism between grandlure and β-bisabolol for the females, since they preferred this combination of insect and plant odorants over β-bisabolol alone ($P < 0.05$).

Synthesis. The results obtained from the laboratory bioassay correlate well with our current knowledge of olfactory-mediated behavior in the boll weevil and help elucidate results previously considered to be enigmatic. Boll weevil populations emerging in the spring consist primarily of overwintered males, and there is some evidence that the first few weevils to emerge are males (Cross, 1983). These males must locate a host suitable for feeding and pheromone production. Both the lower threshold of the male for host odors found in electrophysiological experiments (Dickens, 1984) and its attraction to the major volatile of cotton, β-bisabolol, demonstrated here could facilitate host finding. Once the male is feeding on a suitable host and producing pheromone, then its conspecifics must choose between pheromone + plant odor vs. plant odor alone. When such a choice is offered as with grandlure + β-bisabolol vs. β-bisabolol, both sexes choose the combination of odorants.

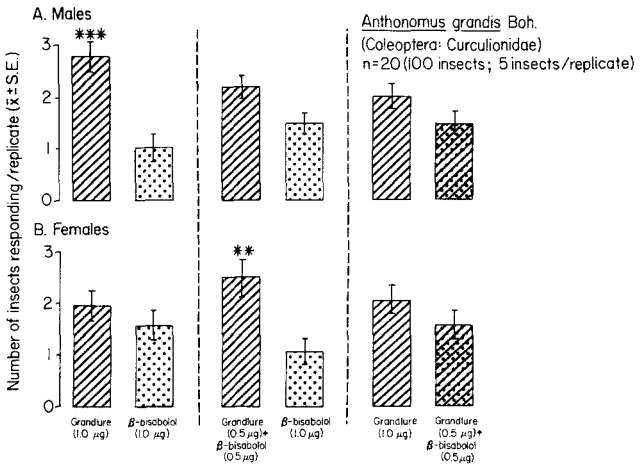

FIG. 4. Mean response of male (A) and female (B) boll weevils in choice experiments involving grandlure and β-bisabolol. Vertical bars represent standard errors. $^{**}P < 0.05$; $^{***}P < 0.01$.

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URINARY SIGNALING PHEROMONE AND SPECIFIC BEHAVIORAL RESPONSE IN TREE SHREWS (*Tupaia belangeri*) I. Basic Investigations for a Bioassay

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Abstract—As soon as they encounter the scent marks made by the urine of fertile male conspecifics, tree shrews of both sexes cover the scent marks with their own sternal-gland secretion by "chinning." The urine of other species, female conspecifics, or castrated male conspecifics does not elicit this behavior; that is, overmarking with sternal-gland secretion is a reaction to a specific chemical signal. The intensity of the overmarking reaction, i.e., the chinning score in response to the urine sample, decreases as the urine is diluted. The urine of different male conspecifics elicits overmarking of varying intensity; an animal's own urine is in general not overmarked. This behavioral reaction meets all the criteria for a bioassay of potential use in isolating the tree-shrew pheromone from urine.

Key Words—Bioassay, chinning, scent marking, signaling pheromone, sternal gland marking, tree shrew, *Tupaia belangeri*, urinary chemosignal.

INTRODUCTION

Tree shrews are small diurnal mammals, distributed throughout the Oriental Region (Lyon, 1913). They live in pairs, occupying territories (Langham, 1982) which they mark with urine as well as with the secretions of skin glands (Kawamichi and Kawamichi, 1979). When they are kept in enclosures, after only a few months the frequently marked branches become covered with intensely scented urine deposit, which may hang down as an orange-colored flange below the branch (Martin, 1968).

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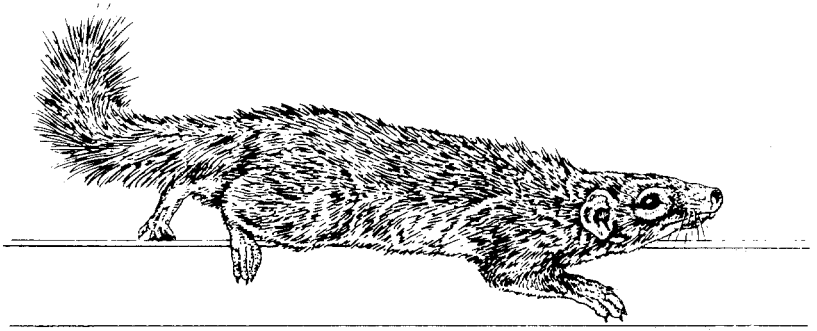

FIG. 1. Chinning response of a tree shrew to urine of a fertile male conspecific (drawn from photograph).

The marks of other animals, whether conspecifics or mammals of other species, are thoroughly sniffed by the tree shrews. After this examination, the urine marks of fertile male conspecifics are overmarked with the secretion from a patch of glands in the jugulosternal region (Figure 1). This behavior is called sternal gland marking or chinning (von Holst and Buergel-Goodwin, 1975). When chinning, an animal presses its sternal region against an object and moves the front of the body back and forth so as to rub the glandular secretion onto the object (Sprankel, 1961).

The urine marks of female conspecifics, unlike those of males, are not usually overmarked by chinning. Hence, overmarking is evidently a specific behavioral response to a specific chemical signal contained in tree-shrew urine.

Specific behavioral responses to chemical signals of conspecifics have rarely been observed in mammals (reviewed by Johnston, 1983). Often the only detectable response is general investigative behavior, and this is usually also elicited by nonspecific stimuli. Where specific behavioral responses to chemical stimuli are found, they can be used as a bioassay, a test by which behaviorally relevant signal substances can be isolated and identified.

In the experiments described here, the degree of specificity of one such behavioral response was measured—overmarking in response to chemical signals in the urine of male tree shrews. Failure of urine other than that of fertile male conspecifics to elicit overmarking would demonstrate the suitability of this behavior as a bioassay for the isolation of a male-specific tree-shrew pheromone.

METHODS AND MATERIALS

Experimental Animals. Twenty-one adult male and 15 adult female *Tupaia belangeri* were studied. Ten of them had been captured as adults in Thailand, and 20 came from our institute's own breeding colony. They were 2–4 years of age. Five of the males had been castrated as adults more than a year previously.

Each tree shrew had a wire-mesh cage to itself (floor area ca. 0.5 m^2; height 0.5 m); neighboring animals were separated physically and optically by wooden partitions. The animals lived in an artificial day/night rhythm (12:12 hr, light–dark), with light phase (activity phase) from 1000 to 2200 hr. They were housed in a climate-controlled room (temperature ca. 25 $\pm$ 1°C, relative humidity 55%; 20-fold air replacement per hour). Food (Altromin diet for tree shrews) and water were present ad libitum.

Sampling Procedures. Immediately after an object is introduced into a tree shrew's cage, the animal marks it with urine. This behavior made it possible to obtain urine without keeping the animals in special cages for metabolic studies and without disturbing them by handling. The urine was collected by placing a PVC rod with a large longitudinal groove diagonally in the cage, so that when it was marked the urine flowed down the groove to one end and, through a hole in that end, into a collecting jar. By replacing this rod with a clean one every hour, as much as 95% of the urine produced in 24 hr was collected. After use, the collecting rods were washed with hot water and dried in air.

Urine from five fertile females and from all the males was collected continuously for several weeks and stored in a deep freezer at −40°C. When the collecting period had been completed, the urine was carefully warmed to the melting point (ca. 0°C) and samples from each individual were pooled separately. In addition, three "group samples" were obtained by pooling urine collected from five fertile males, five castrated males, and five fertile females, respectively. This was done to minimize the effects of individual characteristics and provide urine samples differing only in their group-specific characteristics.

For control tests, urine was obtained from five adult fertile male gerbils (*Meriones unguiculatus*). Beginning two months before the experiments, the gerbil diet had been replaced by tree-shrew diet, to ensure that no differences between the tree-shrew and the gerbil urine would be caused by differences in the food. Urine was collected from the gerbils by putting them in a beaker twice a day; they usually urinated within 1 min, and the fluid was taken up with a pipete and deep-frozen. When 30 ml of gerbil urine had been collected, the samples were thawed (in the same way as the tree-shrew urine) and pooled.

From the pooled urine in each category, several hundred 25-μl samples were deep-frozen in small vials until they were used in the experiments.

Bioassay. The bioassay was carried out with 10 male and 10 female tree shrews. For each test 25 μl of urine was taken into a glass capillary and distributed over ca. 10 cm^2 of the screen forming the front of the animal's cage. As a rule, the animal would come to the screen immediately after this was done, sniff at the sample and, in some cases, then overmark it with the sternal region. (If the animal did not sniff at the urine sample within 30 sec after it was applied, the test was discarded.)

The intensity of the overmarking behavior was expressed in terms of the "chinning score," the number of chinning events (i.e., one forward-and-back-

ward rubbing movement) in the first 2 min after urine application. A given urine sample was tested five times on each animal, each time on a different day.

The sample was tested alternatively with a control sample at an interval of approximately 30 min. The chronological order of the presentation of both samples was changed for each test to counterbalance an eventual effect of the samples' sequence on the chinning reponses of the animals.

From the results of these five tests, the median score was selected for each animal and termed the individual chinning score; this represents the animal's individual response to the particular urine category.

Statistical Analysis. The observed differences among the reactions (i.e., the individual chinning scores) to the various urine categories were tested for significance with the Wilcoxon signed-ranks test for dependent pairs (Siegel, 1959). Differences were regarded as significant when P was less than 0.05 for two-tailed test.

RESULTS AND DISCUSSION

Reaction to Urine of Different Groups. The pooled urine from fertile male gerbils, fertile female tree shrews, and castrated male tree shrews, respectively, did not elicit chinning in most of the experimental animals. The individual chinning scores did not exceed two for each of the three urine categories (Table 1).

In sharp contrast to this result, the pooled urine of fertile male tree shrews was overmarked by 19 of 20 animals. Their individual chinning scores ranged between 1 and 37 (Figure 2). This reaction differs at a highly significant level from those to urine of the other categories ($T = 0$).

This result demonstrates that overmarking with sternal-gland secretion is triggered by a specific chemical signal present in the urine of fertile males tree shrews. The specificity of the signal is determined by taxon (*Tupaia*), sex (male), and physiological state (adult and fertile) of the animal producing it.

Reaction to Urine of Individual Male Conspecifics. Under natural conditions chinning is a response to individual urinary signals. Therefore additional tests were done to check the responses of the animals to different adult males (Table 1).

Male K88 is an animal with a darkly pigmented scrotum. The pigmentation of the scrotum is an indicator of the maturity and the fertility of male tree shrews (von Holst, 1969; Collins et al., 1982); the scrotum of immature or sterile males lacks the dark pigmentation, and the testes are often retracted into the abdominal cavity. The urine of this male elicits significantly more overmarking than does that of castrated conspecifics.

Male K69 is an adult animal of the same species differing from male K88 only in the following respects: its urine lacks the characteristic male odor, the scrotum is not darkly pigmented, and the testes are retracted into the abdominal cavity. Presumably, therefore, this animal is on a very low androgen level or

TABLE 1. MEDIAN CHINNING SCORES OF 20 TREE SHREWS IN RESPONSE TO DIFFERENT CHEMICAL STIMULI.

		Chinning score			Chinning score		
	Test sample[a]	Median[b]	Range[c]	Control sample	Median[b]	Range[c]	T^d
Urine of groups of individuals	fertile male tree shrews	13.5	0–37	fertile male gerbils	0.0	0–1	0.0
	fertile female tree shrews	0.0	0–2	fertile male tree shrews	14.0	0–32	0.0
	castrated male tree shrews	0.0	0–1	fertile male tree shrews	13.5	0–37	0.0
Urine of single individuals	Male K88	7.0	1–25	Male K69	0.0	0–3	0.0
	male animals' own urine	0.0[e]	0–4	Male H38	4.5[e]	0–17	0.0
	Male K88, 100%	7.0	0–27	castrated Male BT36	0.0	0–1	0.0
	Male K88, 10%	0.0	0–5	castrated males	0.0	0–1	37.5
	Male K88, 30%	0.0	0–10	Male K88, 60%	0.5	0–12	12.5
	Male BA1, 100%	6.0	0–17	demineralized water	0.0	0–0	0.0

[a] Each test was done with an aliquot (25 μl) of the pooled urine collected from a group of individuals (or a single individual, respectively) over a period of several weeks.
[b] The median chinning score is the median of the individual scores of all experimental animals.
[c] The individual scores are each the median from the results of five tests on the same sample.
[d] Wilcoxon signed-rank test for dependent pairs; significance level $P < 0.05$.
[e] $N = 10$ males.

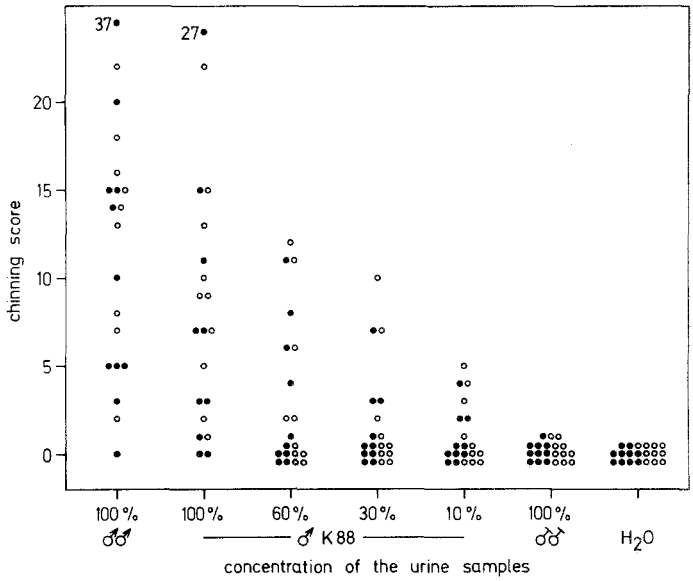

FIG. 2. Individual chinning scores of 20 tree shrews in response to urine of male conspecifics, in one case diluted to various degrees. The individual score (● male, ○ female) is the median from the results of five tests on the same sample.

even sterile. The urine of male K69 is not overmarked by conspecifics significantly more often than the urine of castrated males (Table 1).

The urines of males K88 and K69 represent only two examples of a broad spectrum of conspecific urines. Chinning responses to urine of other males may be within the range between those to the urines of the two animals or even exceed the response to urine of male K88.

Probably the response depends on the concentration of the chinning-eliciting pheromone in the urine. Since this pheromone is androgen-dependent, different pheromone concentrations may result from individual androgen levels of the males. That is, the urine of male tree shrews elicits more chinning the higher the androgen level of its producer. That this is the case has been demonstrated by von Holst and Buergel-Goodwin (1975); when they injected males with different amounts of testosterone propionate, the excretory products of these animals elicited chinning according to the administered doses of androgen.

Androgen level is not the only factor responsible for different pheromone concentrations in the urine of males. Low concentrations of urinary pheromone may be achieved as well by increased water intake and subsequent excretion of diluted urine. To investigate the consequences of urine dilution with regard to behavioral effectiveness, the following experiment was designed.

Reaction to Different Dilutions of Urine from an Individual Male. Urine from male K88 was diluted with demineralized water to give concentrations of

60%, 30%, and 10%. These were tested in order of both ascending and descending concentration. Demineralized water alone was also tested.

The intensity of overmarking decreased in relation to the progressive dilution of the urine (Table 1). Some animals gave very differentiated responses to these small changes in concentration of the pheromone.

The relation between urine concentration and chinning responses is graphed in Figure 2. Similar relations have been found in guinea pigs (Beauchamp et al., 1980) and mice (Nyby et al., 1979).

Figure 2 also illustrates a significant difference ($T = 18$) between the responses of the same animals to 100% urine of male K88 and to the mixed urine of several fertile males. The greater effectiveness of the latter might be due to a higher pheromone concentration. Whether this pheromone concentration has been caused by higher androgen levels of the males or by excretion of a more concentrated urine was not investigated.

On the other hand, the mixed urine from castrated males is even less effective in eliciting overmarking than the 10% dilution of urine from male K88 ($T = 37.5$). This difference cannot be explained by a difference in urine concentration since urine with one-tenth the normal concentration would be pathological when excreted for weeks. Clearly, the pheromone that elicits chinning is not produced by castrates or is produced only in very small amounts.

Urine samples from male gerbils, female tree shrews, and castrated male tree shrews sometimes elicit overmarking (0–2 individual scores) in a few animals. Distilled water was even less effective; it elicited no overmarking at all in 100 tests. A possible explanation is that even nonspecific odor substances trigger overmarking to some slight extent. But this effect is so small in comparison with that of the male pheromone that it is of no practical significance for bioassay.

Reaction to an Animals's Own Urine. It appears biologically plausible that animals would give differentiated responses to the chemical signals of different individual conspecifics. If male tree shrews can distinguish among the urinary signals of various male conspecifics and respond to them in different ways, they should also respond differently to their own urine than to that of fertile male conspecifics. To test this hypothesis, the overmarking reaction of each male to its own urine was observed.

Most of the fertile males failed to overmark samples of their own urine. This reaction is not significantly different from that to the urine of castrated males (Table 1). Inasmuch as the urine of a fertile male must contain its own male pheromone, the lack of a response to it is surprising; the result shows, however, that the animals can distinguish their own signal from those of conspecifics. For such discrimination to be possible, the urine of different males must differ either in qualitative composition or in quantitative composition of its components; I regard the latter as more probable.

From the results of these experiments, we conclude that overmarking with sternal-gland secretion is a specific reaction to a male-specific tree-shrew pher-

omone. A chemical analysis of tree-shrew urine, to isolate and to identify the pheromone, is now in progress.

Acknowledgments—I am grateful to Prof. Dr. D. von Holst for his support. Thanks also to Mrs. W. Deinlein for her technical assistance. The work was funded by the Deutsche Forschungsgemeinschaft Ho 443/16-4.

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CHEMICAL AND BEHAVIORAL ANALYSES OF VOLATILE SEX PHEROMONE COMPONENTS RELEASED BY CALLING *Heliothis virescens* (F.) FEMALES (LEPIDOPTERA: NOCTUIDAE)[1]

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Abstract—Gas chromatographic and mass spectral analyses were conducted on pheromone gland extracts, volatiles collected from excised pheromone glands, and volatiles collected from calling females. In addition to tetradecanal, (Z)-9-tetradecenal, hexadecanal, (Z)-7-hexadecenal, (Z)-9-hexadecenal, and (Z)-11-hexadecenal, four other compounds, tetradecanol, (Z)-9-tetradecenol, hexadecanol, and (Z)-11-hexadecenol, were also identified from gland extracts. Only the six aldehyde components were found in gland and female volatile collections. The mean percentage of components identified from volatiles collected from calling females was 13.0% tetradecanal, 18.1% (Z)-9-tetradecenal, 7.3% hexadecanal, 0.6% (Z)-7-hexadecenal, 1.0% (Z)-9-hexadecenal, and 60.0% (Z)-11-hexadecenal. Bioassays using rubber septa formulated to release the female volatile blend indicated that all six aldehyde components play major roles in close-range male reproductive behavior. Deletion of (Z)-9-hexadecenal from the six-component blend reduced the number of copulation attempts while (Z)-7-hexadecenal exerted subtle effects on all close range behaviors. Tetradecanal affected the number of times males reorient from close range. Deletion of hexadecanal from the six-component blend resulted in a significant reduction in the number of times males landed. Comparison of the six-component synthetic blend (released at somewhat less than 1 female equivalent per hour) with calling females indicated that the six-component blend was indistinguishable from the females in inducing all of the behaviors monitored.

[1] Mention of a commercial proprietory product does not constitute endorsement by either the University of Guelph or the USDA.

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Key Words—Volatile sex pheromone blend, behavior, chemistry, *Heliothis virescens*, Lepidoptera, Noctuidae.

INTRODUCTION

Due to the genetic propensity for rapid development of resistance to pesticides, a number of research groups have been studying alternate methods for control of *Heliothis virescens* (F), the tobacco budworm. A major effort in this direction has been to develop species-specific control techniques using the sex pheromone communication system of this insect. Consequently, the sex pheromone blend produced by female tobacco budworm moths has been under investigation for a number of years. Independent studies by Roelofs et al. (1974) and Tumlinson et al. (1975) indicated that a 16:1 blend of (Z)-11-hexadecenal (Z11-16:Al) and (Z)-9-tetradecenal (Z9-14:Al) was produced by females and was attractive to males. However, based on chromatographic and behavioral data, Tumlinson et al. (1975) suggested that the actual pheromone blend also contained other components. This hypothesis was proven correct when Klun et al. (1980) identified five additional compounds from pheromone gland rinses. The additional compounds identified were: tetradecanal (14:Al), hexadecanal (16:Al), (Z)-7-hexadecenal (Z7-16:Al), (Z)-9-hexadecenal (Z9-16:Al) and (Z)-11-hexadecimal (Z11-16:OH). Initial field studies using this blend dispensed from cotton dental wicks indicated that the seven-component blend was a significantly better lure for tobacco budworm males than was the previous identified two-component blend (Sparks et al., 1979). However, subsequent tests by Hartstack et al. (1980) indicated that the two-component blend was as efficient or better than the seven-component blend.

These results prompted Tumlinson et al. (1982) and Pope et al. (1982) to analyze volatile pheromone components released from the pheromone gland. Neither group found Z11-16:OH in the volatiles. Subsequently, Vetter and Baker (1983) critically evaluated the blend and individual components identified by Pope et al. (1982) plus Z11-16:OH in flight tunnel studies. Their results negated the behavioral function attributed to Z11-16:OH and indicated that Z9-14:Al, Z11-16:Al, and 16:Al were necessary for effective sexual signaling. However, no function could be clearly shown for 14:Al, Z7-16:Al, or Z9-16:Al. Our initial results indicated the presence of 14:Al, Z9-14:Al, 16:Al, and Z11-16:Al in the volatile pheromone blend (Tumlinson et al., 1982). However, males did not respond as well to this four-component blend as they did to virgin females in preliminary bioassays. This prompted us to reevaluate our identification. The following reports the results of chemical analyses of gland extracts, volatile pheromone components released from excised pheromone glands, and volatiles released by calling females. We also report the results of singe- and dual-selection flight tunnel studies used to assess the behavioral functions of the individual components present in the volatile pheromone blend released by females.

METHODS AND MATERIALS

Heliothis virescens used in this study were obtained as pupae from the Bioenvironmental Insect Control Laboratory, USDA, Stoneville, Mississippi. Pupae were sexed and allowed to emerge in isolation from members of the opposite sex. Newly emerged adults were collected daily and allowed to mature for 2 days prior to use in 30 × 30 × 30-cm Plexiglas cages. No insects over 5 days old were used. Both pupae and adults were maintained under a reversed 16:8 hr light–dark cycle at 26° (day) and 24° (night) and a relative humidity of 55%. All cages contained a 10% sucrose solution. Studies were conducted between hours 5 and 8 of the scotophase.

Collection of Pheromone. Gland extracts were prepared by removing the exposed terminal abdominal segments of actively calling females and dipping them in 25 μl of either ethyl ether (Fisher anhydrous reagent grade) or pentane (redistilled Fisher HPLC grade) in a conical microvial for 10–20 sec. Ten microliters of isooctane (Fisher, 99 mole %) containing 10 ng of each of the appropriate internal standards (see below) was then added and the volume was reduced to ca. 5 μl under N_2.

The technique used for collection of volatiles was essentially that described by Tumlinson et al. (1982), except that air from a compressed gas tank was pushed through the system rather than being drawn through by vacuum. Prior to entering the female holding chamber, the compressed air was passed through molecular seive and activated charcoal filters. All air lines were made of copper or Teflon tubing. The silanized glass chamber used to hold females was previously described by Cross (1980). All charcoal microentrainment filters were calibrated using 200 ng of Z9–14:Al, 14:Al, Z11–16:Al, 16:Al, and Z11–16:OH as described by Tumlinson et al. (1982), and the correction factors were incorporated into all calculations. System blanks were obtained daily by passing air through the system at 500 ml/min for a 3-hr period during the photophase immediately preceding volatile collection.

Gland volatiles were collected from the excised terminal abdominal segments of calling females during the mating period. The terminal segments were extended and removed as described previously (Teal et al., 1981a) in the dark using a dim red filtered light. The cut ends of 5–10 preparations were then placed on a 1 × 2-cm stainless-steel planchet so that the tips pointed upwards and the planchet was positioned in the collection device. Air was then passed through the system for 10 min, and the volatiles were recovered as described below. We also collected volatiles from groups of 20–50 tips over a 15- to 30-min period using the same technique.

Volatiles were collected from calling females by placing individuals in collection chambers immediately after the lights went out. Air was continuously passed through the system at 500 ml/min. Although the actual volume of air was high in comparison to that of Pope et al. (1982), the air speed in the cham-

ber was low due to its shape. Calling behavior was not visibly affected by the volume of air passed over the females. When females had entered prolonged calling bouts, the microentrainment filters were fitted onto the outports of the chambers using a Teflon sleeve. Insects were monitored throughout the calling period and, when an individual stopped releasing pheromone, the entrainment filter was removed.

Volatiles were recovered from the entrainment filters using the technique described by Tumlinson et al. (1982). The method was modified to include rinsing the walls of the receiving vessel with 15 μl of redistilled pentane and the addition of 15 ng of each of the appropriate internal standards prior to the addition of 20 μl of isooctane and volume reduction under N_2. Internal standards used for all analyses included the acetates of primary saturated alcohols from 12 to 16 carbons in length.

Chemical Analysis. Capillary gas chromatographic (GC) anslyses of natural products and synthetic standards were performed using Varian 3700 and Hewlett Packard 5792 GCs equipped with splitless injectors and flame ionization detectors. The Hewlett Packard was also equipped with a cool on-column injector. The Varian was interfaced to a Perkin Elmer CIT II system and 3600 data station while the Hewlett Packard was interfaced to a HP 3390 integrator. Capillary columns used for analysis in the Varian included a glass 35-m × 0.15-mm (ID) OV-1 column, a glass 30-m × 0.25-mm (ID) SP2340 column, and a fused silica 60-m × 0.25-mm (ID SP2330 (Supelco) column. Helium was used as a carrier gas in this instrument at a linear flow velocity of 18 cm/sec. Samples were injected at an initial temperature of 80° (injector purge at 60 sec), and the oven temperature was programmed after 2 min at 30°/min to final temperatures of 180° (OV-1). 140° (SP2340), and 165° (SP2330). Columns used in the Hewlett Packard included a fused silica 15 m × 0.15 mm (ID) cross-linked methysilicone (Hewlett Packard), a fused silica 25 m × 0.22 mm (ID) BP20 (SGE) (equivalent to Carbowax 20 M), and a 30 m × 0.25 mm (ID) SPB1 (equivalent to methyl silicone) (Supelco). Hydrogen was used as the carrier gas at 40 cm/sec. Conditions of chromatography were as follows when using the splitless injector: initial temperature = 80°, injector purge at 30 sec, temperature program after 60 sec = 35°/min, final temperature = 155°. Conditions used with the cool on-column injector were: initial oven and injector temperature = 80°, temperature program = 15°/min, final temperatures = 165° (SPB1) and 175° (BP20). In all instances, the retention times of both natural products and synthetic standards were converted to equivalent chain length units (ECL) using the acetate internal standards as the retention index (Swoboda, 1962).

Both methane and isobutane chemical ionization mass spectra were obtained using Finnigan 3200 and VG 1212F chemical ionization mass spectrometers. The Finnigan was interfaced to a Varian 1400 GC equipped with a direct injector and capillary system. Capillary GC columns used in this instrument included a 24 m × 0.25 mm (ID) glass cholesteryl *p*-nitrocinnamate (Heath

et al., 1981), a 26 m × 0.25 mm (ID) glass OV-101, and a 54 m × 0.25 mm (ID) glass SP2300 (Supelco). The VG 1212F was interfaced to a Hewlett Packard 5792 GC equipped with a cool on-column injector. A 50 m × 0.25 mm (ID) DB5 fused silica column (J&W) was used in this instrument. The total effluent from the columns was introduced directly into the ionization source. Columns used with the direct injector were operated at 155° (cholesteryl *p*-nitrocinnamate), 180° (SP2300), and 195° (OV-101). The column used in conjunction with the VG 1212F was operated at 80° until the solvent had eluted and then programmed at 15°/min to a final temperature of 220°. Spectra of peaks eluting from analyses of system blanks and natural product samples were compared with each other and with synthetic standards.

Formulation of Synthetic Blends. All synthetic chemicals used were purchased from Albany International Ltd. The compounds were purified by high-performance liquid chromatography on a 25 × 2.5-cm OD $AgNO_3$–silica column eluted with toluene (Heath and Sonnet, 1980). Hexadecanal was further purified by recrystalization from pentane. All compounds were found to be at least 99% free of isomers by gas chromatographic analyses on both the SP2330 and OV1 capillary columns.

Rubber septa (A.H. Thomas Co., 8153-D22) used as dispensers were extracted with CH_2Cl_2 for 24 hr and air dried prior to formulation. Septa were formulated by loading 500 μg of the total blend for concentration studies and 1000 μg of the blends for dual-selection tests dissolved in 200 μl of hexane into the large end of the rubber septa. Septa were aged two days prior to use, and volatiles emitted from the septa were collected and analyzed at two-day intervals at various flow rates to ensure that the percentage release of each compound and amount released per unit volume of N_2 did not change.

Blend ratios used in formulations were initially based on an empirical technique in which volatiles were collected by passing N_2 over the lures loaded with 2 mg each of 14:Al, *Z*9–14:Al, 16:Al, and *Z*11–16:Al at 500 ml/min for 1 hr as described above and analyzed. Relative differences in the amounts of each of the components released were calculated from the chromatograms of these collections and the correction factor for each compound used to formulate the test blends. Subsequently, a theoretical model of the release of components from rubber septa based on the vapor pressures of the total blend and each of the components was developed (Heath et al., in preparation). Results using the theoretical formula were consistent with the empirical technique. The theoretical model for predicting release ratios accounts for small differences in vapor pressure between *Z*7–16:Al, *Z*9–16:Al, and *Z*11–16:Al and more importantly for large differences between 14- and 16-carbon aldehydes. Therefore, this technique was used for all subsequent formulations.

Bioassays. The system used to dispense the pheromone blends into the flight tunnel consisted of the silanized glass female holding chambers linked through a flow controller to a tank of either N_2 (when comparing synthetic lures)

or air (when comparing lures with females). The outlet of the collection cage was connected via Teflon tubing to a silanized glass tube (2.5 mm ID) bent at 90° positioned in the upwind end of the tunnel. A 3 × 4-cm piece of aluminum screen positioned at the outport of the glass tube provided a landing site for the males. Gas flow through the system was precisely controlled and could be varied, thereby enabling us to precisely control the release rates of the lures.

Bioassays were conducted in a 2.0-m long × 1.0-m wide × 0.5-m high wind tunnel similar to that described by Teal et al. (1981b). The air speed through the tunnel (0.5 m/sec) was regulated by a laboratory fume hood controlled by a variable transformer and exhausted out of the building. Individual males were released at a central point in the wind tunnel 10 cm from downwind end as described by Teal et al. (1981b). The behaviors of the individual males were monitored and recorded as described previously (Teal et al., 1981b) during a 5-min test period. The behaviors used to categorize the responses of males to the blends of pheromone were those defined in mating studies. These behaviors were: directed flight upwind to the source (Tax), arrested advance and partial hairpencil exposure at 5–10 cm from the source (Hov), landing on the screen (On), hairpencil exposure on the screen (Hp), attempted copulation while on the source as indicated by bending the terminal abdominal segments and full hairpencil exposure (Cop), and reorienting after completing the close-range behaviors by moving into a hover pattern no further than 10 cm from the screen and then performing further courtship behaviors (Reo) (Figure 1). Statistical comparisons were made using a χ^2 test of independence and Yates' correction at a 0.05 probability level.

All bioassays were conducted using the volatile blend identified from calling females as the basis from which components were deleted. In initial studies a single dispenser system was positioned in the upwind end of the tunnel. This system was used to establish a concentration gradient between 0.1 and 0.5 female equivalent (FE) by passing different volumes of air over the septa for the six-component volatile blend and a four-component blend which did not contain *Z*7–16:Al or *Z*9–16:Al. Twenty individuals were flown to each treatment at each concentration. Results of the above study differed from those reported by Vetter and Baker (1983) and indicated that the minor components appear to function at close range. Therefore, a dual-selection bioassay system was developed for subsequent studies. In these studies two dispenser systems were positioned equidistant from the upwind walls and each other. The outports were angled such that the plumes formed by $TiCl_4$-impregnated cotton swabs in the holding cages merged 0.75 m downwind from the dispenser outports. The pheromone blends tested in each dual-selection test series were switched between the two dispenser systems daily and an equal number of males flown to each of the blends on each side. Thus, the behavioral sequences of 40 individual males which entered taxis after release were monitored for each pair of blends. Prior to the release of any males, compressed gas was allowed to flow through the

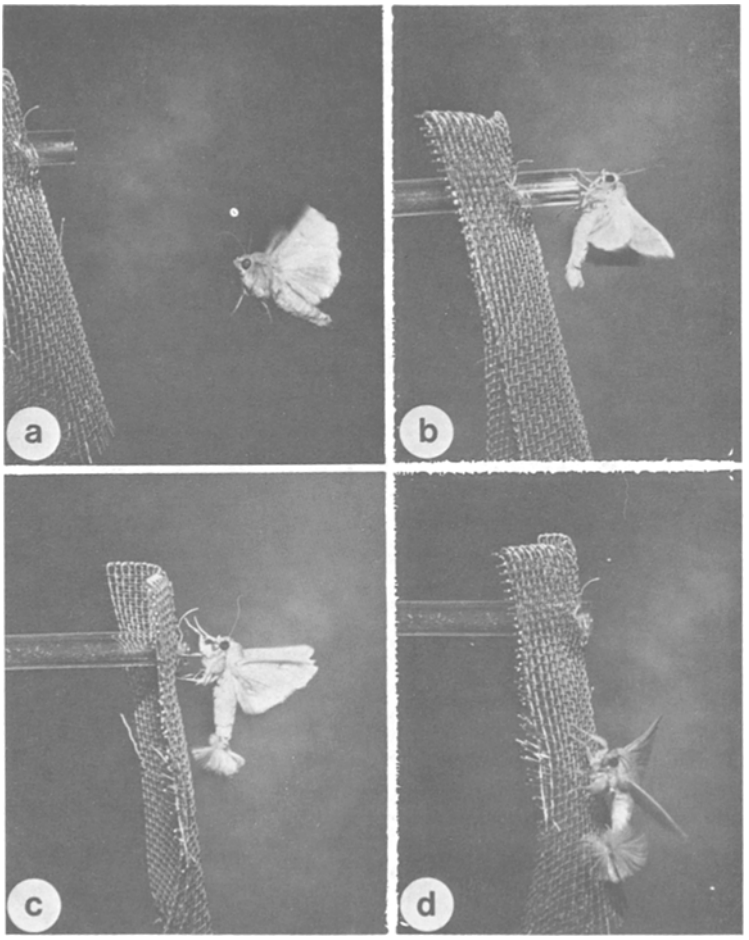


FIG. 1. Close-range behaviors monitored in bioassays. (A) Male hovering ca. 2 cm from the dispenser, note the partially exposed hairpencils. (B) Male landing on the aluminum screen. (C) Male exposing hairpencils but not attempting copulation. (D) Male attempting copulation, note the bent abdominal segments.

dispenser system for 15 min at the appropriate flow rate in order to establish equilibrium. All blends tested were permutations of the six-component blend identified from volatile collection from females and were formulated to release 4, 5, or 6 components at a 1.0 FE rate (ca. 75 ng/hr). In addition, the six- and four-component blends were compared to individual calling females in dual-selection tests. When females were used, air was passed through both dispenser systems for at least 30 min to allow the females to acclimate. Several systems

containing females were set up and, when a female ceased calling another, was immediately connected to the silanized glass outport.

RESULTS

Gland Extracts. Gas chromatographic analyses of pentane gland dips (10 sec) and extracts (30–120 sec) and ethyl ether extracts yielded distinctly different chromatographic profiles. Extracts obtained using the three techniques all contained peaks having equivalent chain-length (ECL) values coincident with 14:Al, Z9-14:Al, 16:Al, Z7-16:Al and Z9-16:Al, and Z11-16:Al (all resolved on BP20, SP2330, SP2340, and SPB1) in the ratio indicated in Table 1. Ethyl ether and pentane extracts also contained components having ECL values equivalent to tetradecanol (14:OH), Z-9-tetradecenol (Z9-14:OH), hexadecanol (16:OH), and Z11-16:OH (Figure 2A, 3A). The only alcohol peak found consistently in pentane gland dips was Z11-16:OH.

Full methane chemical ionization mass spectra (60–300 amu) were obtained using the SP2100 column for peaks corresponding to Z9-14:OH, 16:OH, and Z11-16:OH in ethyl ether extracts of 20 female equivalents (Figure 3A). Although a full mass spectrum of 14:OH was not obtained, the peak having a retention time coincident with 14:OH had fragment ions characteristic of 14:OH including 213 (M−1), and 197 (M+1−18). Additionally, GC mass

TABLE 1. PERCENTAGE OF COMPONENTS FOUND IN GLAND EXTRACTS, VOLATILES COLLECTED FROM EXCISED PHEROMONE GLANDS, AND VOLATILES COLLECTED FROM CALLING FEMALES[a]

Compound	Gland Extract (N = 25)	Gland volatiles 5–10 glands 10 min (N = 20)	Gland volatiles 20–50 tips 15–30 min (N = 15)	Female volatiles (N = 30)
14:Al	2.5 (±0.13)	13.2 (±1.1)	3.8 (±0.3)	13.0 (±0.65)
Z9-14:Al	3.3 (±0.38)	16.9 (±1.1)	4.0 (±0.2)	18.1 (±1.09)
16:Al	8.6 (±0.71)	7.9 (±1.2)	8.3 (±0.5)	7.3 (±0.59)
Z7-16:Al	0.6 (±0.31)	0.6 (±0.3)	0.4 (±0.1)	0.6 (±0.1)
Z9-16:Al	0.8 (±0.15)	0.6 (±0.2)	1.0 (±0.1)	1.0 (±0.1)
Z11-16:Al	79.5 (±0.84)	60.8 (±0.9)	82.6 (±0.3)	60.0 (±1.32)
14:OH	0.3 (±0.12)			
Z9-14:OH	0.3 (±0.22)			
16:OH	0.4 (±0.25)			
Z11-16:OH	4.8 (±0.71)			

[a] ± standard error of the mean.

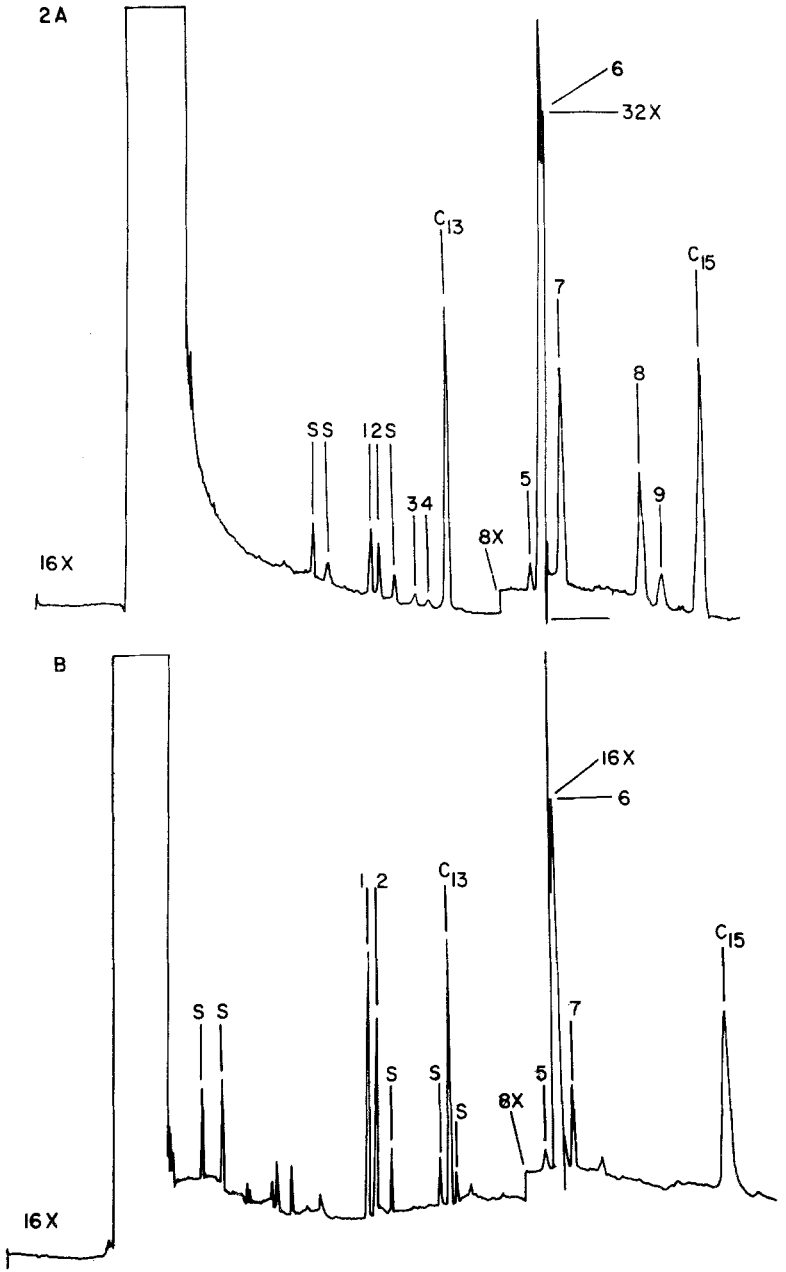
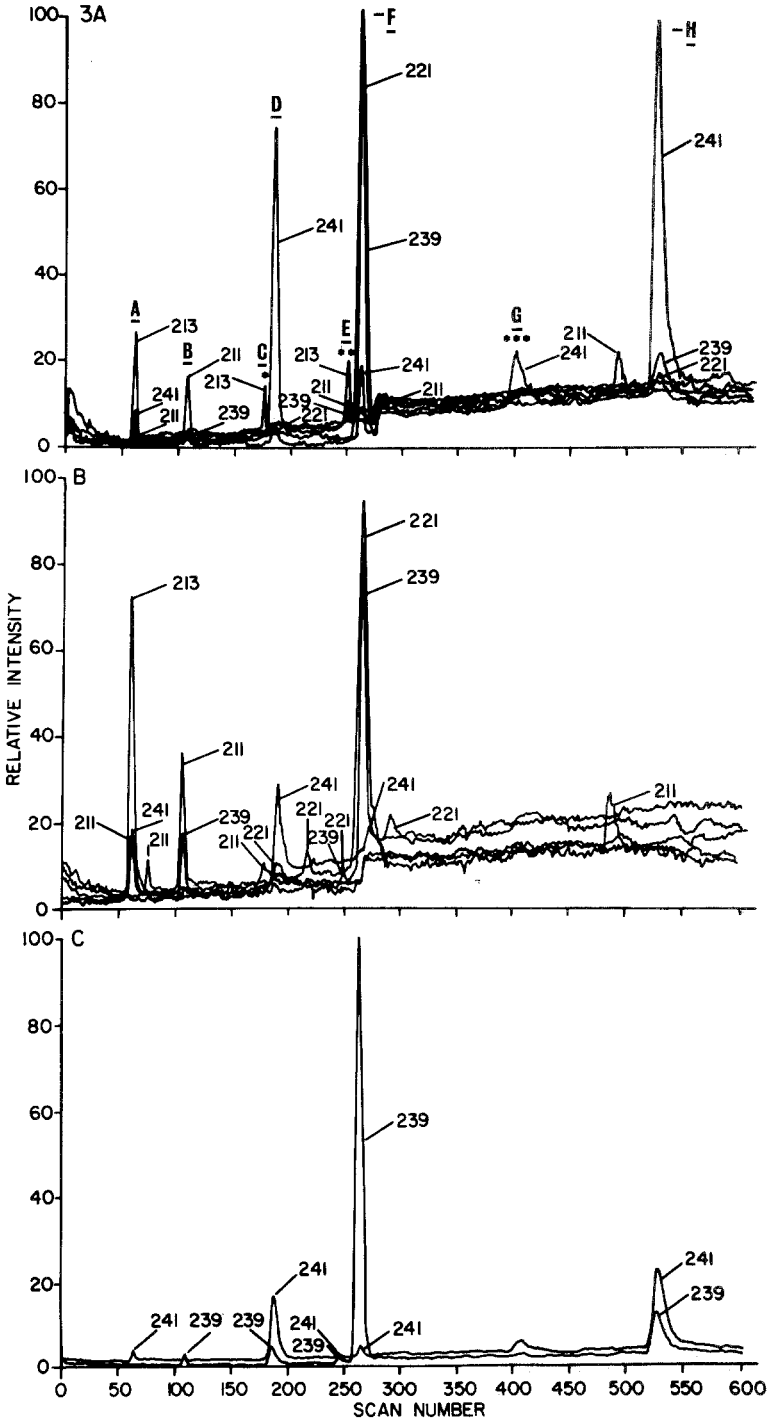


FIG. 2. Chromatographic analyses using the 35-m × 0.15-mm (ID) OV-1 column. S = solvent impurity, 1 = Z9–14:Al, 2 = 14:Al, 3 = Z9–14:OH, 4 = 14:OH, 5 = Z7- and Z9–16:Al (no resolution), 6 = Z11–16:Al, 7 = 16:Al, 8 = Z11–16:OH, 9 = 16:OH, C_{13} = tridecan-1-ol acetate, C_{15} = pentadecan-1-ol acetate. Both chromatograms were run using the same conditions on the same day. (A) Analysis of pentane extract from a single gland from a calling female; (B) = analysis of 1-hr collection from a single calling female.


3A
B
C
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E
F
G
H
213
211
241
239
221
RELATIVE INTENSITY
SCAN NUMBER
0
50
100
150
200
250
300
350
400
450
500
550
600
20
40
60
80
100

spectral analyses using the cholesteryl *p*-nitrocinnamate column and isobutane as the reagent gas indicated that the peak having an ECL value identical with Z9–14:OH had fragments indicative of a monounsaturated 14-carbon alcohol including a small 269 fragment (M+57) and a large 213 ion (M+1). The peak corresponding to 14:OH had fragments corresponding to M+57 (271), M−1 (213), and M+1−18 (197). Fragments indicative of 16:OH, including 299 (M+57), 241 (M−1), 225 (M+1−18), and 224 (M−18), were found in the natural product peak having an identical ECL value with this alcohol.

Gland Volatiles. Gas chromatographic analysis of volatiles collected from excised glands revealed the presence of five peaks on the OV-1 column and six on the SP2330 and SP2340 columns that were not present in system blanks. These peaks corresponded to 14:Al, Z9–14:Al, 16:Al, Z7–16:Al, Z9–16:Al, and Z11–16:Al. No peaks having ECL values corresponding to 14:OH, Z9–14:OH, 16:OH, or Z11–16:OH were found in any of the gland volatiles when analyzed on the OV-1 or SP2340 columns. The SP2330 column did not chromatograph Z11–16:OH under the conditions used. The mean percentages of the identified components are given in Table 1.

Female Volatiles. Gas chromatographic analyses of volatiles collected from calling females on the OV-1, SP2330, and SP2340 all indicated that the same six peaks present in gland volatile samples were released by calling females, and again there was no indication that any of the analogous alcohols identified from gland extracts were present in the volatile collections (Figure 2B). The mean percentages of the six aldehyde components are given in Table 1. As with gland volatile analyses, the percentages of Z7–16:Al and Z9–16:Al were obtained by taking the summed values on all columns and dividing by the mean proportions of each compound identified using the SP2330 (10 replicates) and SP2340 columns (8 replicates). As indicated in Table 1, there was only limited variation in the percentage of each compound released. The total amount of pheromone released per female was 1.3 ± 0.5 ng/min (N = 30).

Full mass spectra (60–300 amu) and ECL values obtained using all three columns indicated that 14:Al, Z9–14:Al, 16:Al, and Z11–16:Al were present in all of the volatile samples. Full spectra required as little as 3.5 ng of each component. None of the columns used in the GC-MS separated Z7–16:Al from

FIG. 3. Selected ion monitoring traces of major fragments obtained from methane chemical ionization mass spectroscopy of components identified from the ethyl ether extract of 20 calling females in (A), a 25 calling female hr of volatile collection obtained from (B) and the seven-component blend identified by Klun et al. (1980) (C) using the SP 2300 column in the GC mass spectrometer. Peak A = 14:Al, B = Z9–14:Al, C = 14:OH, D = 16:Ald, E = Z7- and Z9–16:Al, F = Z11–16:Al, G = 16:OH, H = Z11–16:OH. (A) *M − 1 of 14:OH, **M + 1 of Z9–14:OH, and ***M − 1 of 16:OH.

TABLE 2. PERCENTAGES OF COMPONENTS LOADED ONTO RUBBER SEPTA WITH THEORETICAL AND ACTUAL RELEASE RATES[a]

Compound	Load (%)	Theoretical release (%)	Actual release (%) (N = 20)
14:Al	3.4	13.0	13.9 (±0.65)
Z9-14:Al	4.0	18.1	17.8 (±0.62)
16:Al	9.6	7.3	6.4 (±0.16)
Z7-16:Al	0.9	0.6	0.6 (±0.21)
Z9-16:Al	1.1	1.0	0.8 (±0.10)
Z11-16:Al	81.0	60.0	60.5 (±1.12)

[a] ± standard error of the mean.

Z9–16 : Al; therefore the presence of both compounds cannot be confirmed on the basis of mass spectral evidence. Limited ion monitoring indicated the presence of a peak on both the SP2300 using methane as the reagent gas and cholesteryl *p*-nitrocinnamate columns using isobutane as the reagent gas which had ECL values coincident with Z7/Z9–16 : Al and had fragment ions characteristic of a monounsaturated C_{16} aldehyde. These ions included 239 (M+1), 221 (M+1−18), and 267 (M+29) when reacted with methane or 295 (M+57) when reacted with isobutane. As indicated in Figure 3B there is no indication that Z11–16 : OH was released as a volatile sex pheromone component.

Lure Formulations. Based on the vapor-pressure-release model developed by Heath et al. (in preparation), septa loaded with a 3.4% 14 : Al, 4.0% Z9–14 : Al, 9.6% 16 : Al, 0.9% Z7–16 : Al, 1.1% Z9–16 : Al, and 81.0% Z11–16 : Al should release the blend identified from calling females. The actual percentages released are listed in Table 2. The total amount of pheromone released per milliliter of N_2 from a septa loaded with 500 μg of the six-component blend was found to be linear and followed the equation: ng released = 1.82×10^{-4} ($\times$ ml N_2/min) + 7.68×10^{-2} (Figure 4). This correlation was consistent with values obtained using more than one septum per apparatus and when the concentration per septum was doubled. From the above equation, we calculated that four septa loaded with 1 mg each and aged for two or more days would release 1.1 ng/min at a flow rate of 690 ml/min. Although somewhat below the mean amount of pheromone released by a calling female per minute, this amount was within the range collected per female. These conditions were used in all dual-selection tests.

Bioassays. Results of concentration studies using the single dispenser system indicated that the deletion of Z7–16 : Al and Z9–16 : Al from the six-component blend had little effect on male sexual behaviors at the 0.10 and 0.25 FE levels (Figure 5). However, significantly more males ended the behavioral sequence by hairpencilling or attempting copulation when the six-component lure

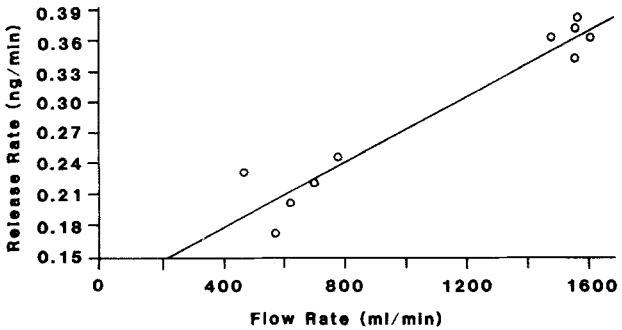

FIG. 4. Amount of the six-component aldehyde blend released from septa loaded with 500 μg at various flow rates. $R^2 = 0.96$ for equation, $y = 1.822 \times 10^{-4}x + 7.682 \times 10^{-2}$.

was presented than when the four-component lure was used at the 0.33 FE level, and all of the behaviors monitored except hovering were observed more often with the six-component than four-component lures at the 0.50 FE level (Figure 5).

Differences noted in the above study strongly suggested that Z7–16 : Al and Z9–16 : Al exert subtle effects on male sexual behaviors either alone or in combination with each other. Therefore, we developed the dual-selection test which enabled us to evaluate male responses to two different blends at the same time. Selection occurred when a male cast from side to side through the two plumes at 30–50 cm from the outports prior to moving to one of the sources. Results of these studies are given in Figure 6. Initially, we assessed the six-component volatile blend and the four-component blend that did not contain Z7–16 : Al or Z9–16 : Al. As indicated in Figure 6, males performed all of the close-range behaviors significantly more often at the six-component station, which supports the results of our single dispenser tests.

Comparison of the six-component blend and the five-component blend which did not contain Z9–16 : Al indicated that the deletion of this component resulted in a general reduction in all of the close-range behaviors with significant reductions occurring in hovering and the number of attempted copulations. Comparison of this five-component blend and the four-component blend which did not contain either Z9–16 : Al or Z7–16 : Al showed that the five-component lure was superior in all respects except selection.

Deletion of Z7–16 : AL from the six-component blend resulted in a significant decrease in the number of copulation attempts and small decreases in all of the other close-range behaviors observed. There were no differences between this blend and the four-component blend that did not contain Z9–16 : Al and Z7–16 : Al.

When 14 : Al was deleted from the six-component blend, there were no

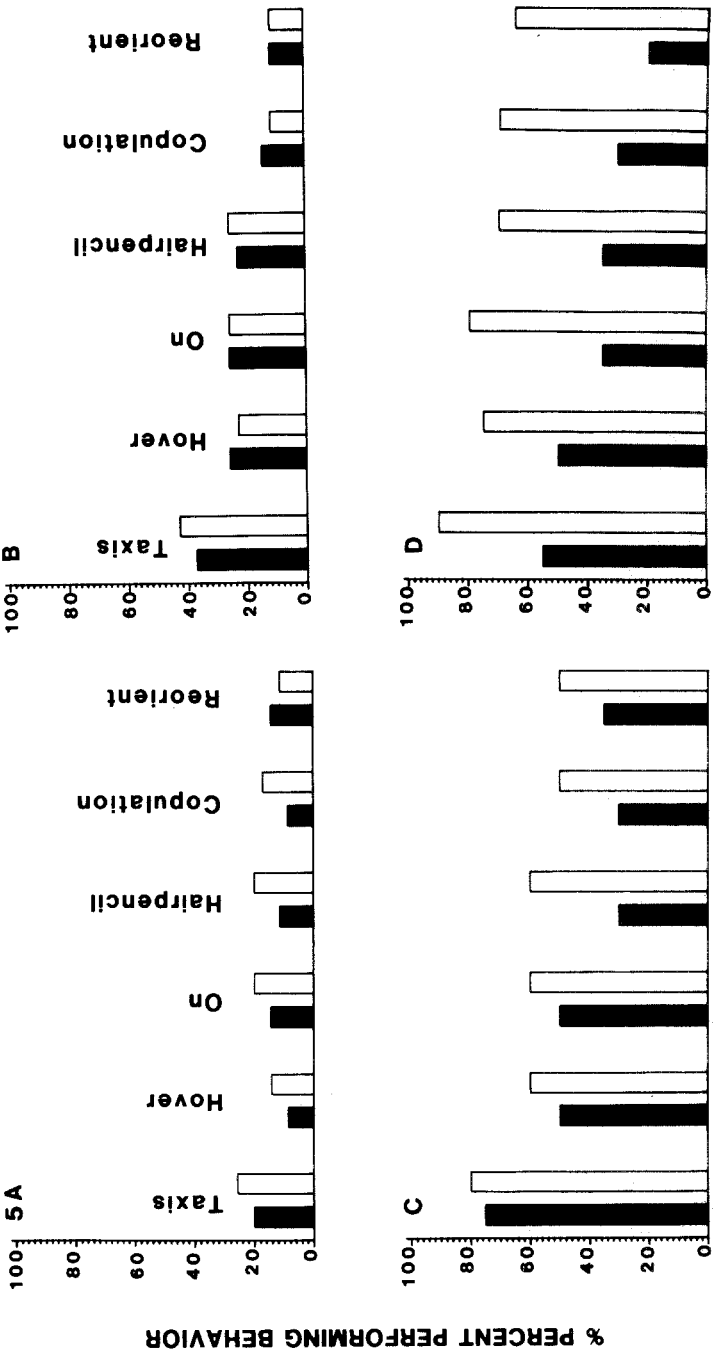

FIG. 5. Results of concentration studies using the single dispenser system. Taxis = upwind taxis, Hover = arrested advance at 5–10 cm from the source, On = landing on aluminum screen, Hairpencil = hairpencil exposure on the source, Copulation = copulation attempt at the source, Reorient = close-range reorientation. (A) = 0.10 FE/min (N = 30); (B) 0.25 FE/min (N = 30); (C) 0.33 FE/min (N = 30); (D) 0.50 FE/min (N = 20 flights to each lure). The six-compound blend (open bars) contained all six aldehydes while the four-component blend (solid bars) did not contain Z7–16:A1 or Z9–16:A1.

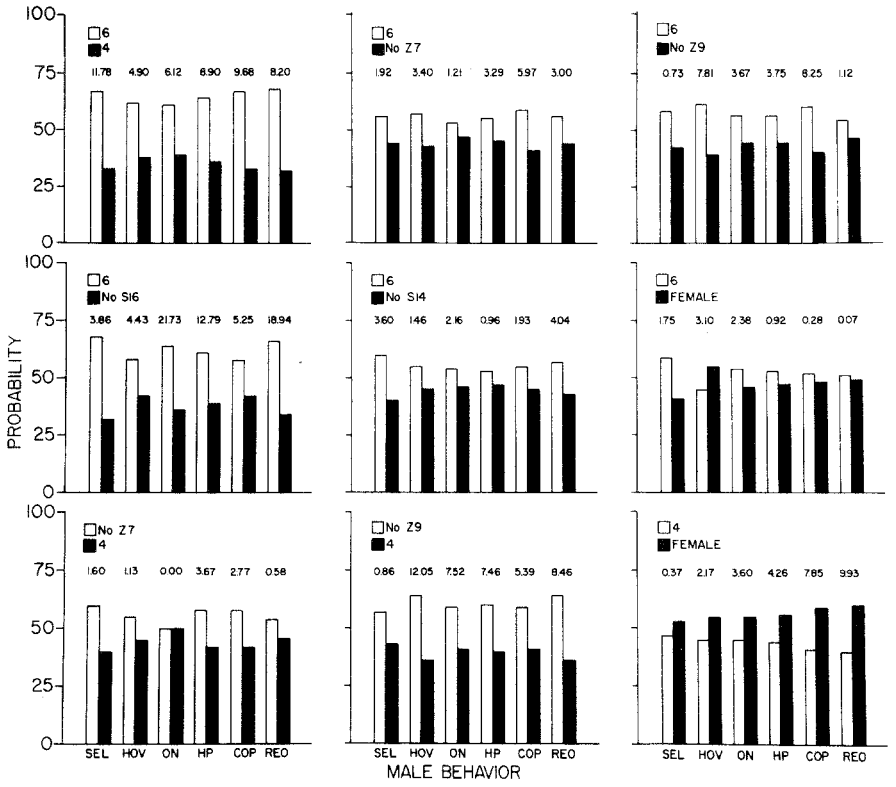

FIG. 6. Results of dual-selection studies. Numbers above figures are χ^2 values calculated using Yates' correction. SEL = selection, HOV = hover at source, ON = lands on, HP = full hairpencil exposure after landing, COP = copulation attempt at the source, REO = close-range reorientation. Four- and six-component blends are as listed in the text and in Figure 5. Blends demarked by "No Z7", "No Z9", "No S16" and "No S14" are five-component blends which do not contain *Z*7–16:Al, *Z*9–16:Al, 16:Al, and 14:Al, respectively. Probabilities are based on the number of times individual behaviors were performed in response to each blend and depend upon the performance of the previous behavior.

significant decreases in those behaviors which are requisite for successful copulation in a single attempt. However, the probability that the male would reorient at close range and perform another series of courtship behaviors was significantly reduced. Deletion of 16:Al from the six-component blend resulted in significant decreases in the number of times each behavior was performed. These overall decreases can be equated to the very low probability of landing on the source when compared to the six-component blend. No differences were found between calling females and the six-component blend released at a 1 FE level from the rubber septa. However, males performed all close-range behav-

iors except selection more often at the female station than at the four-component blend.

DISCUSSION

Results of our analyses of gland extracts support the findings of Klun et al. (1980) and Pope et al. (1982) which indicated that Z11–16:OH is present in pheromone gland extracts. The fact that neither group identified 14:OH, Z9–14:OH, or 16:OH is most probably explained by the minute amounts of these compounds present in the gland, the fact that the 14-carbon alcohols tend to cochromatograph with the 16-carbon aldehydes on high cyano phase columns, and because neither group extracted the glands for relatively long periods in polar solvents. Nonetheless the GC and GC-MS data obtained using a wide variety of columns and both methane and isobutane chemical ionization mass spectroscopy do prove that these alcohols are present within the pheromone gland. The presence of the alcohols and fact that neither Pope et al. (1982) nor we found any of the alcohols in volatile collections strongly suggests that the alcohols serve some function other than acting as pheromone components.

Our hypothesis is that the alcohols are immediate biosynthetic precursors for the aldehydes. In addition to the above features, this hypothesis is supported by the relative instability of aldehydes, ease of biochemical interconversion between alcohols and aldehydes (Snyder, 1972), and ratios between the alcohols which are similar to the aldehyde ratios. Similar ratios have been identified for fatty acids which have been shown to be pheromone precursors in other species (Wolf et al., 1981). Further, there are only limited data to support the hypothesis that Z11–16:OH functions as a pheromone component, and although this alcohol has been identified from gland extracts of all other species of *Heliothis* studied to date, it acts either as a behavioral inhibitor or causes significant decreases in trap captures when released at very low levels (Kehat et al., 1980; Teal et al., 1981a, 1984).

Percentages of the six aldehydes released from the excised glands and calling females were not significantly different when volatiles were collected for short periods. However, when large numbers of glands from both calling and noncalling females were used and the collection time increased to 15 or more minutes, the ratio of components recovered from the filters changed dramatically, becoming more similar to the gland extract (Table 1). It is interesting to note that the mean percentage for the combined short- and long-term collections of each component was very similar to that reported by Pope et al. (1982). Although the differences in the ratios reported by us and Pope et al. (1982) may reflect different geographical strains of *H. virescens*, as such have been reported by these authors, the disparate ratio reported by Pope et al. (1982) may also be

due to collecting from females which were not synthesizing pheromone for the latter part of the collection period.

Comparison of the theoretical and actual release rates from the rubber septa indicate that the release of the six aldehydes does follow the vapor-pressure-release model developed by Heath et al. (in preparation) and that the total amount released is linear over the concentrations and flow rates used. This is of significance because rubber septa can be formulated to release any blend of the six components at a specific ratio without empirical experimentation because the release ratio of these compounds is related to the relative vapor pressures of the components of the blend. It is of interest to note the similarity between the blend of aldehydes loaded onto the septa and that found within the pheromone gland (Table 2). This may indicate that the actual blend released from the surface of the pheromone gland is the result of the same vapor-pressure-release model which governs the release from rubber septa.

Results of the bioassay studies indicate that the four components tested in deletion studies are of importance for effective sexual signaling. Blends without *Z*9–14 : Al or *Z*11–16 : Al were not tested because their function as necessary prerequisites for mate location and inducing close-range courtship sequences was clearly demonstrated by Vetter and Baker (1983). Similarly, none of the alcohols identified from gland extracts were studied because no evidence for their release from the pheromone gland has been found by either us or Pope et al. (1982).

The compound which most obviously affected male reproductive behaviors was 16 : Al. This concurs with the findings of Vetter and Baker (1983) who also suggest that 16 : Al functions to provide an incentive for landing. We also noted a comparatively low probability of reorientation when 16 : Al was deleted. This reduction in reorientation may also reflect the need for 16 : Al to induce landing because, after leaving the source, there was no incentive to land again. Deletion of 14 : Al also resulted in a significant decrease in the probability that close-range reorientation would occur. Therefore, it appears that 14 : Al induces males to continue to perform close-range courtship behaviors when they are unsuccessful in their first attempt. This may account for the larger number of males which reorient to females via close-range ambulation and the higher probability of successful mating when reorienting at close range than from downwind ($P = 0.54$ close range, $P = 0.12$ downwind) (Teal et al., 1981b).

Deletion of either *Z*7–16 : Al or *Z*9–16 : Al from the six-component blend resulted in reductions in the probabilities that males would hover near the source and attempt copulation. This tends to suggest redundancy in the behavioral functions of these two components. However, while the addition of *Z*9–16 : Al to the four-component blend did not result in increases in any of the close range behaviors, the addition of *Z*7–16 : Al to the four-component lure increased the probabilities that all of the close-range behaviors except selection would be

performed. Therefore, it appears that Z7–16 : Al exerts subtle effects on all of the close-range behaviors monitored while Z9–16 : Al has a specific function in inducing the copulation attempt. Although most of the differences between the four- and six-component blends can be explained by simply adding the behavioral effects of Z7–16 : Al and Z9–16 : Al preferential selection of the six- over the four-component blends cannot. This suggests that both Z7–16 : Al and Z9–16 : Al summate to provide a cue which enables males to home in on calling females.

Our results indicate that all of the components in the *H. virescens* pheromone blend appear to exert influences on mating success for this species. While individual components appear to affect specific behaviors, in no case were behaviors eliminated from the sequence when individual components were deleted. Thus, no single compound is responsible for the elicitation of a specific behavior in the mating sequence. Similar situations have been described for both *Grapholitha molesta* (Busck) (Baker and Cardé, 1979) and *Trichoplusia ni* (Hubner) (Linn et al., 1984). In both of those studies all of the behaviors involved in the male reproductive sequence, including preflight behaviors, were affected by each component. Although we did not consider preflight behaviors in single-dispenser studies and could only consider close-range behaviors in dual-selection tests, our single-dispenser studies indicated that two minor components (Z7–16 : Al and Z9–16 : Al) do affect taxis when released at a 1 FE rate. Therefore, as discussed by Linn et al. (1984), the combination of all the secondary components appears to exert effects on all phases of the male reproductive sequence. Hence, while an individual component may influence specific behaviors more than other components, the interaction between all of the compounds is responsible for maximizing male response.

In conclusion, our study strongly indicates that only the six aldehydes previously identified by Klun et al. (1980) are released from the sex pheromone gland of actively calling females. Based on the absence of Z11–16 : OH from volatile samples analyzed by both us and Pope et al. (1982) and identification of the analogous alcohols of the three other major aldehyde components in gland extracts, we suggest that Z11–16 : OH serves as a biochemical precursor for the major aldehyde component released. Further, by developing a dispenser system which releases the same ratio and quantity of compounds released by calling females, we have demonstrated that components previously supposed to have no behavioral function are, in fact, necessary for the successful performance of close-range courtship behaviors by males. In fact, we have shown that males cannot distinguish the synthetic blend from actively calling females when released at the same rate.

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SYNTHESIS OF DIASTEREOMERIC MIXTURE OF 15,19,23-TRIMETHYLHEPTATRIACONTANE, CONTACT SEX PHEROMONE OF TSETSE FLY, *Glossina morsitans morsitans* WESTWOOD[1]

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Abstract—A nine-step synthesis is described for a diastereomeric mixture of 15,19,23-trimethylheptatriacontane, a contact sex pheromone of *Glossina morsitans morsitans* Westwood. The compound has been synthesized by means of double alkylation of diethyl 3-oxoglutarate (DEOG) with 3-methyl-2-heptadecenyl methanesulfonate, which was readily prepared from 1-hexadecene, as the key step.

Key Words—Tsetse fly, *Glossina morsitans morsitans*, Diptera, Muscidae, sex pheromone, branched hydrocarbon, 15,19,23-trimethylheptatriacontane, diethyl 3-oxoglutarate, alkylation.

INTRODUCTION

Several workers have investigated the sex pheromones of tsetse flies, because the flies are major vectors of African trypanosomiases such as sleeping sickness and nagana. Of interest is that many insect pheromones include such compounds as alcohols, acetates, aldehydes, and hydrocarbons (Nelson, 1978; Sonnet, 1984). Carlson et al. (1978) isolated a group of sex pheromones from the

[1] This study constitutes Part XII of "Selective Alkylation of Diethyl 3-Oxoglutarate." Part XI of this series, see Naoshima et al. (1984).

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cuticle of the female tsetse fly, *Glossina morsitans morsitans* Westwood and identified the most active component as 15,19,23-trimethylheptatriacontane (I), a branched hydrocarbon. Compound I was synthesized either as a diastereomeric mixture by Carlson et al. (1978) and Hoshino and Mori (1980) or as the set of four stereoisomers by Helmchen and Schmierer (1983). These methods were complicated and suffered from low overall yields. A recent report on pheromone effects (Helmchen and Langley, 1981) has showed that all of the four stereoisomers of I were equally active on *Glossina morsitans morsitans* Westwood.

A number of pheromones have been prepared from diethyl 3-oxoglutarate, DEOG, through reaction with a variety of alkylating agents (Naoshima et al., 1983, 1984). We now report a synthetic approach toward a diastereomeric mixture of I, which is based on the double alkylation of DEOG with 3-methyl-2-heptadecenyl methanesulfonate (II) derived from 1-hexadecene. Pheromone I was synthesized with an improved overall yield, compared with the value reported previously, by use of both DEOG and 1-hexadecene as the starting materials.

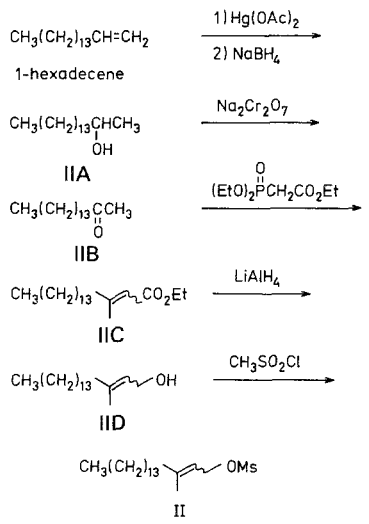
METHODS AND MATERIALS

Column chromatograpy was carried out employing 230–400 mesh silica gel (Merck Kieselgel 60 Art 9385) according to the flash technique described by Still et al. (1978). All solvent systems were expressed in ratios by volume (v/v). IR spectra were determined on a Hitachi model 260-10 spectrometer. [^{1}H]NMR spectra were obtained on a Hitachi model R-24B spectrometer in $CDCl_3$ solutions using Me_4Si as an internal standard. EI and CI mass spectra were recorded on a JEOL model JMS-D300 double-focusing mass spectrometer at 70 eV and 200 eV, respectively, using a direct insertion probe. GLC analysis for the determination of the purity of the pheromone I was made isothermally at 250°C (N_2, 60 ml/min), employing a Hitachi 163 gas chromatograph equipped with a 1-m × 3-mm glass column of 3% OV-1 on 80–100 mesh Chromosorb W AW DMCS. Sodium hydride (Aldrich Chemical Company, Inc., Milwaukee, Wisconsin) was weighed as a 60% dispersion in mineral oil and washed with dry *n*-hexane to remove the oil prior to use. Solvents were purified and dried by standard methods.

2-Hexadecanol (IIA). Compound IIA was readily synthesized from 1-hexadecene with a 97% yield by a oxymercuration–demercuration procedure (Brown and Geoghegan, 1970): mp 45–46°C (mp 41.5°C, see Messer, 1929).

2-Hexadecanone (IIB). Compound IIB was synthesized with a 96.5% yield by oxidizing IIA with sodium dichromate: mp 44–45°C (mp 43°C, see Ruzicka et al., 1928).

Ethyl 3-methyl-2-heptadecanoate (IIC). Compound IIC was synthesized by



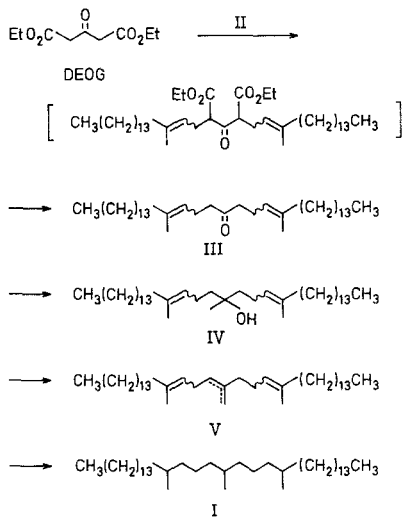
SCHEME 1.

treating IIB (24 g) with triethyl phosphonoacetate in the presence of sodium hydride. Fractionation of the product in vacuo gave IIC as a colorless liquid (29.5 g, 95%), bp 145–147°C at 0.26 mm Hg. IR (neat): 1720, 1650 cm^{-1}. [^{1}H]NMR δ: 0.87 (distorted t, 3H, J = 5 Hz), 4.04 (q, 2H, J = 7 Hz), 5.53 (broad s, 1H). EIMS *m/z*: 310 (M^+, 20), 265 (16), 128 (100). CIMS (isobutane) *m/z*: 311 [$(M+H)^+$, 100].

3-Methyl-2-heptadecen-1-ol(IID). Compound IID was synthesized by reducing IIC (28.6 g) with lithium aluminum hydride. Fractionation of the product in vacuo gave IID as a colorless liquid (21.8 g, 88%), bp 134–138°C at 0.2 mm Hg. IR (neat): 3350, 1660 cm^{-1}. [^{1}H]NMR δ: 0.87 (distorted t, 3H, J = 5 Hz), 4.08 (d, 2H, J = 6 Hz), 5.35 (t, 1H, J = 6 Hz). EIMS *m/z*: 250 (M−18, 3), 71 (100). CIMS (ammonia) *m/z*: 251 [$(M+H-H_2O)^+$, 100].

3-Methyl-2-heptadecenyl Methanesulfonate (II). Compound II was synthesized with an almost quantitative yield by treating IID (50 g) with methanesulfonyl chloride in the presence of triethylamine (Scheme 1). IR (neat): 1640 cm^{-1}. This mesylate was used directly in the next step without further characterization.

15,23-Dimethyl-15,22-heptatriacontadien-19-one (III). To a stirred suspension of sodium hydride (3.6 g, 0.09 mol) and 18-crown-6 (4 g, 0.015 mol) in dry toluene (100 ml) was slowly added a solution of diethyl 3-oxoglutarate (6.06 g, 0.03 mol) in dry toluene (15 ml) and stirring continued for an additional 1 hr at room temperature. To the solution was added a solution of 3-methyl-2-heptadecenyl methane sulfonate II (31.2 g, 0.09 mol) in dry toluene (15 ml) and the mixture was refluxed with stirring for 17 hr. After the solvent had been



SCHEME 2.

distilled off in vacuo from the reaction mixture, the residue was heated in a 15% aqueous solution of sodium hydroxide (350 ml) under reflux for 30 hr. The hydrolyzed mixture was then acidified with conc. HCl and extracted with ether. The ethereal solution was washed with brine and dried over sodium sulfate. After evaporation of the solvent, the residue was purified by column chromatography (120 g) with petroleum ether–ether (50 : 1) followed by removal of the low-boiling impurities in vacuo, giving a colorless liquid of III (5.6 g, 33% from DEOG) (Scheme 2). IR (neat): 2930, 2850, 1720, 1460, 1380, 720 cm^{-1}. [^{1}H]NMR δ: 0.88 (distorted t, 6H, J = 5 Hz), 1.25 (broad s, 48H), 1.57 (m, 6H), 1.75–2.62 (m, 12H), 5.0 (broad s, 2H). EIMS *m/z*: 558 (M^+, 3), 81 (100). CIMS (ammonia) *m/z*: 576 [$(M+NH_4)^+$, 100], 559 [$(M+H)^+$, 70]. Analysis: calcd. for $C_{39}H_{74}O$: C, 83.79; H, 13.34. found: C, 83.48; H, 12.95.

15,19,23-Trimethyl-15,22-heptatriacontadien-19-ol (IV). To a 1.0 M THF solution of methylmagnesium bromide (60 ml, 0.06 mol) was added a solution of the ketone III (5.4 g, 0.0096 mol) in THF (30 ml) at room temperature under nitrogen, and the mixture was refluxed for 6 hr. A saturated aqueous solution of ammonium chloride was added to the reaction mixture under ice cooling, and the mixture was stirred for 1 hr. The mixture was worked up to give IV as a yellow viscous liquid (Scheme 2); column chromatography (90 g) with *n*-hexane-ether (20 : 1) gave a colorless liquid (3.8 g, 68%). IR (neat): 3330, 2930, 2850, 1480, 1375, 1120, 720 cm^{-1}. [^{1}H]NMR δ: 0.82–1.02 (m, 9H), 1.25 (broad s, 52H), 1.50–2.33 (m, 14H), 3.46 (broad s, 1H), 5.05 (broad s, 2H). EIMS *m/z*: 574 (M^+, 2). CIMS (ammonia) *m/z*: [$(M+NH_4)^+$, 68], 574 [$(M+NH_4-H_2O)^+$, 92], 557 [$(M+H-H_2O)^+$, 100].

15,19,23-Trimethylheptatriacontatrienes (V). The *tert*-alcohol IV (3.2 g, 0.0043 mol) was heated in a 20% aqueous solution of H_2SO_4 (60 ml) for 45 hr. The reaction mixture was extracted with *n*-hexane–ether (3 : 1), and the ethereal solution was successively washed with 5% aqueous sodium bicarbonate and brine, and finally dried over sodium sulfate. The solvent was evaporated in vacuo to give a brown substance. Column chromatography (150 g) with *n*-hexane gave V as a colorless liquid (2.12 g, 67%) (Scheme 2). IR (neat): 2930, 2850, 1640, 1380, 890, 720 cm^{-1}. EIMS *m/z*: 556 ($M^{\ddagger}$, 10). Compound V was hydrogenated without further characterization.

15,19,23-Trimethylheptatriacontane (I). Hydrogenation of V (0.9 g, 0.0016 mol) in a mixture of ethanol (40 ml) and *n*-hexane (40 ml) was carried out in the presence of 10% Pd/C catalyst (0.24 g). The reaction mixture was worked up to give pheromone I as a yellow viscous liquid (Scheme 2). The compound was purified by column chromatography on silver nitrate-impregnated silica gel (prepared from a 100 g of SiO_2 and 20 g of $AgNO_3$) with *n*-hexane to give a colorless liquid (0.61 g, 67% from V). IR (neat): 2925, 2850, 1465, 1380, 720 cm^{-1}. [^{1}H]NMRδ: 0.76–0.94 (m, 15H), 1.25 (broad s, 67H). EIMS *m/z*: 547 (M-15, 12), 365 (72), 295 (100), 224 (81). CIMS (ammonia) *m/z*: 579 [$(M+NH_4-H)^+$, 100].

DISCUSSION

The key step in the present approach to the synthesis of pheromone I, as shown in Scheme 2, is the double alkylation of diethyl 3-oxoglutarate, DEOG. Commercially available 1-hexadecene was chosen along with DEOG for the starting material. The alkylating agent, 3-methyl-2-heptadecenyl methanesulfonate II (mixture of *E* and *Z* isomers) was easily prepared in five steps from inexpensive 1-hexadecene with a 78% yield. To shorten the synthesis of the pheromone, DEOG was doubly alkylated with II in the presence of 18-crown-6 using sodium hydride as the base (Naoshima et al., 1981), and the product was hydrolyzed with decarboxylation in an aqueous solution of sodium hydroxide to give the symmetrical long-chain unsaturated ketone III in a 33% yield. Ketone III was then subjected to a Grignard reaction with a large excess of methylmagnesium bromide. The obtained *tert*-alcohol IV (68% yield from III) was dehydrated and subsequently hydrogenated over palladium–charcoal. The product was chromatographed on a silica gel column containing silver nitrate to give a diastereomeric mixture of I.

On a small scale, a column of silica gel coated with silver nitrate is generally used for a cleanup procedure; however, this is seemed not to be suitable for large-scale preparations, since silver nitrate is an expensive reagent. In this case, a column of silver nitrate–silica gel was useful, allowing removal of trace amounts of olefins. GLC analysis of I indicated 92% chemical purity and re-

vealed the presence of impurities which were assumed to be saturated hydrocarbons on the basis of IR and [^{1}H]NMR spectra. The purity for I may or may not be satisfactory for pheromone activities, owing to the presence of the impurities.

Thus a diastereomeric mixture of the pheromone I was synthesized, as depicted in Scheme 2, in four steps from diethyl 3-oxoglutarate, DEOG, with a 10% yield. DEOG itself is easily prepared from citric acid, a natural product. It is produced industrially by the microbiological fermentation of sugars, and thus is a readily available, renewable resource (Sperling and Carraher, 1983). The overall yield of I from 1-hexadecene was actually 7.8% in nine steps. Carlson et al. (1978) described a synthetic method for I employing a Grignard reaction with ethyl acetate, although the overall yield of I was not given. A reported value for the overall yield of I from geranyllinallol was about 0.54% in ten steps (Hoshino and Mori, 1980). The yield by our own synthesis therefore exceeded that by the reported method.

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CHEMICAL IDENTIFICATION AND BEHAVIORAL CHARACTERIZATION OF MALE WING PHEROMONE OF *Ephestia elutella* (PYRALIDAE)

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Abstract—Behavioral and chemical evidence is presented for the identity of the male wing pheromone of *Ephestia elutella* (Hübner) and the role of this pheromone in courtship success is evaluated. Males with the forewing removed experienced a mating success rate less than half of that of either intact males or males that had only the wing gland area remaining of their forewings. GC-MS analysis and microchemical reactions indicated the presence of (*E*)-phytol and a series of saturated γ-lactones in a methylene chloride extract of the wings. Using an assay of female courtship behavioral response, (*E*)-phytol was found to evoke an intermediate level of response in females when presented alone, while the complete array of insect-derived γ-lactones produced no significant response. The combination of either γ-decalactone or γ-undecalactone with (*E*)-phytol in a 1:2 ratio, however, elicited a female response equivalent to that produced by the unfractionated wing extract.

Key Words—courtship pheromone, wing pheromone, male pheromone, *Ephestia elutella*, Pyralidae, phytol, γ-decalactone, γ-undecalactone.

INTRODUCTION

Behavioral studies of members of the stored-product complex of phycitine moths have revealed relatively complex courtship sequences. In addition to the long-range sex pheromone of the female, males of most species have been found to possess pheromone glands on the costal margin of the forewings (Barth, 1937;

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Grant, 1978) and/or the dorsal surface of the eighth abdominal segment (Dickens, 1936; Corbet and Lai-Fook, 1977). Female behavioral responses to conspecific wing pheromones have been demonstrated in three species: *Plodia interpunctella* (Grant and Brady, 1975; McLaughlin, 1982), *Vitula edmandsae* (Grant, 1976), and *Ephestia elutella* (Krasnoff and Vick, 1984). To date, however, no chemicals from these glands have been identified. We report here behavioral and chemical evidence for the identity of the male wing pheromone of the tobacco moth, *Ephestia elutella* (Hübner) and evaluate the importance of this pheromone on courtship success.

METHODS AND MATERIALS

Wing Pheromone Collection. Ephestia elutella were reared on a honey–bran–glycerol diet according to the procedure of Strong et al. (1968). Males and females were separated at the pupal stage, and adults were maintained in separate chambers on 14:10 light–dark cycles at 22–24°C. The wing pheromone was collected from 2- to 5-day-old males by excising the forewings during the period of peak sexual activity (3–6 hr into scotophase) and extracting them with methlene chloride for several hours. Wing extracts were filtered with glass wool, concentrated to 200–300 μl under a gentle flow of N_2, and stored at −15°C.

Chemical Identification. The wing gland extract was fractionated using a Waters Assoc. high-performance liquid chromatograph (HPLC), comprised of two model 6000A solvent delivery pumps, a No. 660 solvent programmer, a RCM-100 radial compression separation module, a No. 450 variable wavelength detector (190–700 nm), and a series R-400 differential refractometer. The extract was chromatographed on a silica gel RCM column using either an isocratic mobile phase of 1% methanol–99% methylene chloride or a linear solvent program of 100% methylene chloride to 10% methanol–90% methylene chloride in 10 min with a flow rate of 1 ml/min. The eluant was collected in 1-ml fractions. The mobile phases were "distilled in glass" spectral grade from Burdick & Jackson (Muskegon, Michigan). Analysis and further fractionation of the extract was carried out on a Varian model 3700 gas chromatograph with two hydrogen flame ionization detectors. A range of polarities in columns was used: 10% Silar 10C (cyanopropyl silicone) on 100–120 mesh ChW Ac (3 m × 4 mm, 4.4 g), 2% SF-96 (methyl silicone) on 100–120 mesh ChW Ac DMCS (2 m × 4 mm, 3.2 g), and Durawax-4 (polyethylene glycol stabilized with methyl silicone) fused-silica capillary (30 m × 0.25 mm, film = 0.25 μm) from J&W Scientific (Rancho Cordova, California). Carbon disulfide, used in GC analysis, was redistilled daily.

Complete mass spectra of active components were obtained using a Finnigan 4021 EI/CI capillary gas chromatograph–mass spectrometer (GC-MS) coupled to a INCOS data system. A Grob injector was used in conjunction with

the following fused-silica capillary columns (Chromatographic specialities, Brockville, Ontario): a 30-m DX-4 temperature programmed from 50°C (2 min hold) to 100°C at 30°C/min, then 10°C/min to 200°C; 30-m DB-5 held at 50°C for 1.5 min programmed at 30°C/min to 120°C, then 10°C/min to 250°C. All EI-MS and CI-MS (methane) were generated at 70 eV.

Microchemical reactions were carried out on behaviorally active HPLC fractions for confirmation of the chemical identities of pheromone components. The products of these reactions were analyzed by GC. Evidence for esters was provided by base hydrolysis (Bjostad et al., 1984). After reducing the sample to near dryness with a gentle stream of N_2, 100 μl of ethanol and 20 μl of 5% KOH were added and the solution was allowed to sit in a 1-ml reaction vial with Teflon-lined screw cap at 50°C for 1 hr. Water was then added and the aqueous solution was extracted three times with hexane. The hexane portion was concentrated under N_2 and was slowly replaced by CS_2. The presence of alcohols was established by acetylation, in which a behaviorally active fraction was taken to near dryness and 2–3 drops of acetyl chloride were added. After 1 min, the acetyl chloride was evaporated under N_2 and replaced with CS_2. For determination of double-bond position by ozonolysis (Beroza and Bierl, 1967), ozone was bubbled for 30 sec into 30 μl of CS_2 followed by the addition of 1–5 μl of the sample. Nitrogen was then bubbled through the solution for 30 sec and 1 μg triphenylphosphine in carbon disulfide was added to break up the ozonide product.

Synthetic γ-lactones were obtained from ICN-K&K Laboratories (Plainview, New York), except for γ-decalactone and (*E*)-phytol which were purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin). The (*E*)-configuration of the commercial phytol was determined by 200-MHz proton NMR, GC, and GC-MS analysis. (*Z*)-Phytol was synthesized by a modification of the scheme of Burrell et al. (1966). A solution of commercial (*E*)-phytol (0.10g) in anhydrous methanol (5 ml) at −78°C was treated with ozone for 15 min. After flushing with N_2, the ozonide was discharged with dimethyl sulfide (0.5 ml) and the solvent was evaporated in vacuo. The crude product was purified by flash chromatography (Still et al., 1978) with hexane–ethyl acetate (9:1) to yield 6,10,14-trimethyl-2-pentadecanone (0.095 g). A solution of this ketone in anhydrous tetrahydrofuran (0.5 ml) was added to a mixture of ethoxyacetylene (0.12 g, 50% in hexane), anhydrous THF (0.5 ml), and *n*-butyl lithium (1.6 ml, 1.2 M in hexane) at 0°C. After 1 hr stirring at this temperature, the reaction was quenched with a saturated NH_4Cl solution (5ml) and extracted into ether (5ml).

Evaporation of the organic phase yielded 2-ethoxyethynyl-6,10,14-trimethylpentadecan-2-ol (0.12g). The acid-catalyzed rearrangement of the adduct was carried out with 10% aqueous oxalic acid (1ml) in ethanol (0.5 ml) at 22°C for 18 hr. Purification of the crude product by preparative TLC (silica gel) gave ethyl phytenoate (0.07g) as a mixture of *E*/*Z* isomers in a ratio of 50:50 as determined by GC (relative retention time *E*/*Z* = 1.19) and NMR [(*Z*)-3-methyl,

δ1.86; (*E*)-3-methyl, δ2.11]. A solution of the ester (0.07 g) in dry toluene (1 ml, distilled from sodium) was treated with Vitride (0.04 ml, 70% in toluene) at 0°C for 7 hr and at 22°C for 10 hr with additional Vitride (0.03 ml). Vitride [sodium dihydrobis(2-methoxy-ethoxy)aluminate] was purchased from Hexcel Specialty Chemicals (Zealand, Michigan). The reaction was quenched with methanol (1 ml), concentrated in vacuo, and purified by preparative TLC to give the following: recovered ester (0.028 g), (*Z*)-phytol (0.011 g), and (*E*)-phytol (0.009 g). These assignments were supported by NMR, GC, and GC-MS analysis.

(E)-*Phytol*. NMR (200MHz, $CDCl_3$): δ0.85 (12H, d, J = 6 Hz), δ1.24–1.54 (20H, m), δ1.65 (3H, s, vinylic CH_3), δ2.00 (2H, t, J = 8 Hz), δ4.14 (2H, m), δ5.40 (1H, broad t, J = 6 Hz); GC (DX-4) retention time ca. 15.4 min; MS (EI 70 eV) *m/e* 296 (M^+), 278, 196, 123, 71 (base peak).

(Z)-*Phytol*. NMR (200MHz, $CDCl_3$): δ0.85 (12H, d, J = 6 Hz), δ1.05–1.34 (20H, m), δ1.72 (3H, d, J = 1 Hz), δ2.03 (2H, t, J = 7 Hz), δ4.14 (2H, m), δ5.40 (1H, broad t, J = 6 Hz); GC (DX-4) retention time ca. 14.4 min; MS (EI 70 eV) *m/e* 296 (M^+, weak), 278, 196, 123, 71 (base peak).

Behavioral Assays. The importance of the wing pheromone in courtship success was investigated by ablation of the gland, using the procedure of Grant and Brady (1975). Within 24 hr of eclosion to the adult, males were cold-anesthetized and either the entire forewing was removed or all of the forewing except the costal wing-gland area. This procedure required that the male be anesthetized <3 min, and the two operations were alternated to control for operator experience. These males were provided sugar water and were tested during their second scotophase, at which time they were introduced into a screen cylinder containing a calling female; the ensuing courtship was video-recorded for more accurate analysis at a later time.

For determination of pheromone components, five male equivalents (ME) of each fraction were tested using a modified version of the Kransnoff and Vick (1984) bioassay, and the responses of the females were compared with those responses elicited by the crude wing-gland extract and clean-air controls. The material to be tested was placed on the inside of a 0.5 cm OD × 10-cm-long glass tube. Air, which was purified by two separated charcoal filters, was passed through this tube at 15 cm/sec. After allowing air to flow through the tube for 10 sec to evaporate the solvent, the end of the tube was presented to a calling virgin female tobacco moth. The use of a gentle airflow ensured that the chemicals were delivered to the female's head. Two- to five-day-old females, which were 3–6 hr into their scotophase, were tested either in small cylindrical cages (2 females/cage) or in a large rearing cage. The cages were backlighted by a 0.2-lux light. Females were considered to show a positive response if they exhibited either a ventral flexion of the abdomen (as described by Krasnoff and Vick, 1984) or turning without forward locomotion. Treatments were presented in a randomized complete-block design, with eight replicates of 10 females per

treatment. Statistical analyses were carried out using Duncan's new multiple-range test or Ryan's (1960) multiple-comparison test for proportions.

RESULTS

Wing Pheromone and Courtship Success. Removal of the wing gland substantially reduced the mating success of male *E. elutella* (Table 1). Males with entire forewings removed recorded a courtship success rate less than half that of either intact males or males with all of the forewing removed except the wing gland. Furthermore, the successful courtships by the glandless males were twice as long as those of males with their wing glands intact; the glandless males met with a much higher frequency of female rejection behaviors, such as turning away from the male or covering the abdomen with their wings, which made copulation more difficult to achieve. Males having all of the forewing removed except for the costal fold were as successful as intact males during courtship. Therefore, courtship success by males appears to depend primarily on the presence of intact costal-fold wing glands and not on mechanical effects such as wind generated by the entire forewings.

Wing Pheromone Identification. Preliminary bioassays (30 females/treatment) of HPLC fractions of male *E. elutella* wing-gland extract indicated that the behaviorally active components were contained in two adjacent 1-ml fractions. Activity elicited by either of these fractions was comparable to that of the complete wing extract (57% and 53% female response for the fractions, respectively, and 63% response for the extract; other fractions evoked $<10\%$ response). These two fractions were characterized by similar complex arrays of peaks on a Silar 10C GC column. Fractional collection of these HPLC fractions from Silar 10C followed by bioassay indicated that full behavioral activity was contained in two adjacent 2.5-min collections. Combining the two GC fractions

TABLE 1. COURTSHIP SUCCESS RATES AND LENGTH OF SUCCESSFUL COURTSHIPS IN MALES WITH FOREWINGS COMPLETELY REMOVED (NO WGS) OR ONLY COSTAL FOLD REMAINING (WGS ONLY) COMPARED TO INTACT MALES

Treatment	*N*	Courtship success (%)	Length of successful courtship (sec ± SD)
No WGs	34	32 b[a]	34.6 ± 10.6 b[b]
WGs only	28	75 a	14.7 ± 4.9 a
Intact males	24	83 a	17.6 ± 4.7 a

[a] Values within the column followed by the same letter not significantly different at $P < 0.05$ by Ryan's multiple-comparison test for proportions.

[b] Values within the column followed by the same letter not significantly different at $P < 0.05$ by Duncan's new multiple-range test.

produced a response of 50% (15/30 females), compared to 53% (16/30) for the combined HPLC fractions. These areas of activity were coincident with two prominent peaks at 8.9 and 11.4 min evident in GC tracings of the original HPLC fractions.

A second HPLC solvent regime (100% methylene chloride to 10% methanol–90% methylene chloride in 10 min) was utilized to fractionally resolve the active components. The component represented by the earlier GC peak was subjected to a number of chemical reagents to aid in its identification. It was unchanged when treated with KOH in EtOH; however, upon treatment with acetyl chloride, the compound (retention time 17.4 min on Durawax-4) was transformed to one with retention time 16.0 min. Base hydrolysis of the acetylated product regenerated the original GC peak. These results strongly suggested the presence of an alcohol. The retention time of this compound on both Durawax-4 and SF-96 was enveloped by those of 1-octadecanol and 1-eicosanol. Ozonolysis of the compound yielded a product with a GC retention time (Durawax-4) and MS fragmentation pattern virtually identical to those of 6,10,14-trimethyl-2-pentadecanone, which was obtained from the ozonolysis of synthetic (*E*)-phytol. Indeed, GC-MS analysis of the putative pheromone component was consistent with that of (*E*)-phytol (3,7,11,15-tetramethyl-2-hexadecenyl alcohol), with identical retention times (*E*/*Z* relative retention time = 1.08 on Durawax-4) and identical fragmentation patterns (Figure 1). (Z)-Phytol was apparently absent from the wing-gland extract. Finally, GC-MS analysis of the online hydrogenation (Beroza and Sarmiento, 1966) of (*E*)-phytol and of the natural component yielded products with matching mass spectra and identical GC retention times on SF-96, Silar 10C, Durawax-4, and DB-5 columns.

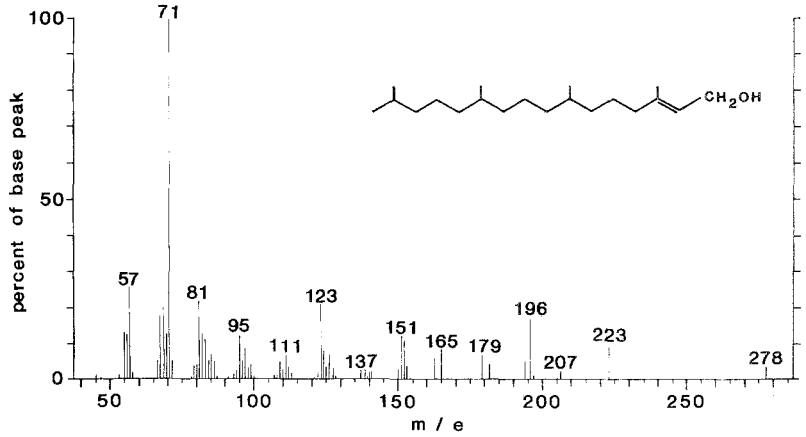

FIG. 1. EI mass spectrum of (*E*)-phytol (3,7,11,15-tetramethyl-2-hexadecenyl alcohol) isolated from *Ephestia elutella* male wing glands.

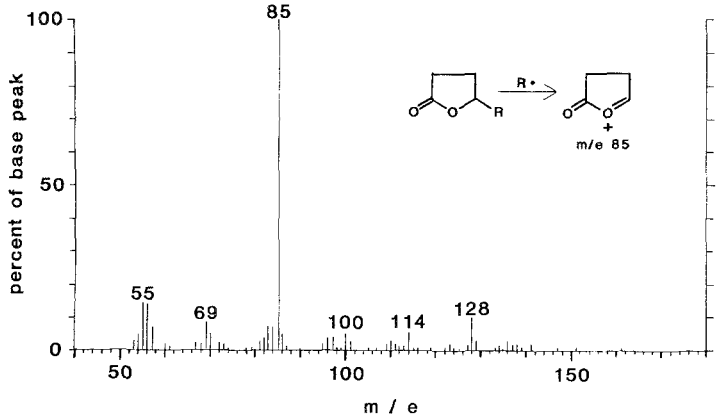

FIG. 2. EI mass spectrum of γ-decalactone isolated from *Ephestia elutella* male wing glands.

The remaining active HPLC fraction, analyzed by GC-MS, contained a series of compounds that exhibited an intense base peak of *m/e* 85 (Figure 2). This fragment is characteristic of the cleavage of the side chain from a γ-lactone (Honkanen et al., 1965). CI-MS (methane) analysis of this series did indeed produce M+1 ions consistent with those of the saturated C_9-, C_{10}-, C_{11}-, C_{12}-, C_{14}-, C_{16}-, and C_{18}-γ-lactones (Figure 3). The identities of two other compounds present with base peaks of *m/e* 85 were not confirmed, although their mass spectra suggested γ-lactones with either unsaturated or substituted side chains. The retention times of the seven wing-extract components matched those of the seven respective synthetic saturated γ-lactones on three GC columns: Silar 10C, SF-96, and Durawax-4. The retention times of C_{14}-, C_{16}-, and C_{18}-γ-lactones were determined by extrapolation of a retention time curve using synthetic C_7-, C_8-, C_9, C_{10}-, C_{11}-, C_{12}-γ-lactones for each GC column.

Behavioral Assays. In the first assay of extracted wing components (Figure 4), a blend of 25 ng each (approx. 5 ME) of synthetic γ-nona-, γ-deca-, and γ-undecalactone was found to elicit a response by females not significantly greater than that produced by clean air; however, when this blend of lactones was combined with 5 ME of the HPLC fraction containing (*E*)-phytol, female response was comparable to that evoked by either the HPLC fraction containing the full complement of natural γ-lactones combined with the (*E*)-phytol-containing HPLC fraction or the unfractionated wing extract. Since the presence of the four longer-chain γ-lactones (C_{12}, C_{14}, C_{16}, and C_{18}) did not appear to increase female response, no further work was attempted on these compounds. When presented alone, the (*E*)-phytol HPLC fraction elicited a response intermediate to that evoked by the clean-air control and the complete wing extract. When individually combined with the (*E*)-phytol fraction (Figure 5), both γ-

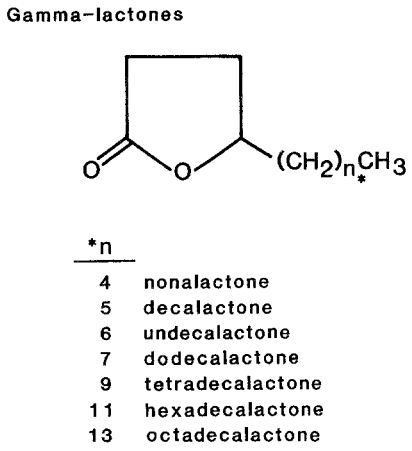

FIG. 3. γ-Lactones determined to be present in *Ephestia elutella* male wing glands.

decalactone and γ-undecalactone increased female response to a level that was significantly greater than the response to (*E*)-phytol alone and not significantly different from that to the full extract. The addition of γ-nonalactone to (*E*)-phytol, on the other hand, had no effect on female response.

Finally, female response to the synthetic (*E*)-phytol (50 ng, 5 ME) was

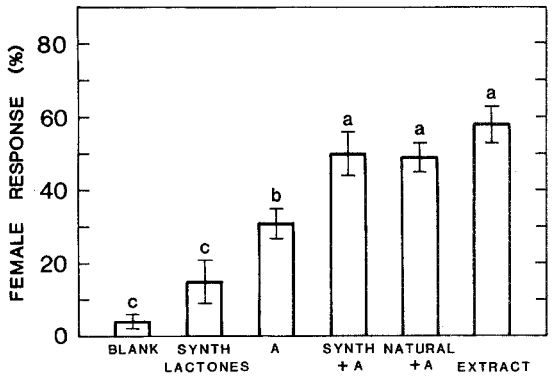

FIG. 4. Response of *Ephestia elutella* females to three synthetic lactones presented alone and in combination with an (*E*)-phytol-containing HPLC fraction (A) of *Ephestia elutella* male wing glands. Activity is compared to the complete wing extract and a recombining of A with the entire complement of lactones isolated from the wing gland (Natural). Different letters indicate significant differences at $P < 0.05$ as determined by Duncan's new multiple-range test of arcsin-transformed values. I bars indicate standard errors.

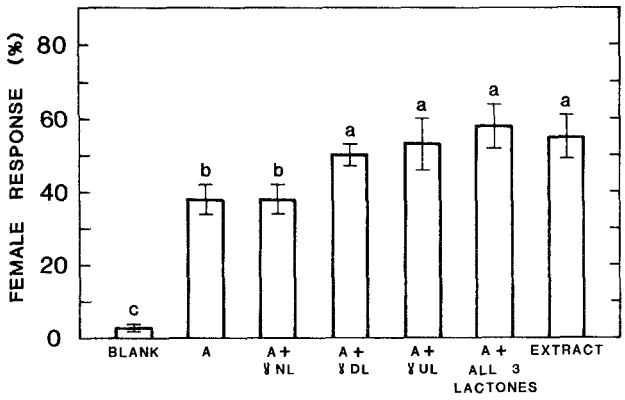

FIG. 5. Response of *Ephestia elutella* females to synthetic lactones when individually added to the (*E*)-phytol-containing HPLC fraction (A) isolated from the male wing gland. Different letters indicate significant differences at $P < 0.05$ as determined by Duncan's new multiple-range test of arcsin-transformed values. I bars indicate standard errors.

assessed (Figure 6). As in earlier assays with the insect-derived (*E*)-phytol, synthetic (*E*)-phytol evoked a response that was intermediate between the clean-air control and the unfractionated wing extract. More significantly, the combination of the synthetic compounds (*E*)-phytol, γ-decalactone, and γ-undecalactone produced a response identical to that elicited by the full wing-gland extract.

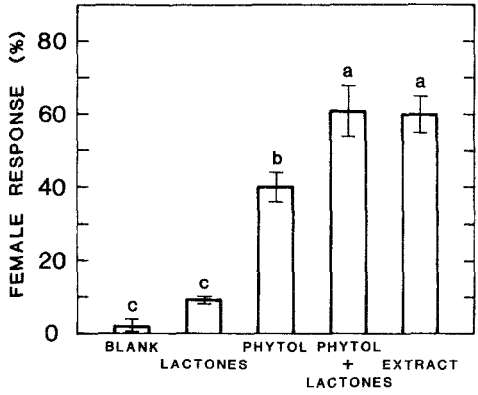

FIG. 6. Response of *Ephestia elutella* females to synthetic (*E*)-phytol alone and in combination with three synthetic lactones. Different letters indicate significant differences at $P < 0.05$ as determined by Duncan's new multiple-range test of arcsin-transformed values. I bars indicate standard errors.

DISCUSSION

We have characterized the importance of the wing pheromone to the mating success of *Ephestia elutella* males. In the past, attempts to identify male pheromones of many species of Lepidoptera have been plagued by the lack of overt behaviors on the part of the female. In *E. elutella*, however, female behaviors important in the courtship sequence are easily observed and can be elicited consistently, using chemicals in the absence of males (Krasnoff and Vick, 1984). Our chemical and behavioral data suggest that the wing pheromone of *E. elutella* consists of a blend of (*E*)-phytol, γ-decalactone, and γ-undecalactone. (*E*)-Phytol appears to be the most important component in that it was the only component that evoked a significicant level of female response when presented alone. The addition of either γ-decalactone or γ-undecalactone to (*E*)-phytol increased the response to a level not significantly different from the complete wing-gland extract. The other lactones identified from the wing glands appeared to be unimportant for female response. Females were not significantly affected by the addition of γ-nonalactone to (*E*)-phytol, and the absence of the longer-chain γ-lactones did not prevent maximum female response from being elicited.

The behavioral activity of (*Z*)-phytol, the stereoisomers of (*Z*)- and (*E*)-phytol, and the enantiomers of the γ-lactones were not studied. Full behavioral activity was evoked by the racemic synthetic compounds; therefore, it would appear that any nonpheromonal enantiomers or stereoisomers present in the synthetic mixture were not behaviorally antagonistic. Complete elucidation of the stereochemistry of this pheromone will require further chemical and behavior testing.

(*E*)-Phytol has long been known as the diterpenoid alcohol moiety of chlorophyll in plants (Willstatter et al., 1919), and both the *Z* and the *E* isomers have been isolated as unbounded molecules from the red algae, *Gracilaria andersonia* (Sims and Pettus, 1976). The present study, however, is the first report of (*E*)-phytol as a volatile component in an animal system, to our knowledge. The report of volatile γ-lactones, on the other hand, is not without precedence. Howard et al. (1983) reported the presence of γ-decalactone in anal droplets formed by the thrips, *Bagnalliella yuccae*. Wheeler et al. (1972) identified γ-dodecalactone from the defensive secretions of several species of *Bledius* rove beetles (Staphylinidae), and Dettner and Schwinger (1982) found γ-decalactone, γ-undecalactone, γ-dodecalactone, and γ-tetradecalactone in the secretions of other staphylinids. In each of these cases, however, the lactones have been found to function in a defensive role, and ours is apparently the first report of γ-decalactone and γ-undecalactone as pheromone components.

Although this bioassay proved to be a convenient method for identifying behaviorally active compounds, it measured only one set of behaviors in the courtship sequence of *E. elutella*. It is possible, therefore, that the roles of other

compounds present in the wing glands have been underestimated. Nevertheless, it seems probable from our behavioral data that these compounds would serve, at most, only a secondary role. This assertion, however, can only be verified by investigating the effect of these synthetic compounds on the complete courtship sequence and on courtship success.

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EFFECT OF COMBINED FEEDING STIMULI ON INGESTION BY THE NEMATODE *Trichostrongylus colubriformis*[1]

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Abstract—Ingestion by the nematode *Trichostrongylus colubriformis* was stimulated by serotonin, histamine, and dopamine. Immune serum inhibited feeding by both sexes while octopamine depressed feeding by only male nematodes. However, the combined influence of these excitatory and inhibitory stimuli is unknown. Feeding in vitro by both sexes of the nematode *T. colubriformis* was similar in millimolar ratios of histamine and dopamine or histamine and serotonin, but not dopamine and serotonin. Feeding by nematodes that was stimulated by histamine, dopamine, and serotonin was decreased by subsequent exposure to octopamine. Conversely, elevated concentrations of histamine, dopamine, and serotonin decreased the inhibitory action of octopamine on helminth ingestion in vitro. Doses of immune serum also decreased the stimulated feeding of nematodes that was induced by histamine, dopamine or serotonin. However, the addition of elevated concentrations of these biogenic amines to immune serum reversed the inhibitory effect. The feeding activity of nematodes may represent a summation of the inhibitory and excitatory stimuli that occur in the chemical environment of the helminth.

Key Words—*Trichostrongylus colubriformis*, Nematoda, parasite feeding, helminth ingestion, biogenic amines, host serum, serotonin, histamine, dopamine, octopamine.

INTRODUCTION

Recent studies of ingestion by nematodes have determined that various chemical stimuli may modulate helminth feeding, based on the in vitro and in vivo uptake

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of fluorescent dye. Histamine and dopamine stimulated in vitro feeding by both sexes of *Trichostrongylus colubriformis* (Bone and Bottjer, 1985b). Octopamine reduced feeding by male helminths, but not females. Other stimuli, such as host bile or chyme, sugars, amino acids, and enzymes had no effect on in vitro ingestion by helminths. Thus, ingestion by nematodes may be regulated by chemical cues from the host rather than nutrients.

The immune status of the host may alter ingestion by intestinal helminths. Serum from an uninfected host had little influence on in vitro feeding by *T. colubriformis*, while immune serum from infected goats decreased feeding by nematodes. Suppression of helminth feeding by immune sera increased with the duration of the host's infection (Bottjer et al., 1985). Indirect immuno fluorescent studies revealed binding of immunoglobulin G_1 to the worms which was associated with an inhibition of feeding by nematodes. Other reports also indicate that serum influenced feeding by nematodes. Dog serum stimulated the in vitro uptake of glucose by *Ancylostoma caninum* (Fernando and Wong, 1964). Roberts and Fairbairn (1965) reported that dog serum was a feeding stimulus for *A. caninum*, but that rabbit serum was an ineffective stimulant of feeding by *Nippostrongylus brasiliensis*.

Nematodes in an intestinal habitat are simultaneously exposed to a variety of multiple stimuli that may alter their ingestive activity. Accordingly, this study examined the combined influences of selected biogenic amines and immune serum on in vitro feeding by *T. colubriformis*. Multiple excitatory and inhibitory stimuli may be more representative of the nematodes' chemical environment and thus provide a greater understanding of the modulation of pharyngeal pumping by helminths in response to the chemical environment.

METHODS AND MATERIALS

Trichostrongylus colubriformis was maintained in male, cross-bred goats according to previous procedures (Bone and Bottjer, 1985a). Animals were free of helminthic infection, based on fecal examination. Helminths were recovered at 21 days postinfection for experimental determination of their feeding, based on the in vitro ingestion of dye.

Triplicate samples of 250 worms of each sex were collected by mechanical pippettor, rinsed repeatedly, and placed in 2 ml of glucose-free Tyrode's solution (GFTS) at 37°C. The fluorescent dye rhodamine B was added at a final concentration of 100 μg/ml. After 30 min for feeding, the worms were rinsed repeatedly to remove the dye. Helminths were macerated in a mechanical tissue grinder prior to fluorometric analysis of ingested dye versus a series of rhodamine B standards. Background fluorescence was corrected by analyses of undyed worms. Feeding was determined as nanograms of ingested dye per microgram of dry body weight of the helminths. Cuticular uptake of dye was not

observed; thus, fluorometry determined only the oral ingestion of rhodamine B (Bottjer and Bone, 1984).

These procedures were used to evaluate in vitro ingestion by nematodes during incubation in various combinations of excitatory or inhibitory stimuli. Blood was taken from goats after 90 days of a primary infection with 50,000 larvae of *T. colubriformis*, allowed to clot, and centrifuged to obtain immune serum. Sera were pooled and frozen at −4°C until use. Biogenic amines were obtained from Sigma. The following treatments were done:

Combined Excitatory Biogenic Amines. Solutions (100 mM) of histamine (diphosphate), serotonin (creatine sulfate complex), and dopamine (hydrochloride) were prepared in Tyrode's solution. The solutions were freshly prepared and had a pH range of 4.85–6.86. The solutions were mixed to produce millimolar ratios of 100:0, 75:25, 50:50, 25:75, and 0:100. Helminths were incubated in histamine and dopamine, histamine and serotonin, or dopamine and serotonin at the above ratios. After 15 min of exposure to these stimuli, dye was added to the worms to determine their ingestion after an additional 30 min.

Combined Excitatory and Inhibitory Biogenic Amines. Helminths were incubated in 100 mM octopamine to which 0–10 mM concentrations of histamine were added. Similarly, worms were treated with 1 or 10 mM histamine that was augmented with 0–100 mM octopamine. The combined effects of octopamine and dopamine were examined by a similar protocol that used 100 mM octopamine with 0–100 mM dopamine or 10 mM dopamine with 0–100 mM octopamine. *T. colubriformis* was incubated also in 100 mM octopamine with 0–100 mM concentrations of serotonin and 100 mM serotonin with 0–100 mM concentrations of octopamine. After 15 min of equilibration in the initial stimulus, the second stimulus and dye were added to determine dye uptake after an additional 30 min for ingestion.

Combined Excitatory/Inhibitory Biogenic Amines and Immune Serum. The effect of immune serum and various excitatory biogenic amines on nematode feeding was examined also. *T. colubriformis* was incubated in immune serum (100%) with 0–500 mM concentrations of histamine, 0–250 mM concentrations of dopamine, or 0–100 mM concentrations of serotonin. Similarly, worms were placed in 10 mM dopamine, 10 mM histamine, or 100 mM serotonin to which 0–100% concentrations of immune serum were added.

The combined effect of the inhibitory stimuli, octopamine and immune serum, was examined also. Feeding by *T. colubriformis* was determined in 100% immune serum that was augmented with 0–100 mM octopamine or in 100 mM octopamine to which 0–100% concentrations of immune serum were added. These experiments allowed a 15-min equilibration in the initial stimulus before exposure of the worms to the second stimulus and dye for a 30-min period of ingestion.

Data was analyzed by linear regression and analysis of variance. The 0.05 probability level was considered significant.

RESULTS

Oral uptake of dye by male and female of *T. colubriformis* in the combined solutions of excitatory biogenic amines is shown in Figure 1. Ingestion of dye by the helminths was similar in the tested ratios of histamine–dopamine or histamine–serotonin. However, uptake of dye declined significantly in male, but not female, nematodes that were incubated in various ratios of dopamine–serotonin, when compared to their uptake in only dopamine (Figure 1A).

The effect of dosages of the feeding inhibitor octopamine on oral ingestion of dye by worms that were initially stimulated by histamine, dopamine, or serotonin is shown in Figure 2. Feeding by male *T. colubriformis* in serotonin (100 mM), dopamine (10 mM), and histamine (1 mM) was significantly reduced by the subsequent addition of 100 mM octopamine to their media. Ingestion by male nematodes in 10 mM histamine was not affected by the addition of 100 mM octopamine. Uptake of dye in vitro by female worms in dopamine, but not histamine or serotonin, was decreased significantly by the addition of octopamine (Figure 2B).

The inhibition of feeding by *T. colubriformis* males that was caused by 100 mM octopamine was reversed by subsequent addition of 100 mM serotonin, 100 mM dopamine, or 10 mM histamine to the media (Figure 3). Ingestion by female nematodes in 100 mM octopamine was increased only slightly by addition of serotonin, dopamine, or histamine to their media.

The addition of doses of immune serum to the worms' media that contained 10 mM histamine, 10 mM dopamine, 100 mM serotonin, or 100 mM octopa-

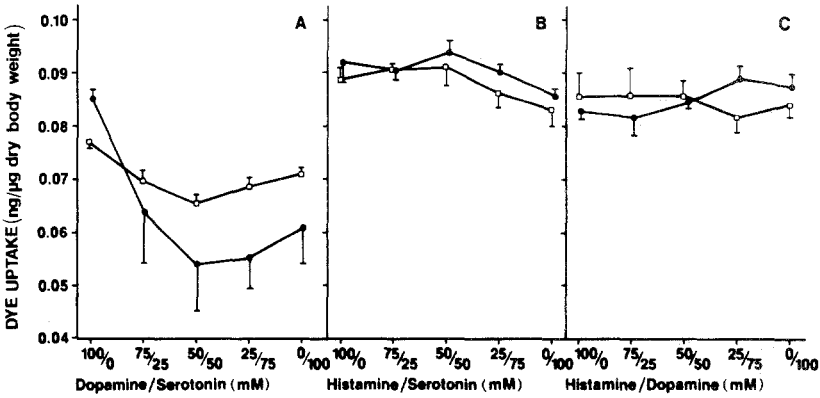

FIG. 1. Uptake of dye (ng/µg dry body weight) by males (●) and females (□) of *T. colubriformis* that were incubated in the indicated millimolar ratios of dopamine–serotonin (A), histamine–serotonin (B) or histamine–dopamine (C).

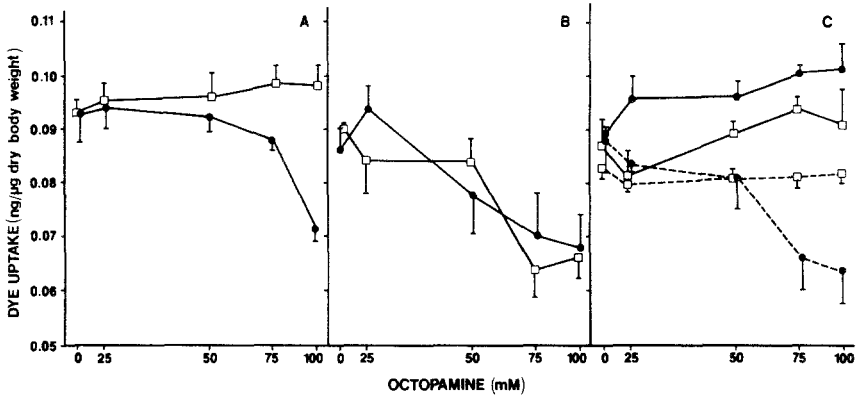

FIG. 2. Uptake of dye (ng/µg dry body weight) by males (•) and females (□) of *T. colubriformis* that were incubated in 100 mM serotonin (A), 10 mM dopamine (B), and 1 mM (---) or 10 mM (—) histamine (C) with subsequent additin of the indicated concentrations of octopamine.

mine reduced dye uptake by both sexes of nematode (Figure 4). Feeding by both helminth sexes in 100% imune serum was increased significantly after the subsequent addition of histamine, dopamine, or serotonin to the serum (Figure 5). Ingestion by female *T. colubriformis* in 100% immune serum was unchanged by the addition of octopamine to the serum, whereas feeding by the males in immune serum was decreased by addition of octopamine (Figure 5D).

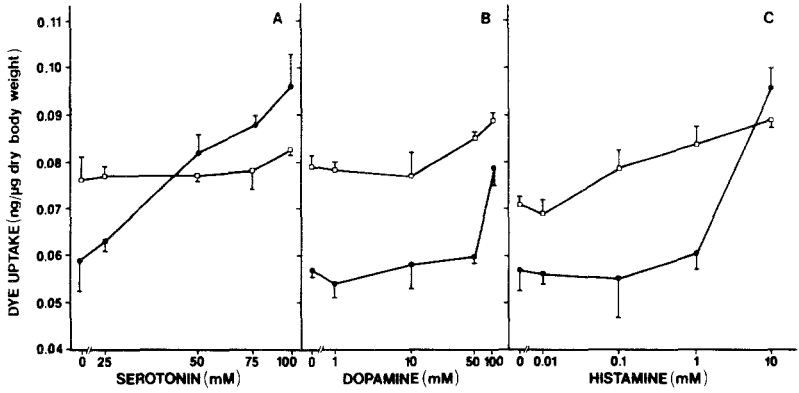

FIG. 3. Uptake of dye (ng/µg dry body weight) by males (•) and females (□) of *T. colubriformis* that were incubated in 100 mM octopamine with subsequent addition of the indicated concentrations of serotonin (A), dopamine (B), and histamine (C).

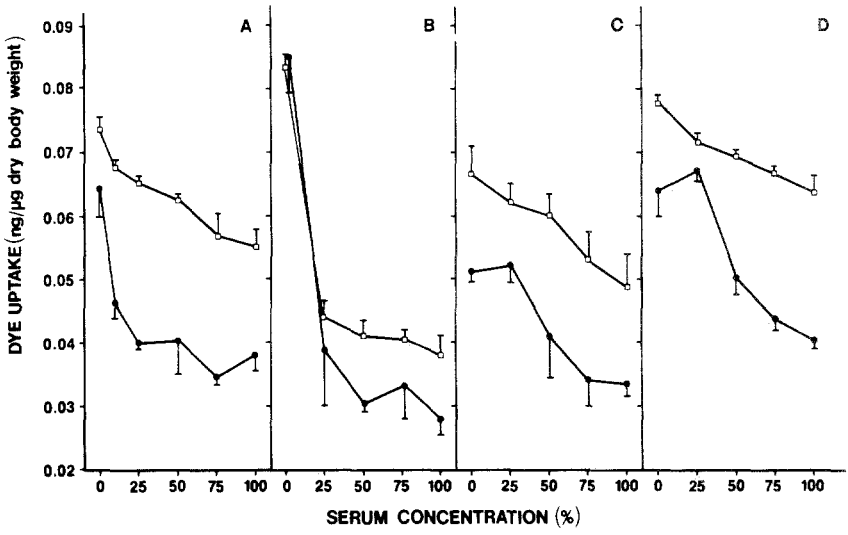

FIG. 4. Uptake of dye (ng/µg dry body weight) by males (•) and females (□) of *T. colubriformis* that were incubated in 10 mM histamine (A), 10 mM dopamine (B), 100 mM serotonin (C), and 100 mM octopamine (D) with subsequent addition of the indicated concentrations of immune serum.

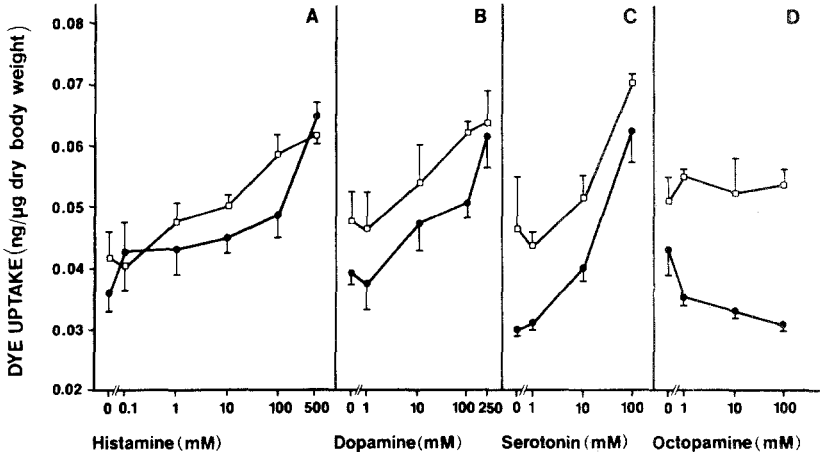

FIG. 5. Uptake of dye (ng/µg dry body weight) by males (•) and females (□) of *T. colubriformis* that were incubated in 100% concentrations of immune serum with subsequent addition of the indicated concentrations of histamine (A), dopamine (B), serotonin (C), and octopamine (D).

DISCUSSION

The biogenic amines dopamine, histamine, octopamine, and serotonin are reported from nematodes (Willett, 1980; Barrett, 1981). Catecholaminergic nerves occur also. Thus, alterations of neuromuscular events, such as pharyngeal pumping should be expected after exposure to these compounds. If ingestion of dye is used as an indicator of uptake from the media, the exposure of worms to a 100 mM solution in vitro results in less than a 0.09 nM internal concentration, based on the organisms dry weight. Horvitz et al. (1982) reported that pharyngeal pumping by the free-living nematode *Caenorhabditis elegans* was stimulated and inhibited, respectively, by serotonin and octopamine. Histamine was found to increase pharyngeal pumping in several free-living nematodes (Croll, 1975).

Treatment of the sexes of *T. colubriformis* with various ratios of histamine–serotonin and histamine–dopamine revealed little alteration in the helminth's ingestion. In contrast, feeding by male nematodes declined as the ratio of dopamine–serotonin was decreased. Thus, some competition between these latter biogenic amines is suggested. Absence of knowledge on the pharyngeal innervation of *T. colubriformis* makes further conjecture difficult.

Octopamine inhibited feeding by *T. colubriformis* even when ingestion was stimulated by serotonin, dopamine, and histamine. Histamine at 10 mM blocked the inhibitory effect of octopamine but failed to stimulate feeding, which suggests some level of inhibition occurred. The bases of these variations in the effects of inhibitory and stimulatory interactions of the biogenic amines is presently unknown. However, qualitative and quantitative differences in the receptors for these biologically active compounds are a likely source of the variation.

Other studies suggest that host immunity may affect feeding of zooparasitic nematodes. The intestinal cells of *T. colubriformis* and *Nippostrongylus brasiliensis* exhibit damage caused by host immunity (Rothwell et al., 1980; Lee, 1969). Alterations of feeding by nematodes may reduce their adenylate energy change as found in immune-damaged helminths (Ballantyne et al., 1978). Jones et al. (1978) reported that histamine was elevated in the host's intestine during infection and may be responsible for immune expulsion of *T. colubriformis* from the gut. Histamine, a vasodilator that facilitates leakage of immunoglobulin into the intestine, may initially cause increased ingestion by nematodes as an anticipatory response to subsequent decreases in feeding that are induced by immune serum.

This study suggests that an interaction of histamine, or other biological amines and immunoglobulin as elements of host physiology may modulate ingestion by nematodes. Histamine-induced feeding was blocked by the binding of a mannose-specific lectin to the anterior of *T. colubriformis* (Bone and Bottjer, 1985a). Thus, these amines may effect surface receptors rather than

acting directly on internal innervation of the pharynx. Bottjer er al. (1985) reported that immunoglobulin G_1 was bound to the cephalic region of the worm. Thus, the immunoglobulin may mask the surface receptors of the nematode. Consequently, higher concentrations of chemicals, as used in this study, are required to permeate the bound immunoglobulin. Zuckerman and Jansson (1984) reported that chemotaxis to a bacterial food source by free-living nematodes was inhibited by lectin blocking or enzyme obliteration of chemoreceptors. Host immunity may have a similar inhibitory influence on the chemical stimuli that induce ingestive function in zooparasitic nematodes.

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CHEMICAL FRACTIONATION OF SHRIMP EXTRACTS INDUCING BOTTOM FOOD SEARCH BEHAVIOR IN COD (*Gadus morhua* L.)

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Abstract—The bottom food search (BFS) feeding behavior in cod (*Gadus morhua* L.), has been used in a bioassay for chemical isolation of the feeding stimulant substances present in shrimp (*Pandalus borealis*). An aqueous methanol extract of ground shrimp was separated into acidic, neutral, and amphoteric/basic fractions by ion-exchange chromatography and into single components by preparative high-pressure liquid chromatography (HPLC). Of the isolated single components, the amino acid glycine was most potent, followed by alanine. Two unidentified substances were also highly potent. There was a synergistic effect between glycine, alanine, proline, and arginine. These four amino acids were more potent than the total amino acid pool found in the shrimp extract, indicating that there may be amino acids in this pool having an antagonistic effect.

Key Words—Shrimp, *Pandalus borealis*, cod, *Gadus morhua*, food search, behavior, amino acids.

INTRODUCTION

The consumption of food in cod is composed of a series of distinct behavior patterns which add up to the total consumatory act. The behavior patterns involved in food search activity are orientation, approach, nibbling, biting, rushing, snapping, and swallowing (Tilseth and Solemdal, 1977). It seems reasonable to assume that different sensory stimuli are responsible for eliciting parts or sequences of the behavior patterns. Evidence for this assumption has become more tenable when it comes to olfactory stimuli as Døving and Selset (1980) showed that two distinct phases of the food search activity can be elicited via the olfactory system. These two behaviors are also observed in free-swimming cod (Brawn, 1969; Pawson, 1977).

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During the initial phase, called orienting reaction, the fish swims around making sharp turns to the side, and up and down in the water. During the second phase, a benthic food search is displayed as an arrest of swimming close to the bottom, then the fish swims backwards using the pectoral fins and tail with the head down, trailing the barbel and the pelvic fin rays along the bottom. These behaviors are qualitatively different and can be elicited by electrical stimulation of the lateral and medial part of the lateral olfactory tract, respectively, demonstrating that the behaviors are released via different neural pathways. As the behaviors are elicited by different nervous pathways, it is reasonable to assume that the chemical stimuli must be different.

In the present study we have focused on the chemical basis for eliciting one particular component of the food search behavior, viz., the bottom food search (BFS) behavior pattern. Following a new line of chemical isolation procedures, we have isolated fractions and components releasing this behavior pattern in the cod. Chemical analysis shows that several substances with similar physical/chemical properties are involved in releasing the BFS, among them are the amino acids alanine and glycine. Two components, as yet unidentified, are also potent agents releasing BFS behavior.

METHODS AND MATERIALS

Maintenance of Fish. Cod (*Gadus morhua* L.) were caught in fish pots at Drøbak 30 km south of Oslo and transported to the aquarium facilities at the University of Oslo. The fish were 25–35 cm long and had been kept in captivity in the observation tanks for one week to two months prior to experimentation. During the test period, feeding the fish and cleaning the aquaria were regularly done on Fridays after the experimental series. The tests were performed the five first days of the week. The test fish were calm at the time of testing. They showed no signs of alarm reaction and moved quietly around in the observation aquaria. Sick or aggressive fish were removed from experiments and replaced by healthy, nonaggresive fish. A total of 28 fish were used during the period of bioassay.

Procedure. The cod were observed in four glass aquaria, 120 × 60 × 50 cm each, with a flow of 3 liters/min of seawater. The water temperature was 10 ± 2°C. Four cod marked with individual colored tags were placed in each aquarium. Test solutions were introduced at the bottom of the aquaria via two separate glass tubes (ID 1.5 mm). Teflon tubes of the same internal diameter as the glass capillaries provided connections between the delivery syringes and the glass tubes. The outlet from these glass tubes were hidden in the sand covering the bottom. The dead volume of the delivery lines was 5 ml. The stimuli were presented from syringes placed in an adjustable infusion pump. The solutions were injected into the aquarium at a rate of 2 ml/min during a period of

10 min. Stimulations were performed once a day. The test solutions were introduced in parallel with seawater blanks. Each test solution was given at five different concentrations. The aquarium seawater was used for dilution of all solutions to be tested in the bioassay.

Behavior Patterns. A benthic food search is observed in the cod when the olfactory tract is stimulated electrically and also when the fish is searching for a food source (Brawn, 1969; Pawson, 1977). Initial activity consists of an abrupt cessation of forward swimming, the cod descending to the bottom and moving backwards with the barbel and rays of the pelvic fins trailing the bottom. Each backward swim over the tube outlet was counted as a bottom food search (BFS). The number of fish doing BFS and the total number of BFS counted for the fish during a stimulation period of 10 min were used as a measure of the potency of a test solution. Other parts of the food search behavior pattern were also noted but not included in the present study. These were: orienting reaction, in the sense used by Pavlov (1927); aggression, as described by Brawn (1961); snapping, a rapid opening and closing of the jaws; and bellying, the fish making a quick swim towards the bottom, turning to its side, flashing its belly and swimming along sideways close to the bottom.

Extracts and Synthetic Solutions. Shrimp was ground twice in an electric grinder and then extracted with 3 vol of methanol–distilled water (75:25 v/v) by stirring at 800 rpm for 30 min (Franz Morat, Eisenbach, Hochschwarzwald, FRG, electrical stirrer, type R-16). The liquid phase was separated from the protein phase by vacuum filtration through a Whatman No. 1 filter. The filtrate was evaporated to dryness on a rotary evaporator at 40°C. The residue was dissolved in distilled water to a "dry-weight" content of 100 g/liter. This solution (stock solution) was used for chemical analysis, fractionation, and bioassays. Synthetic solutions were prepared with distilled water so that the concentration of each individual component was the same as that measured in the stock solution.

Chemicals. Homarine HCl was obtained from Carl Roth KG, Karlsruhe, FGR, dimethylthetine HCl from ICN Pharmaceuticals, Inc., Plainview, New York. Other chemicals were from Sigma Chemicals, St. Louis.

Characterization of Stimulants and Analytical Procedures. Analyses and characterization of the stimulants were conducted on the stock solution. Amino acid concentrations were measured on a Biotronic LC 5000 automatic amino acid analyzer. The concentration of trimethylamine was determined by a microdiffusion technique (Conway and Bryne, 1933). Trimethylamine oxide was determined as trimethylamine after reduction with titanium trichloride (Hjorth-Hansen, 1952).

Characterization and purification of stimulants are summarized in Figure 1.

By ion-exchange chromatography, the compounds were separated into three fractions: (1) acidic compounds, adsorbed on the anion exchanger and eluted

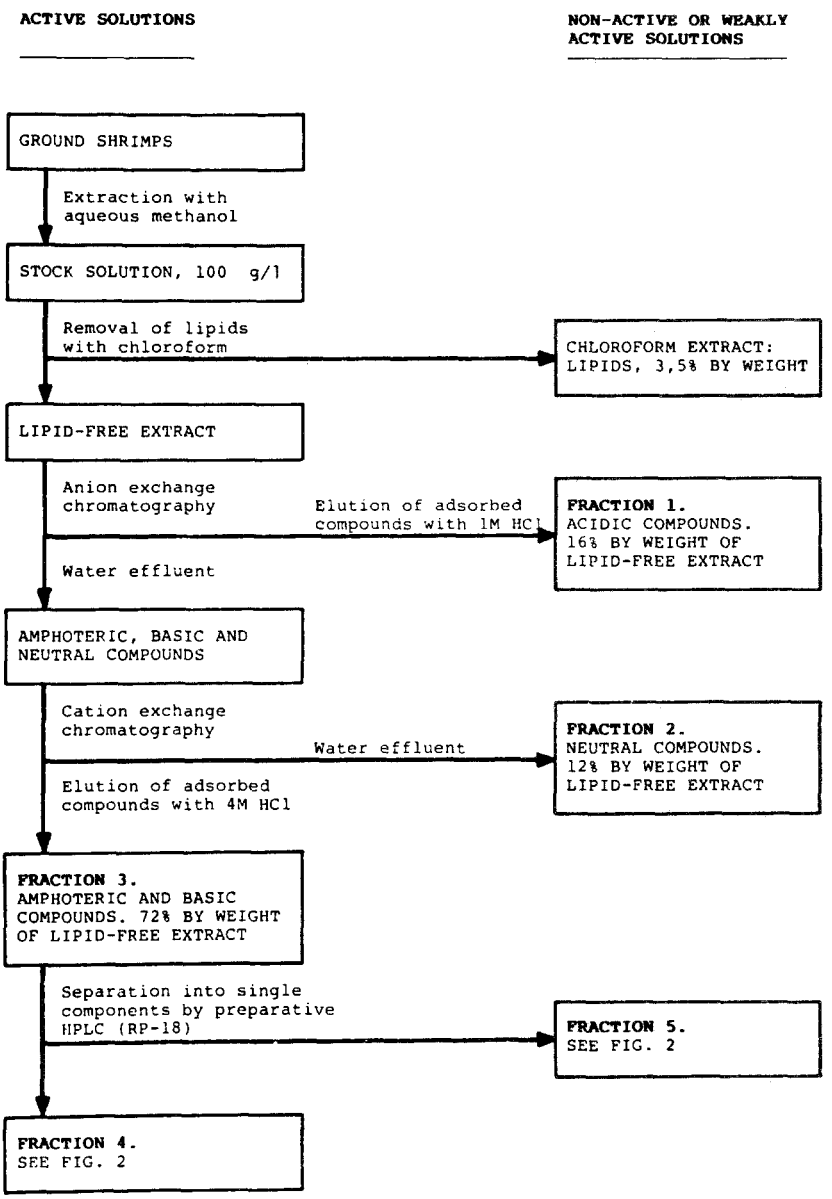

FIG. 1. Flow chart indicating the procedure followed for fractionation of food-search-inducing compounds in extract from shrimp.

with excess 1 M HCl; (2) neutral compounds, eluted from the cation exchanger with excess water; and (3) amphoteric and basic compounds, eluted from the cation exchanger with excess 4 M HCl.

Solutions with the same concentration as in the stock solution were made up from fractions 1, 2, and 3 and tested in bioassays. Test solutions made up from the stock solution and fraction 3 were also tested in equal concentrations.

Traces of fats and pigments (ca. 3.5% by weight) were removed with chloroform before chromatography. One volume of stock solution was extracted twice with two volumes chloroform. Defatted material (30 g) was dissolved in 50 ml distilled water, pH adjusted to 7.0. This solution was applied to an anion exchange column (Amberlite IRA-401, Cl^- form, 16–40 mesh, Koch-Light Laboratories, England, 2.5 × 100 cm). The column was washed with 1500 ml distilled water and adsorbed substances were eluted with excess 1 M HCl (1500 ml). The effluent and washings were combined, concentrated, and dissolved in 50 ml distilled water. The pH was adjusted to 7.0, and the sample was applied to a cation-exchange column (Dowex 50-X8, H^+ form, 16–40 mesh, Dowex, Switzerland, 2.5 × 100 cm) and rinsed with 1000 ml distilled water. The adsorbed substances were eluted with excess 4 M HCl (1500 ml).

The eluate was concentrated, dissolved in buffer, and the pH was adjusted to 4.0 before separation into single components by preparative HPLC (Perkin-Elmer, Norwalk, Connecticut, Series 3B liquid chromatograph with a Perkin-Elmer column C-18 bounded phase, 10 μm particle size, 1.8 × 25 cm). The initial eluent was 0.05 M KH_2PO_4, pH 4.0, and the final eluent methanol. Buffer was used during the first 20 min, then a linear gradient of 0–100% final eluent was used over the next 45 min. Methanol was run as a final eluent for 20 min. The flow rate was kept constant at 2 ml/min. The eluate was monitored at 210 nm (Perkin-Elmer LC 75, spectrophotometric detector).

The components separated by HPLC gave the chromatogram shown in Figure 2; they were collected in two fractions. Fraction 4 contained the substances giving rise to peaks 1–6, and fraction 5 contained all components eluted thereafter. Both fractions were tested in the bioassay at equal concentrations. Single components eluted in fraction 4 were also collected. Before testing, the buffer was removed by the following procedure: Water was removed on a rotary evaporator and the residue dried at 40°C to a constant weight. The dried, water-free material was then dissolved in excess water-free methanol, and buffer was separated from the methanol soluble components by filtration through a Millipore filter, Millipore Corp., Bedford, Massachusetts (45-μm pore size).

RESULTS

The bioassays were performed from September 1983 to September 1984. In all experiments, samples of stock solution were tested as a reference for other solutions applied to the fish (see Tables 2, 4, and 5). This control was partic-

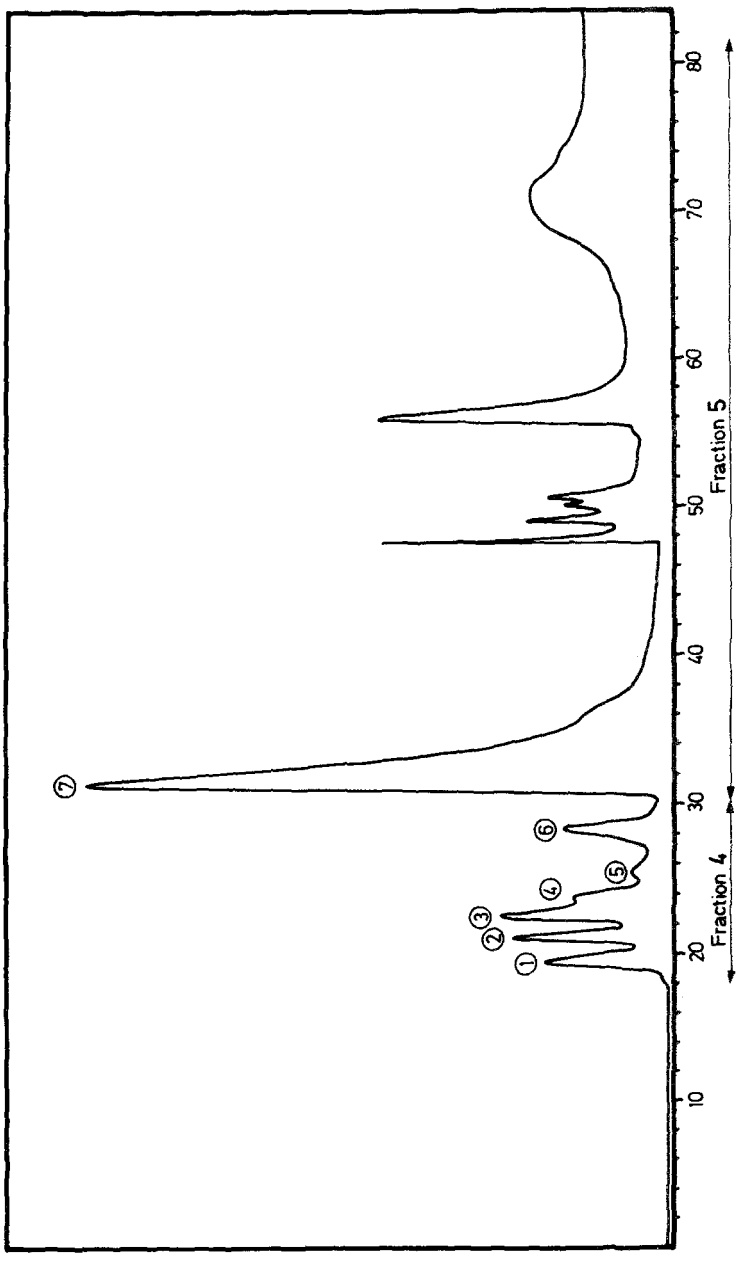

FIG. 2. Chromatogram showing the separation of amphoteric/basic components, fraction 3, by preparative HPLC. Amount injected: 50 mg. The experimental conditions are given in the text.

ularly important as the behavior and threshold for eliciting BFS varied over the year.

Comparison between Shrimp Extracts and Synthetic Solutions. The concentrations of amino acids, trimethylamine oxide, and trimethylamine found in the stock solution are given in Table 1. When a synthetic solution based on the concentration of amino acids found in the stock solution was prepared and tested, it was observed that the stock solution was 100–200 times as potent by weight as the synthetic solution (Tables 2A and B). The four amino acids (glycine, alanine, proline, and arginine) which were most abundant in the stock solution seemed more efficient in evoking the BFS behavior than the total amino acid pool (Tables 2B and C), and the results indicated that these four amino acids were only 10 times less potent than the stock solution (Tables 2A and C). Two amino acids, glycine and alanine alone, did not seem as potent as the four amino acids taken together, but were still more potent than the total amino acid pool (Tables 2B and D). When the four main amino acids were tested individually in equimolar concentrations (Table 3), glycine appeared to be most potent followed by alanine.

A solution containing the amino acids with five carbon atoms, viz., valine,

TABLE 1. CONCENTRATIONS OF AMINO ACIDS, TRIMETHYLAMINE, AND TRIMETHYLAMINE OXIDE IN STOCK SOLUTION[a]

Substance	Concentration	
	mmol/liter	g/liter
Aspartic acid	1.28	0.17
Threonine	5.13	0.61
Serine	3.17	0.33
Glutamic acid	4.47	0.66
Proline	21.31	2.46
Glycine	58.76	4.41
Alanine	12.52	1.12
Valine	5.25	0.62
Methionine	2.19	0.33
Isoleucine	4.26	0.56
Leucine	5.72	0.75
Phenylalanine	2.94	0.49
Histidine	2.63	0.41
Lysine	5.69	0.83
Arginine	14.50	2.53
Taurine	27.47	3.44
Trimethylamine	2.13	0.13
Trimethylamine oxide	75.07	5.63

[a] Dry weight content: 100 g/liter.

TABLE 2. COMPARISON OF BFS ACTIVITY INDUCED BY SAMPLES OF STOCK SOLUTION AND SYNTHETIC SOLUTION OF AMINO ACIDS[a]

	Dilution factor	Concentration,	Number of fish performing BFS	Number of BFS counted
A. Stock solution,	$\times 10^4$	g/liter $\times 10^{-4}$		
1		100	16	40
2.5		40	13	31
5		20	10	20
10		10	7	18
15		5	2	5
B. Total amino acid pool,	$\times 10^2$	g/liter $\times 10^{-2}$		
0.5		40	16	43
1		20	12	33
5		4	9	25
10		2	5	13
15		1	1	3
C. Glycine, alanine, proline, and arginine,	$\times 10^3$	g/liter $\times 10^{-3}$		
0.5		21.0	16	53
1		10.5	13	46
2.5		4.2	10	27
5		2.1	7	17
10		1.05	3	5
D. Glycine and alanine,	$\times 10^3$	g/liter $\times 10^{-3}$		
0.5		11.0	9	31
1		5.5	9	30
2.5		2.2	8	27
5		1.1	8	33
10		0.55	5	10

[a] The amino acid concentrations are in the proportion found in the stock solution. The entries give the number of fish out of 16 doing BFS and the total counts of BFS performed during the injection period. Concentrations are in g/liter of material injected. Test period: September–December 1983.

proline, methionine, and glutamic acid, induced no BFS activity when tested at the same concentrations as that found in the stock solution. A combination of the basic amino acids lysine, arginine, and histidine, scored only a few BFS patterns at the highest concentrations used (1/50 of the concentration in stock solution). The combination of methionine, the five-carbon amino acids, and the basic amino acids induced BFS activity at the same rate as did the basic amino acids.

TABLE 3. COMPARISON OF BFS ACTIVITY INDUCED BY INDIVIDUAL SUBSTANCES TESTED IN EQUIMOLAR CONCENTRATIONS[a]

Substance	Concentration (mol/liter)	Number of fish performing BFS	Number of BFS counted
Glycine	10^{-2}	10	19
	10^{-3}	7	9
	10^{-4}	6	8
	10^{-5}	2	6
Alanine	10^{-2}	6	18
	10^{-3}	4	11
	10^{-4}	3	9
	10^{-5}	0	0
Arginine	10^{-2}	6	14
	10^{-3}	4	8
	10^{-4}	0	0
	10^{-5}	0	0
Proline	10^{-2}	3	4
	10^{-3}	0	0
	10^{-4}	0	0
	10^{-5}	0	0
Dimethylthetine	10^{-2}	5	9
	10^{-3}	0	0
	10^{-4}	0	0
	10^{-5}	0	0

[a] The entries give the number of fish doing BFS and the total counts of BFS performed during the injection period. Concentrations are in mol/liter of material injected. Test period: February–April 1984.

Trimethylamine oxide and trimethylamine HCl were tested in the same concentrations as found in the natural extract. They did not induce any BFS activity when tested alone. They did not affect the activity scores when tested in combination with the amino acids.

Comparison between Chemical Fractions of Stock Solution. As seen from Figure 1, the weight ratio of the adsorbed compounds was 16:12:72 for fractions 1, 2, and 3, respectively. Only the acidic fraction (fraction 1) and the fraction containing amphoteric and basic components (fraction 3) scored significantly on the bioassay tests as demonstrated in Table 4. When the stock solution and fraction 3 were compared in equal concentrations, the latter was slightly more potent (Table 5).

Further separation of fraction 3 by HPLC (Figure 2), yielded 60% by weight of the material in fraction 4, while components in fraction 5 accounted for 40%. No BFS was induced by fraction 5, while the activity induced by fraction 4 was virtually the same as the activity induced by fraction 3. Of the single components eluted in fraction 4, most of the activity was located in peaks

TABLE 4. COMPARISON OF BFS ACTIVITY INDUCED BY SAMPLES OF STOCK SOLUTION AND FRACTIONS 1, 2, AND 3 ISOLATED BY ION-EXCHANGE CHROMATOGRAPHY[a]

Concentration (g/liter)	Number of fish performing BFS	Number of BFS counted
A. Stock solution		
10	16	65
1	11	36
0.5	10	20
0.1	7	15
0.01	4	10
B. Acidic fraction, 1		
1.6	9	13
0.16	2	4
0.032	0	0
C. Neutral fraction, 2		
1.2	3	4
0.12	2	3
0.024	0	0
D. Basic/amphoteric fraction, 3		
7.2	16	40
0.72	12	28
0.144	8	15
0.072	5	8
0.0072	3	4

[a] The entries give the number of fish out of 16 doing BFS and the total counts of BFS performed during the injection period. Concentrations are in g/liter of material injected. Test period: August–September 1984.

TABLE 5. COMPARISON OF BFS ACTIVITY INDUCED BY STOCK SOLUTION (SS) AND BASIC/AMPHOTERIC FRACTION, 3[a]

Sample	Concentration (g/liter)	Number of fish performing BFS	Number of BFS counted
SS	1	10	20
3	1	16	40
SS	0.1	7	12
3	0.1	8	14
SS	0.01	6	10
3	0.01	5	10
SS	0.005	5	8
3	0.005	3	7
SS	0.001	1	2
3	0.001	2	4

[a] The entries give the number of fish out of 16 doing BFS and the total counts of BFS performed during the injection period. Concentrations are in g/liter of material injected. Test period: May 1984.

1, 2, and 3. Peaks 4 and 5 were less active when tested in equal concentrations (1 g/liter).

DISCUSSION

The chemicals emanating from prey induce feeding behavior in cod. In the present study we have shown that shrimp contain amino acids and substances with amphoteric/basic properties which are effective inducers of a particular part of the feeding behavior, viz., the BFS. We discuss the results of our experiments in light of previous studies on cod and attempts to isolate feeding substances for cod and other fish species. The methods developed for the chemical isolation procedure are specifically considered since they might be useful in forthcoming analyses.

The four amino acids in the stock solution, glycine, alanine, proline, and arginine, appeared to be more potent than the total amino acid pool. These results demonstrate that the amino acid pool in a shrimp extract may contain amino acids which have an antagonistic effect on BFS induction in cod. The four main amino acids (glycine, alanine, proline, and arginine) act highly synergistically. This is demonstrated from the results presented in Table 2C and 3. At a total amino acid concentration of 10.5×10^{-3}, 46 BFS were performed by 13 fish when glycine, alanine, proline, and arginine were tested in combination (Table 2C). When the same four amino acids were tested individually at a concentration of 10^{-4} mol/liter (ca. 10^{-3} g/liter), glycine induced eight BFS (performed by six fish); alanine, nine BFS (performed by three fish); proline and arginine did not induce BFS at this concentration (Table 3). Of the individually tested amino acids, glycine appeared to be most potent, this observation being in agreement with Pawson (1977). Johnstone (1980) determined detection threshold values to nine amino acids in cod by a conditioning method and found that tyrosine was most potent, followed by cysteine, phenylalanine, and glycine. The difference between threshold values was small.

Tilseth and Solemdal (1977) examined the preference of cod to a wide variety of natural baits derived from invertebrates and fish tissue. The relative effectiveness of 10 different natural baits in initiating biting of a bag indicated that unconditioned cod clearly responded best to extracts of tissue from crustaceans such as euphasids, shrimp, and deep-sea prawns. Attempts to characterize the effective components in aqueous extracts of crustacean-based baits indicated a reduction in activity at pH below 4 or above pH 10. On adjustment of pH to 11 in a diluted solution of the stock solution, we observed a similar reduction in activity. This treatment was associated with the release of dimethyl sulfide and a precipitate. Boiling of the same solution for 3 hr at pH 1 only resulted in a small decrease in food search activity.

The fractionation procedure used in this work for isolating new food-search

stimulants, other than amino acids, was a modified procedure used for isolation of quaternary bases (Abe and Kaneda, 1975; Konusu and Hayashi, 1975; Hayashi et al., 1978). These authors used the OH^- form of an anion exchanger. The anion exchanger has a higher selectivity for Cl^- than OH^-; the OH^- form is therefore more effective in exchanging acidic compounds than the Cl^- form.

In our preliminary studies, we also applied the lipid-free stock solution on a column with the anion exchanger in the OH^- form. This procedure resulted in a highly alkaline effluent, and the release of dimethyl sulfide and a precipitate. When this sample was analyzed by HPLC after passing through the cation exchanger, the chromatogram was totally different from that seen in Figure 2. Peaks 2 and 3 did not appear. Therefore, the anion exchanger was used in the Cl^- form in the fractionation procedure.

The activity associated with the acidic fraction (fraction 1) should be related to adsorbed amino acids and to nucleotides which also adsorb onto the Cl^- form of an anion exchanger. (Hayashi et al., 1978). The activity associated with the neutral fraction (fraction 2) was regarded as insignificant and was neglected in later work. Since the amphoteric/basic fraction (fraction 3) was the most potent of the three tested fractions and even had a higher activity by weight than the stock solution, further work on identification of food-search stimulants was mainly limited to this fraction.

Most of the activity induced by single components eluted by HPLC was located in substances giving rise to peaks 1, 2, and 3 in Figure 2. The substance giving rise to peak 1 was identified as the amino acid glycine, possibly containing a small amount of alanine [identical IR and mass spectra with authentic glycine, NMR (60 MHz, D_2O): δ = 3.6 ppm (s, CH_2), 1.5 ppm (d, J = 3.5, CH_3 of alanine, very weak signal, shift position and coupling constant identical with authentic alanine)].

Peaks 2 and 3 are still unidentified. These components carry the very distinctive smell of shrimp and are very labile to alkali, releasing dimethyl sulfide on treatment with cold aqueous 1 M NaOH. The presence of dimethyl-β-propiothetine (DMPT) in several species of multicellular algae is well documented (Challenger, 1959; Ishida, 1968; Noda and Horiguchi, 1975; Larher et al., 1977). The decomposition of this substance by cold alkali, yielding dimethyl sulfide, is believed to be a specific reaction (Ackman and Dale, 1965). An indirect determination of DMPT, as dimethyl sulfide, is based on this assumption. Such analyses have been carried out for marine multicellular algae (Ackman et al., 1966), for Labrador cod (Ackman et al., 1967), for brackish water algae and baltic herring (Granroth and Huttula, 1976), and for antarctic krill (Tokunaga et al., 1977). From these data it would seem possible that either peak 2 or 3 might be DMPT. The IR spectra and NMR spectra [60 MHz, D_2O, signals at δ = 3.8 ppm(s) and 3.3 ppm(s)] were not in accordance with published IR spectra (Ishida, 1968; Noda and Horiguchi, 1975) and NMR spectra (Ishida, 1968; Larher et al., 1977). Weak signals in the NMR spectrum at δ =

3.0 ppm and 3.6 ppm might suggest the presence of DMPT as a minor component in peak 3.

Due to the lack of verified structures, it is impossible to determine the concentration in the stock solution, although there are areas in HPLC comparable to glycine. The reduction in activity on alkali treatment of aqueous extracts of crustacean-based baits, as observed by Tilseth and Solemdal (1977) and in this work, obviously should be related to the alkali-labile components giving rise to peaks 2 and 3 in Figure 2. As a representative for thetines, dimethylthetine was tested in our bioassay. This compound does not occur in marine organisms, but it has been described as potent in inducing food consumption in juvenile Dover sole (Mackie et al., 1980). As demonstrated in Table 3, this compound even induced BFS when tested in our bioassay.

Peak 4, observed as a shoulder on peak 3, has been identified as glycine–betaine [superimposable IR and mass spectra, NMR (60 MHz, D_2O): δ = 3.3 (s, 9H, 3X $N-CH_3$) and 4.2 ppm (s, 2H, CH_2)]. The importance of glycine–betaine as a BFS inducer in cod is very doubtful. When glycine–betaine was tested in the same concentrations as the amino acids glycine, alanine, arginine, and proline, no BFS could be observed. The exact concentration of this compound is difficult to determine in our chromatographic system due to low concentration and lack of separation from peak 3. The concentration was determined to be approximately 0.05 g/liter in the stock solution. We were not able to show that glycine–betaine potentiated the stimulation power of amino acids when this component was included.

Components 5 and 6 are still unidentified. Compared to the substances giving rise to peaks 1, 2, and 3, they induced only a very weak degree of BFS.

The main peak in the chromatogram, peak 7, has identical retention time with homarine. No attempt has been made to identify this component by spectroscopy as neither the isolated component nor authentic homarine induced BFS-activity.

The variability of response to the stock solution could be expected due to the cyclic feeding activity of cod found in the coastal zone. Experiments were made both during the most intense feeding period and during breeding season when feeding activity is low even among juvenile cod. Variability of the seawater at the aquarium facilities could also be responsible for the changes in behavioral activity although precautions were taken to keep the water quality constant. The ordinary control routines of the water quality were unaltered during the testing period.

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FATE OF INGESTED IRIDOID GLYCOSIDES IN LEPIDOPTERAN HERBIVORES

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Abstract—Thin-layer chromatography was used to follow the fates of iridoid glycosides ingested by four species of lepidopteran herbivores. These four species differed in their feeding strategy, ranging from generalist to monophagous specialist; and in their predator avoidance strategy, ranging from cryptic and palatable to aposematic and unpalatable. The fates of the iridoid glycosides ranged from sequestration by the unpalatable specialist, *Euphydryas phaeton* (Nymphalidae); to passage into the hemolymph and eventual elimination in the meconium by the specialists *Junonia coenia* (Nymphalidae) and *Ceratomia catalpae* (Sphingidae); to elimination of the intact compounds in the feces of the generalist feeder, *Lymantria dispar* (Lymantriidae).

Key Words—Iridoid glycoside, Lepidoptera, *Euphydryas phaeton*, Nymphalidae, *Junonia coenia*, *Ceratomia catalpae*, Sphingidae, *Lymantria dispar*, Lymantriidae, chemical ecology, insect–plant interaction, unpalatability, insect defense strategy.

INTRODUCTION

Plant allelochemicals may meet one or the other of several possible fates when ingested by an insect: (1) they may be metabolized in some way (Rosenthal et al., 1977; Brattsten, 1979; Duffey, 1980; Blum, 1983; Ivie et al., 1983; Bull et al., 1984); (2) eliminated without being metabolized (Self et al., 1964a,b); (3) used as building blocks for pheromones or defensive compounds (Schneider et al., 1975; Conner et al., 1981); (4) sequestered and used in the insect's own defense (Parsons, 1965; Teas, 1967; Brower et al., 1968; Duffey and Scudder, 1972; Rothschild et al., 1972; Roeske et al., 1976; Isman et al., 1977; Duffey, 1980; Blum, 1981, 1983). Some combination of these fates is also possible. A guild of insect herbivores feeding on plant species with a common group of

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plant allelochemicals may process these compounds in very different ways (e.g., Rothschild, 1973; Isman et al., 1977; Blum, 1981).

In this paper, we compare the fates of a group of plant allelochemicals, the iridoid glycosides (Figure 1), in the various life stages of four lepidopteran herbivore species which have ingested these compounds: *Euphydryas phaeton* Drury (Nymphalidae), *Junonia coenia* Hbn. (Nymphalidae), *Ceratomia catalpae* Bdv. (Sphingidae), and *Lymantria dispar* (L.) (Lymantriidae) (Table 1). These four species show different degrees of dietary specialization, ranging from generalist to extreme specialist; as well as very different predator avoidance strategies, ranging from palatable, solitary, and cryptic, to unpalatable, gregarious, and aposematic (Table 1). The three specialist species feed only on plants that contain iridoid glycosides, while *L. dispar* is a generalist.

Butterflies in the genus *Euphydryas* specialize on plants that contain iridoid glycosides and use these compounds as larval feeding stimulants and attractants (Bowers, 1981, 1983). Feeding experiments with birds have shown that the *Euphydryas* species, in general, are unpalatable and earlier work by Bowers (1979, 1981) indicated that iridoid glycosides can play a role in this unpalatability. Larvae of the buckeye, *J. coenia*, also use iridoid glycosides as larval feeding stimulants and are restricted to feeding on plants that contain these compounds (Bowers, 1984). *Junonia coenia* is cryptic on the underside, although there are conspicuous eyespots on the dorsal surface. The catalpa sphinx, *C. catalpae* is restricted to feeding on species of *Catalpa* (Bignoniaceae), which also contain iridoid glycosides, and appears to use iridoid glycosides as feeding stimulants (Nayar and Fraenkel, 1963). The larvae have a conspicuous black and white coloration and are gregarious, whereas the adults are cryptic. In contrast to these three specialists, the gypsy moth, *L. dispar*, is a relatively generalist feeder, and the adults seem to be cryptic and palatable (Leonard, 1974).

The iridoid glycosides (Figure 1) are a group of cyclopentanoid monoterpene-derived compounds found in over 50 families of plants (Bobbitt and Segebarth, 1969; Jensen et al., 1975; Hegnauer and Kooiman, 1978; El-Naggar

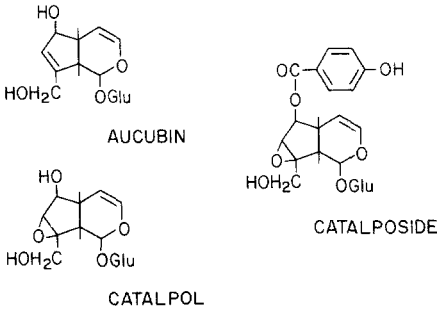

FIG. 1. Iridoid glycosides used in this study.

TABLE 1. FEEDING STRATEGIES, HOST PLANTS, AND PREDATOR AVOIDANCE STRATEGIES OF INSECTS

Insect	Feeding strategy	Host plants	Predator avoidance strategy	
			Larvae	Adult
Euphydryas phaeton (Nymphalidae)	specialist	Scrophulariaceae Plantaginaceae Caprifoliaceae Oleaceae	aposematic unpalatable gregarious	aposematic unpalatable sedentary
Junonia coenia (Nymphalidae)	specialist	Scrophulariaceae Plantaginaceae Acanthaceae Verbenaceae	cryptic palatability unknown solitary	cryptic unpalatable solitary
Ceratomia catalpae (Sphingidae)	specialist	*Catalpa* sp. (Bignoniaceae)	aposematic unpalatable gregarious	cryptic palatable solitary
Lymantria dispar (Lymantriidae)	generalist	many families		

and Beal, 1980; Dahlgren et al., 1981; Gershenzon and Mabry, 1983). These compounds are known as "bitter substances" because of their bitter taste to humans (Thomas, 1961; Hegnauer, 1966; Kubota and Kubo, 1969; personal observation), and plants containing them have been used as natural insecticides (Hegnauer, 1966). In addition, they have been shown to be important in the interactions of insects and the plants that contain them (Nayar and Fraenkel, 1963; Bowers, 1979, 1981, 1983, 1984; Sha'aban et al., 1980; Bernays and DeLuca, 1981; Stephenson, 1981, 1982; Chang and Nakanishi, 1983).

We used thin-layer chromatography to qualitatively assay plant material, eggs, larvae, pupae, adults, and larval frass for evidence of the iridoid glycosides, aucubin, catalpol, and catalposide, which are the major identified iridoid glycosides found in the host plants we studied (El-Naggar and Beal, 1980).

METHODS AND MATERIALS

Insects and Plants. Euphydryas phaeton were reared from eggs from a wild-collected female from Leverett, Massachusetts, and during prediapause instars were fed on *Chelone glabra* L. (Scrophulariaceae) leaves, the primary host plant of this species (Bowers, 1980). In the postdiapause instars, one group of larvae were fed on *C. glabra* and another on *Plantago lanceolata* L. (Plantaginaceae), an alternative host plant (Bowers, 1980). These two plant species were chosen because adult *E. phaeton* butterflies are unpalatable and emetic to birds when fed as larvae on *C. glabra*, but are relatively palatable when larvae are fed *P. lanceolata* in the postdiapause instars (Bowers, 1980).

Junonia coenia were from a laboratory culture derived from eggs from several wild-collected females from Columbia, South Carolina. They had been maintained in the laboratory on *P. lanceolata* leaves, a commonly utilized host plant (refs. in Bowers, 1984), for only a few generations.

Ceratomia catalpae were reared from eggs derived from wild-collected females from Cape May County, in southern New Jersey. Larvae were fed in the laboratory on leaves of *Catalpa bignonioides* Walt.

Lymantria dispar larvae were reared on one of two artificial diets (Bowers, 1983): either a diet containing 3.6% dry weight catalposide or a diet containing no iridoid glycosides. These larvae were from eggs obtained from the USDA Gypsy Moth Rearing Facility at Otis Air Force Base, Massachusetts.

Thin-Layer Chromatography. Plant and insect samples were prepared using a slightly modified procedure of Wieffering (1966). Quantities of all materials were adjusted relative to the starting dry weight of the sample. For example, 100 mg dried plant or insect material is ground with an equal weight of sand. Ten milliliters of distilled water are added with 50 mg polyvinyl pyrrolidone to remove phenolics. After 15–20 min, the mixture is filtered and 50 mg sand and 50 mg kieselguhr are added. The mixture is evaporated to dryness on a rotary

evaporator. The residue is extracted overnight with 4 ml 96% ethanol, filtered, and concentrated. The concentrated samples were spotted onto prepared silica gel glass plates (Baker). The solvent system was butanol, prepared by mixing it with acetic acid and water in a ratio of 4:1:5, and then drawing off the butanol layer. The running time for these plates was about 4 hr. The spots were visualized by spraying the plates with a 1 N solution of H_2SO_4 in methanol and heating them at 105°C for 5 min (Wieffering, 1966). We have also used a *p*-anisaldehyde reagent made up of 90 ml 50% ethanol, 10 ml concentrated H_2SO_4, and 0.5 ml *p*-anisaldehyde, which produces different colors, but also identified the iridoid glycosides we are interested in. We compared the color and R_f of spots from our samples with those of standards to determine the presence or absence of particular iridoid glycosides in plants and insects.

Standards of aucubin, catalpol, and catalposide were run with the samples on each plate, and on each individual plate the R_fs of the samples and standards were virtually the same. There was minimal variation in the R_fs of the standards from one plate to another. The R_fs of the standards were as follows: aucubin: 0.35–0.39, catalpol: 0.27–0.31, catalposide: 0.55–0.58.

RESULTS

We found that *E. phaeton* larvae sequestered iridoid glycosides from both *C. glabra* and *P. lanceolata*, and that these compounds were retained through to the adult stage (Table 2). However, there were some interesting differences in the results with insects reared on these two plants. *Euphydryas phaeton* larvae reared on *P. lanceolata* produced adults that contained more aucubin than catalpol, as indicated by the size and intensity of the spots. When larvae were fed on *C. glabra*, which appeared to contain more catalpol than aucubin, the adult contained more catalpol than aucubin. Preliminary results with high-pressure liquid chromatography (HPLC) have substantiated this qualitative result: there was from six to 20 times as much aucubin as catalpol in *P. lanceolata*, and four to five times as much catalpol as aucubin in *C. glabra* (Bowers and Puttick, unpublished). The larvae, pupae, and eggs of *E. phaeton* also contained iridoid glycosides (Table 2). In addition, HPLC showed that *E. phaeton* butterflies reared as larvae on *C. glabra* have two to five times as much catalpol as aucubin. Those reared on *P. lanceolata* appear to selectively sequester catalpol, or convert aucubin to catalpol, as the butterflies contain approximately equal amounts of aucubin and catalpol, while the plants had much more aucubin. Stermitz (unpublished), using gas–liquid chromatography (GLC), found that wild-collected *Euphydryas anicia* (Doubleday and Hewitson) contained as much as 4% dry weight iridoid glycosides. We could not detect aucubin or catalpol in the frass of *E. phaeton* larvae reared on either *C. glabra* or *P. lanceolata*.

Junonia coenia, in contrast, did not sequester iridoid glycosides through

TABLE 2. RESULTS OF THIN-LAYER CHROMATOGRAPHY TO DETECT PRESENCE OF IRIDOID GLYCOSIDES IN PLANT, LARVAE, PUPA, AND FECES (FRASS)[a]

Insect	Food	Iridoid glycosides in food	Results of TLC					
			Eggs	Larva	Pupa	Adult	Frass	Meconium
J. coenia	*P. lanceolata* leaves	auc & cat	—	auc & cat	auc & cat	0	auc & cat	auc & cat
C. catalpae	*C. bignonioides* leaves	cat, cat'side & at least 3 others (C1, C2, C3)	—	cat	cat	0	cat, cat'side, C1, C2, C3	cat
E. phaeton	*C. glabra* leaves	cat & auc	auc & cat	auc & cat	auc & cat	auc & cat	0	cat
	P. lanceolata leaves	auc & cat	auc & cat	auc & cat	auc & cat	auc & cat	0	auc & cat
L. dispar	AD + 3.6% cat'side	cat'side	—	0	0	—	cat'side	—
	AD	none	—	0	0	—	0	—

[a] "0" = tested and no iridoid glycosides found; "—" = not tested; auc = aucubin; cat = catalpol; cat'side = catalposide; AD = artificial diet; C1, C2, C3 = unknown iridoids. R_f's: aucubin: 0.35–0.39, catalpol: 0.27–0.31, catalposide: 0.55–0.58, C1: 0.39, C2: 0.51, C3: 0.57.

to the adult stage, although curiously, the pupa did contain iridoid glycosides (Table 2). *Ceratomia catalpae* larvae appeared to sequester selectively one iridoid glycoside, catalpol, from an array of at least five iridoid glycosides detected in the leaves of *C. bignonioides*. Although the pupa also contained catalpol, the adult did not contain any iridoid glycosides. We did not detect catalposide in either the larva or adult of *L. dispar*, although it was clearly evident in the frass. However, it is possible that our methods may not have been sensitive enough to detect the catalposide in the food material in the gut.

We were surprised to find iridoid glycosides in the pupae of *J. coenia* and *C. catalpae*, since the adults of these species did not contain the compounds. Because wastes are not eliminated during pupation but only at adult eclosion, we hypothesized that the iridoid glycosides might be in the meconium, the substance containing the waste products accumulated during pupation which is eliminated upon emergence. Nishio (1980) found that the meconium of *Danaus plexippus* L. (Nymphalidae), which fed on *Asclepias humistrata* Walt. (Asclepiadaceae), contained cardenolides. Thin-layer chromatography of the meconium of *J. coenia* revealed the presence of substantial amounts of aucubin and catalpol, the two primary iridoid glycosides found in *P. lanceolata*, and the same two which had been detected in the pupa (Table 2). Likewise, the meconium of *C. catalpae* contained catalpol (Table 2). Despite the fact that *E. phaeton* adults contained iridoid glycosides, the meconium of this species was also found to contain these compounds: catalpol if the larvae had fed on *C. glabra*, and aucubin and catalpol if the larvae had fed on *P. lanceolata*.

DISCUSSION

The fate of the ingested iridoid glycosides appears to be related to the predator avoidance strategy of the specialist herbivores. Thus, *E. phaeton*, which is unpalatable (Bowers, 1980), sequesters iridoid glycosides during larval feeding, and they are retained in the adult stage. Although we clearly need to feed pure iridoid glycosides to birds for final proof, the evidence is overwhelming that iridoid glycosides are responsible for the unpalatabilty of the *Euphydryas* (Bowers, 1980, 1981). In contrast, in *J. coenia*, iridoid glycosides are found in the larva and pupa, but not in the adult. During pupation, the iridoid glycosides pass into the meconium and are ejected at adult eclosion. The adults of *J. coenia* seem to be palatable as is suggested by their wing pattern and coloration. In addition, adult *J. coenia* were taken and presumably eaten when offered to wild birds at an outdoor feeding station and were eaten readily by a caged California scrub jay.

The pattern of sequestration in *C. catalpae* is particularly interesting. The larvae appear to selectively sequester one iridoid glycoside, catalpol, from a range of at least five iridoid glycosides which we were able to detect in the host

plant. Alternatively, they could be converting the other iridoid glycosides into catalpol. There may be other iridoid glycosides in the plant and larva that we cannot detect (see Nayar and Fraenkel, 1963). Interestingly, catalpol is one of the most bitter of the iridoid glycosides (personal observation). In addition, the results with *E. phaeton* suggest that it is catalpol that is responsible for the emeticity of adult butterflies which fed as larvae on *Chelone glabra* (Bowers, 1980) (Table 2). The larval stage of *C. catalpae* is very unlike the other species of *Ceratomia* and those of the closely related genus *Manduca* in North America. *Ceratomia catalpae* larvae are gregarious, the females laying eggs in batches of as many as 1500. The early larval stages are white with black spots, while the last three instars are usually black and yellow. There is also a predominantly yellow larval morph. In contrast, larvae of related sphingid species are solitary and cryptic. The adult of *C. catalpae* is a drab yellowish gray, very similar to the adults of related species (Hodges, 1971). Although controlled feeding experiments with birds need to be performed, anecdotal evidence from offering larvae and adults to birds at a feeding station suggests that the larvae are unacceptable to birds, while the adults are palatable (Bowers, personal observation). This suggests that utilization of *Catalpa* species as the larval host plant of *C. catalpae* with consequent unpalatability of the larvae has selected for the evolution of aposematism in this species.

We were intrigued by the presence of iridoid glycosides in the meconium of the specialist insects. This interest was piqued when we disturbed a resting *C. catalpae* and it squirted us with meconium from a distance of two feet. This suggested that the presence of iridoid glycosides in the meconium may be another line of defense against predators, a role not previously accorded to it. In general, the meconium is considered to contain the waste products accumulated during the pupal stage. Nishio (1980) found cardenolides, another group of bitter allelochemicals, in the meconium of *D. plexippus* which had fed on milkweeds. Stephenson (1982) found that iridoid glycosides were deterrent to ants when included in a sugar solution, and ants may be important predators of helpless, teneral adult lepidopterans. The meconium is usually eliminated soon after eclosion, often when the insect is disturbed. Thus it may be a defense during a very vulnerable time when the newly eclosed adult is unable to fly.

In summary, the fate of ingested iridoid glycosides differed in four species of lepidopterans fed on plants or artificial diets containing these compounds, ranging from sequestration by the unpalatable specialist, *E. phaeton*; to passage into the meconium and eventual elimination via the meconium of *J. coenia* and *C. catalpae*; to elimination of the intact compounds in the frass of the generalist feeder, *L. dispar*. These fates appeared to be related to the predator avoidance strategy of these herbivores.

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REINVESTIGATION OF ANHYDROSERRICORNIN, (2*S*,3*S*)-2,6-DIETHYL-3,5-DIMETHYL-3,4-DIHYDRO-2H-PYRAN, AS A SEX PHEROMONE COMPONENT FOR MALE CIGARETTE BEETLE

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Abstract—Reinvestigation of the pheromonal activity of anhydroserricornin, (2*S*,3*S*)-2,6-diethyl-3,5-dimethyl-3,4-dihydro-2H-pyran, showed that the magnitude of its activity was less than $1/10^3$ of that of serricornin, 7-hydroxy-4,6-dimethyl-3-nonanone, the sex pheromone of the cigarette beetle *Lasioderma serricorne* (F.). Neither a synergistic nor an inhibitory effect of anhydroserricornin addition on the action of serricornin was observed.

Key Words—Sex pheromone, cigarette beetle, *Lasioderma serricorne* (F.), *Coleoptera*, *Anobiidae*, serricornin, 7-hydroxy-4,6-dimethyl-3-nonanone, anhydroserricornin, 2,6-diethyl-3,5-dimethyl-3,4-dihydro-2H-pyran.

INTRODUCTION

The cigarette beetle *Lasioderma serricorne* (F.) is a common, destructive pest for not only cured tobacco leaves but also various foodstuffs. The sex pheromone component produced by female adults was isolated and identified as 7-hydroxy-4,6-dimethyl-3-nonanone (Chuman et al. 1979a) and named serricornin (Chuman et al., 1979b). Recently, M. Mori et al. (1982a,b) determined the absolute structure to have a 4*S*,6*S*,7*S* configuration, and the remarkable inhib-

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itory action of the 4*S*,6*S*,7*R* isomer was also demonstrated (M. Mori et al., 1985).

Levinson et al. (1981) reported a second pheromone component (2*S*,3*S*)-2,6-diethyl-3,5-dimethyl-3,4-dihydro-2H-pyran, namely anhydroserricornin, isolated from solvent extracts of the female adults. Their observation in the "glass jar" bioassay using a synthetic diastereoisomeric mixture of anhydroserricornin suggested a much higher pheromonal activity than that of a diastereoisomeric mixture of serricornin. Chuman et al. (1982a), however, could not reproduce their findings using a synthetic enantiomeric mixture of anhydroserricornin in two kinds of bioassay systems. After that, Levinson et al. (1982) reported again on the high pheromonal activity of the diastereoisomeric mixture of anhydroserricornin in the "Petri dish" bioassay system, and Chuman et al. (1982b) confirmed their own earlier result using optically active anhydroserricornin. This difference between two groups on the evaluation of anhydroserricornin as the sex pheromone component has remained unresolved (Fletcher et al., 1984).

We report here the results of our recent laboratory bioassay using optically active anhydroserricornin and serricornin to solve the above question and to elucidate whether anhydroserricornin plays a synergistic or an inhibitory role to the pheromonal action of serricornin.

METHODS AND MATERIALS

Handling of Insects and Pheromonal Bioassay. The same system was adopted in the same manner as described before (M. Mori, et al., 1985.) Thus the evaluation of pheromonal activity of each test substance was achieved through the following three parameters: (1) attractiveness (accumulated number of times that males visited the source of test substance), (2) sex stimulation (accumulated number of attempted matings induced by the test substance), and (3) maximum aggregation number (maximum number of males staying simultaneously on the source of test substance).

(4S,6S,7S)-7-Hydroxy-4,6-dimethyl-3-nonanone (Serricornin). Optically active serricornin (100% EE) was synthesized by the described procedure of K. Mori and Watanabe (1985).

(2S,3S)-2,6-Diethyl-3,5-dimethyl-3,4-dihydro-2H-pyran (Anhydroserricornin). The optically pure sample of serricornin (100 mg) was refluxed in chloroform for 1 hr, followed by removing the solvent under atmospheric pressure to afford almost pure anhydroserricornin. When the dehydration of serricornin was performed by using a catalyst such as *p*-toluenesulfonic acid (Hoffman et al., 1982), many minor byproducts were revealed to be formed, some of which were difficult to remove in the subsequent purification procedure. This mild treatment with chloroform gave a satisfactorily pure sample of anhydroserricornin. Further purification was achieved by preparative GC (5% OV-1

glass column, 1 m) to obtain the pure sample (30 mg). $[\alpha]_D^{25}$ −83.1° (c = 0.42, n-hexane); lit. $[\alpha]_D^{23}$ − 53.1° (c = 0.065, n-hexane) (Chuman et al., 1982b), $[\alpha]_D^{23}$ −65.5 ± 0.5° (c = 2.28, chloroform) (Hoffmann et al., 1982), and $[\alpha]_D^{20}$ −80.7° (c = 2.54, chloroform) (Hoffman et al., 1984). High-resolution [^{1}H]NMR spectrum (500 MHz) showed that the sample was quite free from its diastereoisomer.

RESULTS AND DISCUSSION

Pheromonal Activity. The pheromonal activity of anhydroserricornin was measured with respect to the three parameters, and the data obtained were compared with those of serricornin (Tables 1–3). Small inversions of data are seen in some of these tables; however, such levels of fluctuations should be ignored because of the individual variations of insects used. As seen in Table 1, the attractiveness of anhydroserricornin is significantly lower than that of serricornin; the magnitude for anhydroserricornin is $\frac{1}{2}$ to $\frac{1}{3}$ of that for serricornin at the respective dose levels within 10^{-1}–10^{-3} μg. Table 2 shows the lower sex stimulatory activity of anhydroserricornin compared to serricornin; the accumulated number of times of attempted matings with anhydroserricornin is $\frac{1}{2}$ to $\frac{1}{3}$ of that obtained with serricornin at the respective dose levels. The third parameter, maximum aggregation number, for anhydroserricornin was also observed to be smaller than that for serricornin (Table 3); the maximum number of simultaneously responding males was $\frac{1}{2}$ to $\frac{1}{3}$ of that measured with serricornin at the same dose levels.

On the above measurements of pheromonal activities, a dose–response dependency was observed in every case. This dependency was further confirmed by measurement of the attractiveness over the range of 10^{-1}–10^{-8} μg dose levels (Figure 1). As seen in Figure 1, it is suggested that the magnitude of the pheromonal activity of anhydroserricornin is less than $1/10^3$ of that of serricornin.

TABLE 1. ATTRACTIVENESS OF ANHYDROSERRICORNIN AND SERRICORNIN TO MALE CIGARETTE BEETLES[a]

Dose (μg)	Anhydroserricornin	Serricornin	t	P
1.0×10^{-1}	44.6 (±33.6)	117.3 (±29.2)	5.16	<0.001
1.0×10^{-2}	28.0 (± 9.6)	89.5 (±48.7)	3.91	<0.01
1.0×10^{-3}	19.6 (±10.0)	82.0 (±31.1)	6.85	<0.001

[a] Accumulated numbers of times that 10 males visited the source of the test substances within 10 min. Each test was replicated 10 times, and the standard deviations are shown in parentheses.

TABLE 2. SEX STIMULATORY ACTIVITY OF ANHYDROSERRICORNIN AND SERRICORNIN TO MALE CIGARETTE BEETLES[a]

Dose (μg)	Anhydroserricornin	Serricornin	t	P
1.0×10^{-1}	3.4 ($\pm$2.5)	8.3 ($\pm$1.7)	5.13	<0.001
1.0×10^{-2}	3.0 ($\pm$1.8)	8.2 ($\pm$2.8)	4.94	<0.01
1.0×10^{-3}	2.6 ($\pm$1.7)	7.2 ($\pm$2.3)	5.09	<0.001

[a] Accumulated numbers of times of attempted matings induced by the test substances among 10 males within 10 min. Each test was replicated 10 times, and the standard deviations are shown in parentheses.

These results agree with those which we already reported briefly for the enantiomeric mixture of anhydroserricornin (Chuman et al., 1982a) and for optically active anhydroserricornin (Chuman et al., 1982b); however, these are considerably different from those reported by Levinson et al. (1981, 1982) for a diastereoisomeric mixture of anhydroserricornin. Their "Petri dish" bioassay system (Levinson et al., 1982) is somewhat different from that applied in this study, so we further examined the pheromonal activities of anhydroserricornin and serricornin by modifications of our system, using Petri dishes of the same size used by them (75 mm ID, 30 mm height), awakened insects instead of resting insects, 30 min instead of 10 min for the observation time, and without using the filter paper screen as the source of test substance. The result is shown in Table 4, in which every number is relatively large compared with that in Table 1, mainly owing to the prolonged observation time. The data for attractiveness of anhydroserricornin in this table are essentially the same as those reported by Levinson et al. (1982) for a diastereoisomeric mixture of anhydroserricornin. But the data for serricornin in Table 4 are quite different from those obtained by Levinson et al. (1982); their value was only 20 or less at the 10^{-1} μg dose level (read from their figure), which is small compared with that obtained in this study (322, Table 4). For this reason, we consider that the serri-

TABLE 3. MAXIMUM AGGREGATION NUMBERS FOR ANHYDROSERRICORNIN AND SERRICORNIN[a]

Dose (μg)	Anhydroserricornin	Serricornin	t	P
1.0×10^{-1}	4.2 ($\pm$1.9)	7.7 ($\pm$1.2)	4.93	<0.01
1.0×10^{-2}	4.3 ($\pm$1.8)	8.3 ($\pm$1.8)	4.97	<0.01
1.0×10^{-3}	2.6 ($\pm$0.5)	6.8 ($\pm$1.1)	10.99	<0.001

[a] Maximum numbers of males staying simultaneously on the source of test substances among 10 males within 10 min. Each test was replicated 10 times, and the standard deviations are shown in parentheses.

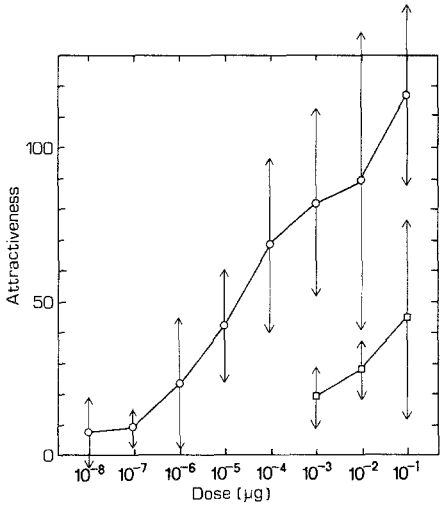

FIG. 1 Dose-response curves for attractiveness of serricornin (—○—) and anhydroserricornin (—□—). The attractiveness is expressed by the accumulated number of times that 10 males visited the source of the test substance within 10 min. Each test was replicated 10 times, and the standard deviations are shown by arrows.

cornin sample used in their bioassay contained some of the 4*S*,6*S*,7*R* isomer which is known as the inhibitory component of conventionally synthesized serricornin product (M. Mori et al., 1985).

Therefore it should be concluded that the magnitude of pheromonal activity of anhydroserricornin is $1/10^3$ of that of serricornin at the highest, and that anhydroserricornin is not an important component as the sex pheromone.

Effect of Anhydroserricornin Addition on Pheromonal Activity of Serricornin. It has been clarified above that the pheromonal activity of anhydroserricornin is very low by itself, so next we examined whether it plays a synergistic or inhibitory role to the action of serricornin. The same bioassay system was

TABLE 4. BIOASSAY RESULTS FOR ANHYDROSERRICORNIN AND SERRICORNIN AT 1.0×10^{-1} μg DOSES ACCORDING TO LEVINSON *et al.* (1982)[a]

Parameter	Anhydroserricornin	Serricornin	*t*	*P*
Attractiveness	186.5 (±40.3)	321.8 (±80.9)	4.73	<0.01
Sex stimulation	8.0 (± 2.8)	23.4 (± 6.2)	7.16	<0.001
Maximum aggregation	5.5 (± 0.7)	8.3 (± 1.5)	5.35	<0.001

[a] All parameters were examined for 10 awakened males within 30 min. Each test was replicated 10 times, and the standard deviations are shown in parentheses.

TABLE. 5. ATTRACTIVENESS OF MIXTURES OF SERRICORNIN AND ANHYDROSERRICORNIN TO MALE CIGARETTE BEETLES[a]

Mixing ratio serricornin–anhydroserricornin	Dose (μg)		
	1.0×10^{-1}	1.0×10^{-2}	1.0×10^{-3}
99:1	109.0 (±30.2) a	77.2 (±31.2) a	69.6 (±11.0) a
90:10	110.3 (±30.5) a	58.3 (±30.0) ab	66.8 (±22.3) b
50:50	72.0 (±18.0) b	48.8 (±33.3) ab	69.6 (±37.0) c
10:90	66.0 (±16.5) b	47.7 (±22.5) b	37.6 (±19.3) d
1:99	44.6 (±18.5) c	17.0 (±12.1) c	15.8 (± 9.9) e

[a] Accumulated numbers of times that 10 males visited the source of the test mixtures within 10 min. Each test was replicated 10 times, and the standard deviations are shown in parentheses. Values in each column with different letters are significantly different ($P < 0.05$) by the Student's t test.

applied using 99:1, 90:10, 50:50, 10:90, and 1:99 mixtures of serricornin and anhydroserricornin as the test substances. The dose level of each test mixture was fixed at 1.0×10^{-1}, 1.0×10^{-2}, and 1.0×10^{-3} μg, respectively. The results are shown in Tables 5–7 according to the individual parameters.

As seen in Table 5, the attractiveness of serricornin was reduced with the increase of anhydroserricornin concentration. The magnitude of sex stimulation due to serricornin was also lowered with the increase of anhydroserricornin concentration as seen in Table 6. The same tendency was observed for the maximum aggregation number (Table 7). In these cases, however, the rate of activity loss never exceeded the decrease of serricornin concentration.

TABLE 6. SEX STIMULATORY ACTIVITY OF MIXTURES OF SERRICORNIN AND ANHYDROSERRICORNIN TO MALE CIGARETTE BEETLES[a]

Mixing ratio serricornin–anhydroserricornin	Dose (μg)		
	1.0×10^{-1}	1.0×10^{-2}	1.0×10^{-3}
99:1	8.4 (±4.3) a	6.4 (±2.1) a	8.6 (±4.0) a
90:10	5.1 (±4.7) ab	9.5 (±3.3) b	6.0 (±2.0) b
50:50	4.5 (±3.0) bc	3.6 (±2.9) c	5.4 (±3.6) c
10:90	2.8 (±2.2) c	3.5 (±2.5) c	5.6 (±5.3) c
1:99	3.2 (±2.6) c	3.3 (±1.7) c	3.4 (±2.6) d

[a] Accumulated numbers of times of attempted matings induced by the test mixtures among 10 males within 10 min. Each test was replicated 10 times, and the standard deviations are shown in parentheses. Values in each column with different letters are significantly different ($P < 0.05$) by the Student's t test.

TABLE 7. MAXIMUM AGGREGATION NUMBERS FOR MIXTURES OF SERRICORNIN AND ANHYDROSERRICORNIN[a]

Mixing ratio serricornin–anhydroserricornin	Dose (μg)		
	1.0×10^{-1}	1.0×10^{-2}	1.0×10^{-3}
99:1	7.5 (±1.0) a	7.4 (±0.6) a	7.8 (±1.9) a
90:10	7.1 (±1.4) a	6.0 (±1.8) b	6.5 (±1.0) b
50:50	5.6 (±0.5) b	4.4 (±2.4) b	6.4 (±1.7) ab
10:90	4.2 (±1.3) c	4.8 (±1.5) b	3.8 (±1.3) c
1:99	3.4 (±1.8) c	2.8 (±1.3) c	2.8 (±1.9) c

[a] Maximum numbers of males staying simultaneously on the source of test mixtures among 10 males within 10 min. Each test was replicated 10 times, and the standard deviations are shown in parentheses. Values in each column with different letters are significantly different ($P < 0.01$) by the Student's t test.

Anhydroserricornin is thought to exist together with serricornin in nature in relatively small quantities (Levinson et al., 1981). We have also confirmed the existence of anhydroserricornin in the *n*-hexane extract of adult females by GC in the ratio of approximately 9 : 1 (serricornin–anhydroserricornin). But the results shown in Tables 5–7 do not suggest any significant effect of the anhydroserricornin addition at around this region of the mixing ratio.

These facts indicate that anhydroserricornin plays neither a synergistic nor an inhibitory role on the pheromonal activity of serricornin. Since the reversible transformation between serricornin and anhydroserricornin occurs even under mild conditions (M. Mori et al., 1984), the role of anhydroserricornin may be speculated to be a precursor of serricornin in the process of biosynthesis or an inactivated form of serricornin, although no evidence has been given yet.

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VOLATILE FATTY ACIDS IN URINE AND VAGINAL SECRETIONS OF COWS DURING REPRODUCTIVE CYCLE[1]

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Abstract—Levels of volatile fatty acids (VFA) in vaginal secretions (VS) of cows were nearly independent of concentration changes of acids in the gastrointestinal tract, while levels in urine showed marked dependence. During three-week cycles, the VFA concentrations in VS increased shortly before heat and decreased to one third on days 19–22. Some cows showed an immediate increase after heat while in others the increase was slow and gradual. Moderately increased levels at midcycle were probably related to interovulatory follicle growth. Content of VFA in VS of pregnant cows was significantly above the level of the postestrus drop. Some pregnant cows in the first two thirds of gravidity had substantially higher levels of VFA than the other cows. Changes in VFA levels have a potential value for estrus detection and pregnancy diagnosis in cows. The gas chromatographic method used is too slow and tedious for practical application, but a reliable semiquantitative test for rapid proof of VFA or acetic acid in VS would be a very useful device for field use. It would have a substantial impact on the economy of cattle production by minimizing the loss of time due to delayed breeding.

Key Words—Volatile fatty acids, urine, vaginal secretion, sexual pheromones, cow, estrous cycle, estrus detection, gravidity, pregnancy diagnosis, gas chromatography.

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INTRODUCTION

Recent observations associating volatile fatty acids (VFA) levels in vaginal secretions (VS) with reproductive functions in minks (Sokolov and Khorlina, 1976), monkeys (Bonsall and Michael, 1971; Curtis et al., 1971; Goldfoot et al., 1976; Michael et al., 1971), apes (Fox, 1982), and women (Bauman et al., 1982; Huggins and Preti, 1976; Michael et al., 1974, 1975; Sokolov et al., 1976; Waltman et al., 1973) have raised hopes that these compounds are mamalian reproductive pheromones. So far, this possibility has not been unequivocally confirmed or rejected, nor has any compound been identified as a true pheromone with all its predicted attributes in the above species. Regardless of whether VFA are true pheromones or not, these acids may be related to the reproductive functions and, therefore, deserve closer study both from the point of view of reproductive and pheromonal physiology and for possible diagnostic utilization (Hradecký, 1978).

Study of VFA in VS and urine of ruminants in relationship to reproduction required preliminary study of influences of acids from the gastrointestinal tract on the levels in other body fluids. Large amounts of acids are ingested with silage and are also produced by fermentation in ruminant forestomachs and large intestine. Diurnal changes in concentrations of VFA increase with grain content in the ration (Wheaton et al., 1970). Concentration in the gastrointestinal content are reflected by concentrations in portal blood due to extensive absorption of acids, but variations in peripheral blood are largely reduced due to liver uptake of acids for energetic purposes. After the relationship of gastrointestinal acids to the levels of VFA in urine and VS was ascertained, the study of VFA in relationship to reproduction was undertaken.

METHODS AND MATERIALS

For evaluation of the influence of gastrointestinal VFA levels on the acid levels in urine and VS, the samples of rumen fluid, feces, blood, urine, and VS were collected from two healthy diestrous cows in the clinic every four hr for 28 hr. For the study of VFA levels in urine and vaginal secretions during the three-week estrous cycle, samples were collected from these two cows and six cycling cows in a farm about twice a week for a period of 36 days. Cows in the clinic had been open (nonpregnant) for a long time; cows at the farm were 1–2 months after calving. Samples of urine and vaginal secretions for determination of VFA levels in pregnancy were collected at the farm from eight cows in the first trimester, seven cows in the second trimester, and six cows in the third trimester of gravidity. The cows were fed twice a day in the clinic with grass hay and grain mix and on the farm with silage (sugarbeet tops) supplemented with hay, straw, and grain mix.

VS were collected with cylindrical cellulose dental tampons 5 cm long and

1–1.5 cm in diameter wrapped in 1–2 layers of gauze. A 70-cm-long silk string with a loop was attached to one end of each tampon. Tampons were extracted with methanol, dried at 70°C, placed in 10-ml plastic vials, and weighed with vials prior to use. For sample collection, the loop was hooked in a groove made at the end of a long mare urinary catheter and the tampon was introduced into the fornix vaginae. Tampons with absorbed secretion were removed after 1 hr by pulling the strings and were placed back into original vials. The strings were cut off and the vials with tampons were weighed. After correction for the cut off string the weight of absorbed secretion in grams was determined and regarded as equal to volume in milliliters. Tampons were processed upon arrival at the laboratory or frozen until processing.

Sample processing included soaking of tampons in vials in a mixture of two volumes of acetone and three volumes of methanol for one day. Then the tampons were squeezed in a syringe and the expressed fluid was collected. Additional portions of solvent mixture were drawn through the tampon into the syringe and squeezed out until the volume of 20 ml was obtained. This extract was concentrated to 3 ml in a rotary evaporator.

The 3-ml sample was acidified with 0.5 ml of 85% H_3PO_4, mixed with one drop of antifoaming agent (silicone oil), and steam distilled until 30 ml of distillate were obtained. The distillate was adjusted to pH 10 with KOH and evaporated to dryness at 80°C in the rotary evaporator. The dry sample was transferred into 5-ml vials by washing the evaporator flask with four 1-ml portions of methanol and evaporated again at 80°C in a water bath. The resultant dry samples were dissolved in 100 μl of distilled water and 20 μl of 5 M H_3PO_4. For gas chromatographic analysis, 10 μl were used.

Blood was obtained by jugular venipuncture, urine by catheterization, and rumen fluid by a stomach tube. Samples of blood plasma and urine (3 ml) were acidified, mixed with antifoaming agent, and distilled with water vapor and further processed as samples of VS. Rumen fluid was analyzed directly after centrifugation and acidification of 80 μl of sample with 20 μl of 5 M H_3PO_4. Fecal samples were mixed with water and supernatant was treated in the same manner as rumen fluid; results were calculated on dry matter basis.

Gas chromatographic analyses were performed on CHROM 4 instrument (Laboratory Instruments, Prague, Czechoslovakia) equipped with FID and glass column (120 cm × 3 mm ID) packed with Porapak P 80–100 mesh (Waters Associates, Framingham, Massachusetts) treated with 3% *o*-phosphoric acid. Injector temperature was 190°C; column temperature was programmed from 120°C to 200°C at 6°C min, and the flow rate was 44 ml N_2/min.

Analyses separated acetic, propionic, isobutyric, butyric, isovaleric, and valeric acids. For quantitation, external standardization with a known VFA mixture in combination with recovery factors was used. Recovery factors were determined in advance by the method of standard addition of the known VFA mixture to a series of samples to eliminate the impact of extensive sample pro-

cessing on final results (Novák, 1975). Recovery factors ranged from 0.90 to 0.36 in urine and from 0.73 to 0.29 in VS for acetic and valeric acids, respectively. Reproducibility in a series of six analyses of the same sample were around 10% for urine and blood plasma, 15% for VS (six tampons inserted simultaneously into vagina of a cow), and 3% for rumen fluid. Blank processing of the tampons prepared for sample collection showed no measurable amounts of VFA contributing to the final results.

RESULTS AND DISCUSSION

Quantitative sampling of VS using the same method throughout the investigation remains an unresolved problem due to changes in the amount and viscosity of secretion. Secretion is copious and watery near estrus and very scarce and viscous during diestrus and pregnancy. Changes in technique for secretion sampling during different parts of the reproductive cycle may require modifications in subsequent sample processing and cause errors due to changes of uncontrollable factors.

The tampon technique used in this study was relatively easy. The maximum amount of fluid absorbed by the tampons at full soaking was about 5 g. Collection from 21 pregnant cows yielded an average of 1.6 ± 0.12 g secretion, while the amounts collected from cycling cows were higher and extremely variable. However, the secretion collected was not pure VS, since small amounts of secretion from vestibulum vaginae were collected during insertion and removal of tampons. Different physical properties of secretion might also cause different extractability of acids from the tampons.

Steam distillation of herbivore urine also yielded variable amounts of benzoic acid originating from microbial and acidic hydrolysis of hippuric acid (benzoylglycine). Presence of large quantities of benzoic acid in final samples to be injected for analysis was damaging since sudden crystallization in the drawn sample froze the microsyringe and might cause its destruction during injection.

VFA in rumen fluid exhibited large diurnal changes in concentrations due to microbial fermentation and content in feeds (silages). In the two observed cows, the total concentrations of VFA varied in the range 75–150 mM in rumen fluid and 0.20–0.37 mmol/g of dry matter of feces (average dry matter was $17.7 \pm 0.3\%$; $N = 16$). Fermentation in the large intestine partially compensated the effect of ruminal changes since the correlation of ruminal and fecal concentrations was $r = -0.33$ ($+0.18$ to -0.38 for individual acids).

Peripheral blood concentrations showed generally low correlations with gastrointestinal concentrations (r in the range ± 0.2) except branched-chain acids (isobutyric and isovaleric) which showed $r = \pm 0.2$ with rumen fluid and $r = -0.5$ with fecal values, and valeric acid ($r = \pm 0.2$ with rumen fluid and $r = -0.3$ with feces). These acids are used mainly by gastrointestinal microflora for amino acid synthesis, and their liver uptake is apparently less effective.

Urinary concentrations of VFA were influenced both by gastrointestinal and blood concentrations. Moderate correlations of the total VFA in urine and rumen fluid ($r = +0.67$) or blood ($r = +0.4$) indicated that any changes in urinary VFA related to reproductive functions would be obscured by dietary factors.

Concentrations of VFA in VS showed only low correlations with concentrations in other fluids (r in the range ± 0.2). With respect to the reproducibility of the sampling and processing procedure, the levels of VFA in VS could be regarded as independent of gastrointestinal production of acids and could reflect changes associated with reproduction without substantial interference of gastrointestinal acids. Table 1 gives comparative values of concentrations of the total VFA and their molar composition in the samples collected for the study of influences of gastrointestinal acids on the levels of VFA in blood plasma, urine, and VS.

Dynamics of VFA in VS of cycling cows was closely related to the estrous cycle, especially around the time of estrus (Figure 1). Several days prior to estrus, the concentrations gradually increased and then rapidly decreased to about one third. This pattern was especially clear in the two cows at the clinic (Nos. 1 and 2) that were observed daily for heat and sampled more frequently around estrus. Overall dynamics of VFA reflect to some degree the dynamics of both blood or milk progesterone (Hoffmann et al., 1976; Thibier, 1976) and estradiol (MacDonald et al., 1982) during estrous cycle and, therefore, there is probably not any simple relationship of VFA levels to a particular ovarian steroid.

The concentration decrease was observed between days 19 and 22 of the cycle, but its exact relationship to the day of estrus and optimal breeding time could not be determined because of long intervals between sample collections. The days of sampling at the farm were unrelated to the days of heat and, therefore, the results do not necessarily include maximal changes occurring during estrus. The average values obtained from samples collected before and after

TABLE 1. DIETARY INFLUENCES ON VFA IN URINE AND VS ($N = 16$)[a]

Sample	Total VFA	C-2	C-3	isoC-4	C-4	isoC-5	C-5
Rumen fluid	101.00	72.8	15.6	1.04	8.57	1.06	0.98
Feces	0.30	76.8	14.8	1.90	3.29	1.86	1.40
Blood plasma	3.08	94.0	3.30	0.49	0.43	1.19	0.55
Urine	1.97	90.6	4.55	1.01	1.32	1.85	0.70
Vaginal secretion	2.28	92.2	5.35	0.29	0.82	0.68	0.68

[a] Average concentrations of total VFA (mM, mmol/g DM in feces) and molar percentage composition (mol%) of the individual acids (C-2, acetic; C-3, propionic; isoC-4, isobutyric; C-4, butyric; isoC-5, isovaleric; C-5, valeric) in samples collected for study.

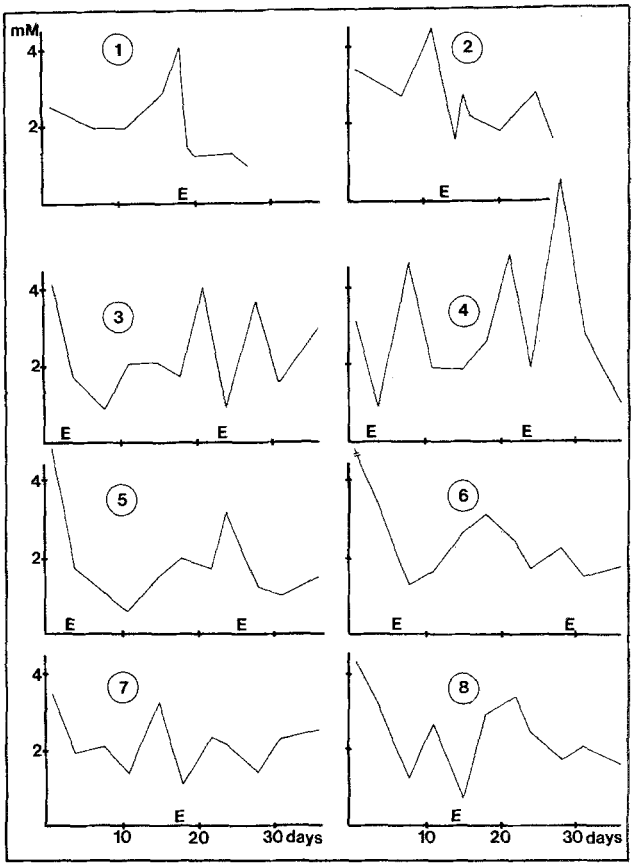

FIG. 1. Dynamics of the total VFA concentrations (mM) in VS of the eight cows during the 36-day study. E = estrus.

heat and from diestrous (all others) samples are given in Table 2. Differences among these values for VS are highly significant ($P < 0.01$, t test).

After heat, the VFA concentrations in about half the cases sharply increased while remaining low in the other half. The reason for this increase and its nearly equal distribution is so far unclear. It is interesting that a similar double peak of VFA around estrus has been observed in VS of monkeys (Goldfoot et al., 1976). Some cows exhibited increased VFA concentrations in VS also during midcycle; these might be related to the hormonal changes associated with interovulatory follicles (Schneebeli, 1984). These follicles may induce estrous behavior in cows and increase their attractivity for bulls (Hradecký et al., 1983).

Acetic acid composed 85–95 mol% of VFA in VS of cycling cows, and

TABLE 2. AVERAGE CONCENTRATIONS OF TOTAL VFA IN SAMPLES OF VS AND URINE COLLECTED BEFORE AND AFTER HEAT AND DURING DIESTRUS

Samples	N	Concentration (mM, $\bar{X} \pm s_{\bar{X}}$)	
		Vaginal secretion	Urine
Samples before heat	12	3.68 ± 0.25	6.54 ± 1.40
Samples after heat	12	1.35 ± 0.11	3.74 ± 0.60
Diestrous samples	61	2.31 ± 0.15	4.81 ± 0.43

this percentage increased slightly around estrus. Therefore, the dynamics of acetic acid closely corresponded to the dynamics of total VFA. The peak of acetic acid, the major component of VFA, lasted up to six days around estrus and was detected in all cases of heat. Peaks of the minor acids were probably of narrower duration since they were not consistently detected in every heat by the used frequency of sample collection. Dynamics observed for the minor acids is therefore less consistent than for acetic acid. In cases where the estrous peak and drop of minor acids were observed, the concentration changes were often larger than in acetic acid. In individual cases, propionic acid concentration dropped 30 times, butyric 33 times, isovaleric 14 times, and valeric 8 times. These changes approached the 20- to 30-fold changes observed in VS of minks (Sokolov and Khorlina, 1976). Another study of vaginal VFA in cows has demonstrated no cyclic variations in acid levels, but that may be due to differences in methods used or other factors not mentioned (Quinn and Simmons, 1980).

Dynamics of VFA in urine of cycling cows showed similar changes around estrus but also numerous other changes unrelated to the estrous cycle. The six farm cows had the same housing and feeding conditions, but the VFA changes in their urine were not synchronous. These changes were probably indirect consequences of dietary factors (quality of silage and hay) influencing diuresis, gastrointestinal function, and energetic status (subclinical ketosis). Some cows with large changes also had frequent positive reaction for oxidized ketone bodies in urine which can be frequently seen in high producing cows without any clinical sign of disease (Hradecký and Kudláč, 1984). Average values obtained for urinary samples from cycling cows are given also in Table 2. Urinary changes around estrus were not significant ($P > 0.05$).

Values of VFA in VS and urine of pregnant cows are given in Table 3. Of the values in VS, only the difference between the second and third trimester was significant ($P < 0.05$). The group of pregnant cows in the first and second trimester had an average concentration of total VFA in VS 4.31 ± 0.47 ($\bar{X} \pm s_{\bar{X}}$) but showed clear distribution of values into two subgroups, one with eight cows and one with seven cows. The subgroups had 5.74 ± 0.43 mM and

TABLE 3. AVERAGE CONCENTRATIONS OF TOTAL VFA IN VS AND URINE COLLECTED FROM PREGNANT COWS

Stage of pregnancy	N	Concentration (mM, $\bar{X} \pm s_{\bar{X}}$)	
		Vaginal secretion	Urine
1st trimester (47–84 days)	8	3.44 ± 0.43	3.30 ± 1.36
2nd trimester (117–170 days)	7	5.31 ± 0.73	1.25 ± 0.19
3rd trimester (210–269 days)	6	2.48 ± 0.24	2.44 ± 0.53
Total	21	3.79 ± 0.38	2.37 ± 0.56

2.67 ± 0.09 mM of VFA, respectively. All three of these values were significantly higher than post estrous values of nonpregnant cows (1.35 ± 0.11 mM). Differences in urinary values among trimesters and between pregnant and nonpregnant cows were not significant. Molar percentage of acetic acid in VS during the first two thirds of pregnancy was about 95 mol% and slightly decreased during the third trimester, while in urine it was about 90 mol% and slightly increased by the end of gravidity.

Origin of the VFA in VS as possible mammalian reproductive pheromones was discussed in relation to hormonal influences on vaginal symbiotic microflora (Bonsall and Michael, 1971; Doty et al., 1975; Hradecký, 1978; Preti and Huggins, 1975), but this possibility was not definitely proved or rejected. A possible role of microorganisms in generating VFA and other odorous compounds in VS that are related to reproduction and act as pheromones is supported by clinical experience with passing a stomach tube in ruminants. Cattle often show flehmen when the tube is being withdrawn and the rumen fluid on its surface is smeared over the sensitive area of the vomeronasal organ in the oral palate. This reaction can seldom be elicited by direct mechanical stimuli, but rubbing with rumen fluid or ingesta containing microbial products can elicit flehmen. The flehmen reaction in a bull has been shown to be related to reproductive functions and probably to pheromones in cows (Hradecký et al., 1983).

Apparent dietary influences on VFA levels in urine practically preclude use of these acids for monitoring reproductive functions in cows. However, the low dependence of the concentration changes of VFA in VS on dietary factors and their association with reproductive functions have promising diagnostic value especially in recently inseminated cows. A drop in acid concentrations around days 19–22 after breeding (natural or artificial insemination) would indicate conception failure and return into heat. Rapid determination of acid levels in VS would permit rebreeding of cows in three weeks after previous breeding with the possibility of optimal timing for insemination. On the other hand, the persistence of high acid levels would indicate probable conception. My preliminary investigation indicated that by days 20–21 after artificial insemination and

conception, the acid levels were already elevated to the gravidity values and no drop in concentration occurred. Some cows may also show behavioral signs of gestational estrus during early pregnancy (Erb and Morrison, 1958; Donoho and Rickard, 1955; Patil et al., 1982; Singal et al., 1978) and avoiding artificial insemination of these cows could eliminate probable induced embryonic death.

Gas chromatographic determination of VFA levels in VS is a valuable research tool, but it is too tedious and slow for practical application. Because all acids involved are a homologous series with similar chemical reactions and the major acetic acid represents about 90 mol%, it should be possible to develop a reliable semiquantitative test for rapid proof of VFA (or acetic acid) levels in VS for field use. This device would minimize the loss of time due to delayed breeding and conception and would have a substantial impact on the economy of cattle production.

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SPECIES, INDIVIDUAL AND KIN SPECIFIC BLENDS IN DUFOUR'S GLAND SECRETIONS OF HALICTINE BEES
Chemical Evidence

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Abstract—The compositions of Dufour's gland secretions of four sympatric halictine bee species were chemically studied by combined gas chromatography–mass spectrometry. The secretion is composed of C_{16}–C_{24} macrocyclic lactones, isopentenyl esters, and hydrocarbons. Comparative analyses have revealed that the blends of compounds are species specific. Studies of individual glands belonging to bees of the species *Evylaeus malachurum* have demonstrated that each bee exhibits a specific blend in its Dufour's gland. Nestmate bees (considered as sister bees), however, are more similar to each other than nonrelated bees. The relative importance of species specificity, individual specificity, and similarities between kins are discussed.

Key Words—Halictine bees, Hymenoptera, Halictidae, *Evylaeus melachurum*, *E. carneiventre*, *E. politum*, Dufour's gland secretion, gas chromatography–mass spectrometry, macrocyclic lactones, species specificity, individual specifity, kin specificity.

INTRODUCTION

Dufour's gland, present in halictine bees, is exceedingly large and has attracted the attention of many researchers. Its oily secretion has been analyzed in many species revealing the omnipresence of macrocyclic lactones ranging from 16-hexadecanolide to 26-hexacosanolide (Anderson et al., 1967; Hefetz et al., 1978; Bergström and Tengö, 1979; Duffield et al., 1981). In all cases the secretion also contained hydrocarbons, and in a few species small amounts of isopentenyl esters accompanied them. The secretion is used to line the brood cells and tunnels of the nest (Cane, 1981; Brooks and Cane, 1984), where the

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lactones, at least in part, are transformed to polyesters that create the waterproof lining.

A comparative analysis of the relative amounts of the various lactones have been conducted in 16 halictine species, demonstrating species specificity (Johansson et al., 1982). This suggests that Dufour's gland secretion may serve a more complex role, perhaps communicative, rather than merely creating a hydrophobic lining for the brood cells. Indeed, in *Lasioglossum zephyrum*, several studies suggest that odors play an important role in mate selection (Barrows et al., 1975) and nestmate recognition (Bell et al., 1974), although nothing is known of the source or the nature of the secretion involved. A communicative function of Dufour's gland secretion was demonstrated in the solitary anthophorid bee *Eucera palestinae* (Shimron et al., 1985). In this species, the secretion is utilized to mark the entrances of the nest, helping the bees to locate their own nest in a dense aggregation. Since Dufour's gland secretion is smeared at the nest entrance of at least one halictine species (Brooks and Cane, 1984), it is possible that in halctines it has a similar function in nest recognition. If the secretion produced by Dufour's gland of halictine bees indeed functions as a discriminator, then it should convey individuality. Yet in the social species, odors common to nestmates are pertinent and discrimination should take place between nests. The large size of Dufour's gland in halictine bees makes it possible to analyze individual glands and enables us to test if these characteristics hold true.

METHODS AND MATERIALS

The four species of halictine bees investigated, *Halictus resurgens*, *Evylaeus malachurum*, *E. politum*, and *E. carneiventre* nest sympatrically and were studied at Beith Elazari, on the coastal plain of Israel. Collection of the bees took place during the months April–June, 1983 and 1984, at their nesting sites. Each nest was marked, and the foraging behavior of its occupants was followed prior to their collection. The bees were collected while leaving the nest by placing a glass vial on the top of the nest. They were chilled on ice until brought to the laboratory for dissection.

All the bee species were identified by Dr. A. W. Ebmer, Linz, Austria. Voucher specimens are kept in the Entomological Collections of Tel Aviv University.

In the laboratory, the bees were dissected under cold water, and the ovarian development and the state of the spermatheca (full/empty) were recorded. Dufour's glands of the bees were placed in pentane for extraction and further analysis. In order to analyze individual glands of *E. malachurum*, each gland was put in pentane in a separate vial. Chemical characterization of the glandular exudates were carried out at the Ecological Station of Uppsala University at

Öland, Sweden, using an LKB 2091 combined gas chromatograph–mass spectrometer. The samples were run on a WG11 capillary (polar) column (0.5 mm × 25 m), temperature programmed from 150°C to 200°C at 8°C/min, then run isothermally. The different components were identified by their mass spectra.

Individual variations in the glandular components of *E. malachurum* were studied by two replicate injections of samples of individual gland extracts and comparing the relative amounts of the various components in each case. Sample analyses were carried out using an OV-1 column temperature programmed as described earlier. The data were then subjected to cluster analysis of cases based on the differences between the relative amounts of the components in each sample. An amalgamation distance between samples was determined by pseudo-nearest-neighbor algorithm. Initially each case was considered a separate cluster, then two cases with the smallest amalgamation distance were joined. This is a stepwise process continuing until all cases are combined into one cluster (Dixon, 1981). The smaller the amalgamation distance is, the more similar are the exudates involved. An example of such a clustering is depicted in Figure 2. For the statistical analysis the amalgamation distances before clustering were considered. Since the data were not normally distributed, a Wilcoxon two-sample test was used, and the results were presented as medians (Table 2). In this way nestmates and nonnestmates were compared in four nests.

Nests of *E. malachurum* were excavated and the brood cells removed. They were washed to remove adhering pollen and their cell linings were extracted with pentane. In addition, nest entrances (top 2 cm) of 12 nests were collected and similarly extracted in pentane. Both extracts were analyzed by gas chromatography using either AT 1000 or OV-1 columns programmed from 150°C to 250°C at 8°C/min. The presence of the lactones in these extracts was deduced from their respective retention times and from coinjections with Dufour's gland extracts.

RESULTS

Comparative Chemical Analysis

Dufours' gland secretion of four species of halictine bees was analyzed by combined gas chromatography–mass spectrometry. The secretions contained saturated and unsaturated unbranched macrocyclic lactones, branched macrocyclic lactones, isopentenyl esters, and saturated straight-chain hydrocarbons (Figure 1A–D). The lactones were identified by their characteristic mass spectra, including ions at M−18 (loss of water), M−60 (loss of acetic acid), a corresponding ion at *m/z* 60, and smaller ion peaks at M−43 (loss of CH_3CO) and M−46 (loss of HCOOH). These lactones were 16-hexadecanolide (mol wt 254), 18-octadecanolide (mol wt 282), 20-eicosanolide (mol wt 310), 21-hen-

eicosanolide (mol wt 324), 22-docosanolide (mol wt 338), 23-tricosanolide (mol wt 352), and 24-tetracosanolide (mol wt 366). In addition, monounsaturated unbranched lactones were present. These characteristically had a larger M^+ ion than that at M−18, and included 18-octadecenolide (two isomers, mol wt 280), 20-eicosenolide (mol wt 308), 22-docosenolide (mol wt 336), and 24-tetracosenolide (mol wt 364). No attempts to elucidate the position of the double bond or the nature of the two isomers of 18-octadecenolide were made.

Dufour's gland secretion of three of the species apparently also contained dimethyl branched lactones. This was deduced from the slightly altered mass spectra of these compounds as compared to the unbranched lactones of the same molecular weight, but mainly from their respective retention times. In *E. malachurum*, a lactone with a molecular weight of 310 corresponding to a C−20 lactone, had a retention time, under the conditions of the separation (see Methods and Materials), of 27 min. In the same analysis, 18-octadecanolide eluted 1 min earlier, while 20-eicosanolide eluted after 34 min. In addition, the compound in question had a different mass spectrum when compared to 20-eicosanolide. The compound was tentatively identified as dimethyloctadecanolide, but since we did not have any standards to authenticate this structure, this identification remains speculative. Similarly, a C−22 lactone (mol wt 338) had a retention time of 39 min while 20-eicosanolide and 21-heneicosanolide had respective retention times of 34 and 42 min. By the same line of reasoning it was assigned as dimethyleicosanolide.

The isopentenyl esters present in Dufour's gland of the species investigated were similar to those published for other halictine bees (Duffield et al., 1981) and included 3-methyl-2 (or 3)-butenyl octadecanoate, 3-methyl-2 (or 3)-butenyl eicosanoate, and 3-methyl-2 (or 3)-butenyl docosanoate. On the basis of the mass spectrum alone we could not distinguish between the two isomers. Dufour's gland secretions also contained homologous series of paraffins ranging from nonadecane to octacosane.

Species Specificity

Four sympatric species were studied: *H. resurgens*, *E. politum*, *E. carneiventre*, and *E. malachurum*, each of which produced a specific blend of chemicals in its Dufour's gland (Figure 1 and Table 1). This is demonstrated best if we inspect the various groups of compounds separately.

Lactones. H. resurgens was conspicuous in having 16-hexadecanolide and 24-tetracosenolide which were absent from the secretion of the three *Evylaeus* species. The secretion of *H. resurgens* was also the only one to be largely dominated by 24-tetracosanolide. The differences between the three *Evylaeus* species were less distinct. The major unbranched lactones were omnipresent, but *E. politum* stood out in having odd-carbon-number macrocyclic lactones including 21-heneicosanolide and 23-tricosanolide. These compounds, found

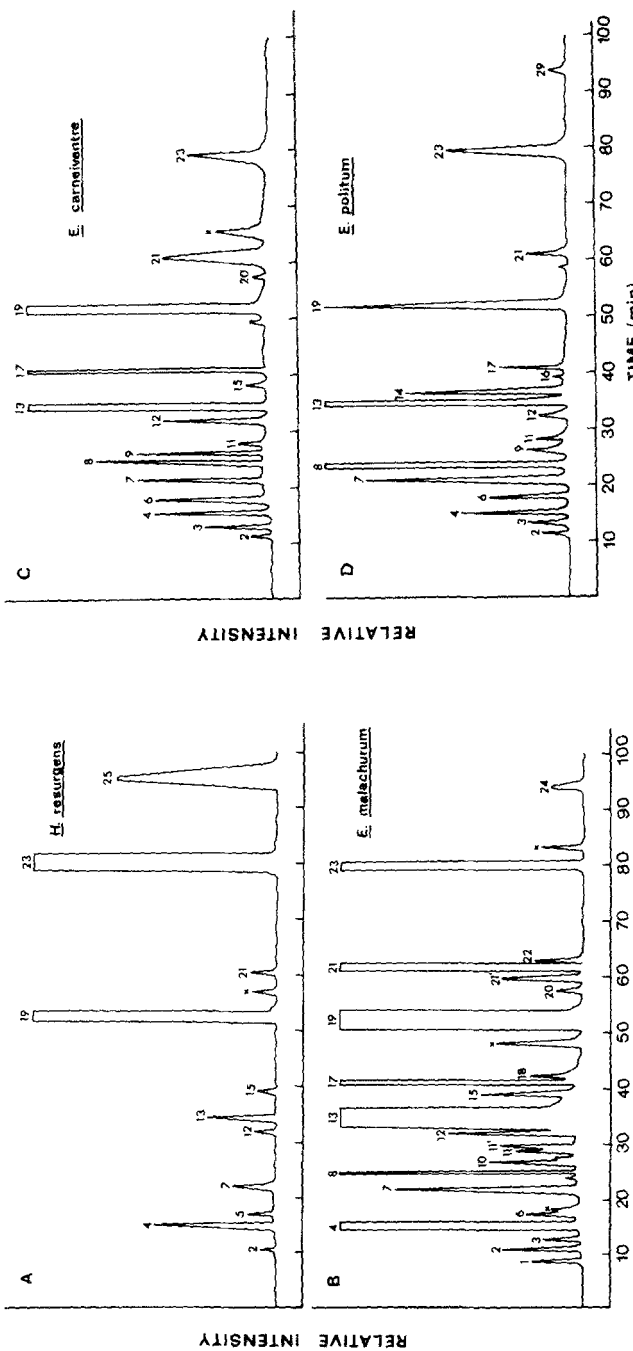

FIG. 1. Gas chromatograms of Dufour's gland extracts of four halictine species: *Halictus resurgens*, *Evylaeus malachurum*, *E. carnieventre*, and *E. politum*. Samples were run on a WG11 column programmed from 150°C to 200°C at 8°C/min, then run isothermally in an LKB 2091 combined GC-MS. The compounds identified are as follows: (1) nonadecane; (2) heinecosane; (3) docosane; (4) tricosane; (5) 16-hexadecanolide; (6) tetracosane; (7) pentacosane; (8) 18-octadecanolide; (9) hexacosane; (10) dimethyl octadecanolide; (11) 18-octadecenolide (1st isomer) (11') 18-octadecenolide (2nd isomer); (12) heptacosane; (13) 20-eicosanolide; (14) 3-methyl-2 (or 3)-butenyl octadecanoate; (15) dimethyleicosanolide; (16) octacosane; (17) 20-eicosenolide; (18) 21-heneicosanolide; (19) 22-docosanolide; (20) 3-methyl-2 (or 3)-butenyl eicosanoate; (21') 22-docosenolide; (21) 22-docosenolide (2nd isomer); (22) 23-tricosanolide; (23) 24-tetracosanolide; (24) methyl-2 (or 3)-butenyl docosanoate; (25) 24-tetracosenolide. Compounds marked with an x were not identifiable by their mass spectra.

TABLE 1. COMPARATIVE ANALYSIS OF DUFOUR'S GLAND SECRETION OF 4 HALICTINE SPECIES[a]

Compound	Mol wt	R_t (min)[b]	Species			
			H. resurgens	*E. politum*	*E. carneiventre*	*E. malachurum*
Lactones						
16-Hexadecanolide	254	17	t			
18-Octadecanolide	282	24		+++(D)	+	+
Dimethyloctadecanolide[c]	310	27				t
18-Octadecenolide	280	28		t	t	t
20-Eicosanolide	310	34	+	+++	+++	+++(D)
Dimethyleicosanolide[c]	338	39	t		t	t
20-Eicosenolide	308	41		+	++	++
21-Heneicosanolide	324	42				t
22-Docosanolide	338	52	++	++	+++(D)	+++(D)
22-Docosenolide	336	61	t	+	++	++
23-Tricosanolide	352	62				t
24-Tetracosanolide	366	79	+++(D)	++	++	++
24-Tetracosenolide	364	96	++			

Isopentenyl esters						
3-Methyl-2(3)-butenyl octadecanoate	352	36		++		
3-Methyl-2(3)-butenyl eicosanoate	380	57			t	t
3-Methyl-2(3)-butenyl docosanoate	408	98		+		t
Hydrocarbons						
Nonadecane	268	9				t
Heneicosane	296	11	t	t	t	t
Docosane	310	13		t	+	t
Tricosane	324	15	+	+	+	++
Tetracosane	338	18		+	+	t
Pentacosane	352	22	+	++	+	+
Hexacosane	366	26		t	+	
Heptacosane	380	32	t	t	+	t
Octacosane	394	39		t		

[a] Each sample was run on a WGll capillary column temperature programmed from 150°C to 200°C at 8°C/min then run isothermally. The relative amounts are indicated as follows: t = trace; + = small amounts; ++ = fair amounts; +++ = large amounts; D = dominant peak.
[b] Under the condition described in footnote *a*.
[c] The identity of the methyl branched lactones is only tentative.

only in trace amounts, are the first reported from the Halictidae. The differences in the apparent branched lactones is harder to depict since we do not know their exact structures. Nonetheless, *E. malachurum* posessed at least two unbranched lactones while in *E. carneiventre* only dimethyleicosanolide appeared. The latter compound was also present in the secretion of *H. resurgens* but absent in *E. politum*.

Isopentenyl Esters. The same species specificity existed when we compared the abundance of the isopentyl esters in the various species. *E. politum* was unique in having isopentenyl octadecanoate that was accompanied by isopentenyl docosanoate. In *E. carneiventre* only isopentenyl eicosanoate was present, while *E. malachurum* has, in addition to the latter compound, isopentenyl docosanoate. In Dufour's gland secretion of *H. resurgens* we could not detect any isopentenyl esters.

Hydrocarbons. Even though the hydrocarbons were present in relatively small amounts in Dufour's gland secretions, several features could be pointed out. *H. resurgens* produced only odd-carbon hydrocarbons, two of which, tricosane and pentacosane, appeared in fair amounts. In general, these two paraffins were present in fair amounts in all four species. Another distinct feature is the unique presence of nonadecane in *E. malachurum* and octacosane in *E. politum*.

Individual Variation and Genealogical Relatedness

Evylaeus malachurum was the dominant species in the study area and is a social species (Michener, 1974). We chose this species to conduct analyses of individual bees in order to assess if individual blends in Dufour's gland secretion exist. We further analyzed bees occupying the same nest as compared to randomly collected bees. The nestmates analyzed were all foragers, had undeveloped ovaries, and were unfertilized. We assume these nestmates to be sister bees. Each Dufour's extract was injected twice into the gas chromatograph, and the relative amounts of the various components were recorded by an interfaced integrator. We then compared the injections by cluster analysis of cases. Table 2 summarizes the amalgamation distances between injections of the same extracts, injections from extracts belonging to sister bees, and injections from extracts belonging to nonnestmate (= strange) bees, for four nests that were assayed. The amalgamation distance between injections of extracts belonging to nestmate (= sister) bees was significantly lower ($P < 0.001$) than that between injections of extracts belonging to nonnestmate bees and higher than that between injections belonging to the same extract. This suggests that although individual blends exist, the variation between nestmate bees is lower than that of nonnestmates.

TABLE 2. VARIATION IN COMPOSITION OF DUFOUR'S GLAND SECRETIONS OF INDIVIDUAL *E. malachurum* BEES[a]

	Median Amalgamation Distance			
Nest	Two samples of the same glandular exudate	Samples of glandular exudate from nestmate (sister bees)	Samples of glandular exudate from nonnestmates (strange bees)	*P*
1	4.99 ($N = 14$)	30.96 ($N = 8$)	54.2 ($N = 6$)	<0.001
2	4.78 ($N = 15$)	30.36 ($N = 9$)	52.02 ($N = 6$)	<0.001
3	7.68 ($N = 15$)	20.7 ($N = 9$)	52.62 ($N = 6$)	<0.001
4	6.10 ($N = 11$)	34.14 ($N = 5$)	52.26 ($N = 6$)	<0.001

[a] Samples originating from nestmates (sister bees) and nonnestmates (strange bees) were compared by cluster analysis of cases. The data of each nest are presented as median amalgamation distance calculated before the clustering of the cases.

Analysis of Cell Linings of E. malachurum

Analysis of cell lining extracts revealed the presence of the lactones at the same ratio as present in Dufour's gland secretion. During field observations, it was noted that the bee constantly smears the top of its tunnel near the nest entrance with its abdominal tip and glossae. These parts of the tunnels later became white from the material deposited by the bees. Extraction and analysis of the coated soil revealed that the secretion laid there originated from the Dufour's gland. This coating was characteristic to the top of the nest. Extraction of lower parts of the tunnel failed to demonstrate the presence of Dufour's gland compounds.

DISCUSSION

The research on Dufour's gland secretions of halictine bees has expanded over many species, making it the most studied family in this respect. This family was also extensively studied behaviorally mainly because it exhibits all known degrees of social evolution (Michener, 1974).

Most of the studies pertaining to Dufour's gland concentrated on the glandular structure (Lello, 1971) or the chemistry of its secretion (Duffield et al., 1984, and references therein). The three major groups of compounds, e.g., macrocyclic lactones, isopentenyl esters, and hydrocarbons, present in the species studied so far, are also present in the four species investigated in this study. In the present study, we show evidence of the existence of four macrocyclic lactones that were not reported earlier: 21-heneicosanolide and 23-tricosanolide

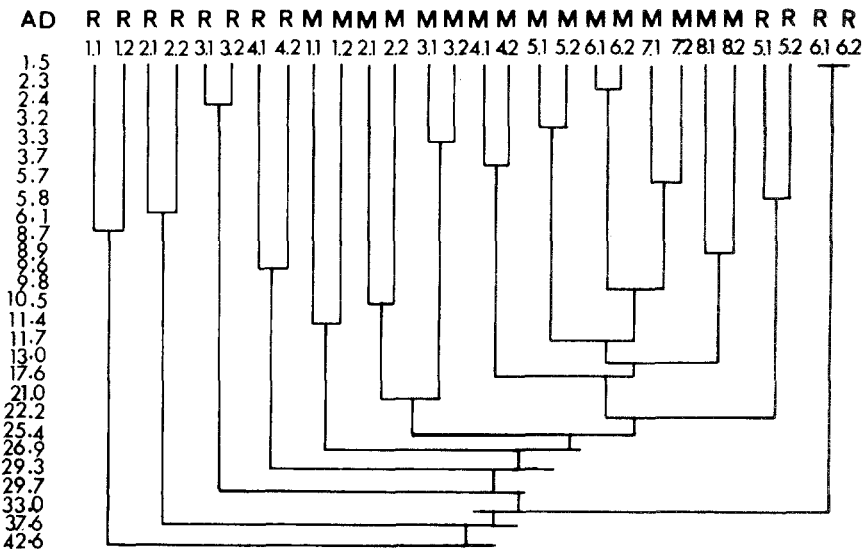

FIG. 2. Dendrogram representing the degree of similarity in the composition of Dufour's gland secretions of 14 *E. malachurum* bees. Eight of the bees were nestmates (marked by M), the other six were nonnestmates collected at random in the nesting area (marked by R). Each glandular exudate was injected twice. Accordingly, case R 1,2 is the second injection of extract of the first bee collected at random, while case M 3,2 is the second injection of the third bee collected from nest M. The amalgamation distances depicted to the left of the dendogram are calculated after the clustering procedure.

and two branched lactones, dimethyloctadecanolide and dimethyleicosanolide, all present in trace amounts only.

The chemical studies of halictine Dufour's gland secretions have largely demonstrated that the composition of the glandular secretion is species specific (Hefetz et al., 1978; Duffield et al., 1981; Johansson et al., 1982). Species specificity is also evident from the analyses of the four species presented here and, as in the other species, it is expressed usually in differences between the lactones that dominate the secretion. Our study, however, further demonstrates that individual variability exists among conspecific bees in addition to species specificity. In *E. malachurum* bees, the two lactones, 20-eicosanolide and 22-docosanolide, are always the dominant lactones conveying the species specificity, but their relative amounts with respect to the other components in the secretion varies among individuals. Thus we have a species-specific marker and demonstration of individually at the same time. Furthermore, in the social species *E. malachurum* (Figure 2 and Table 2), nestmate bees are more similar in their Dufour's gland composition than nonnestmates although individuality can also be seen among sister bees. An interesting question is how these features effect the biology and social behavior of the bees.

Several studies with *Lasioglossum zephyrum* have pointed out the importance of olfactory cues in its social behavior, mostly with respect to nestmate recognition. Nonresident bees are often recognized by the nest guard as aliens by their odor and are not allowed to enter the nest (Bell et al., 1974). Further combined behavioral and hybridization studies revealed that these individual odors are genetically controlled (Greenberg, 1979) but that learning components also exist (Buckle and Greenberg 1981). Although Dufour's gland secretion was not implicated in contributing to these individual odors, there are arguments in favor on this supposition. Dufour's gland secretion is extensively deposited in the nest walls of several halictine species including the brood cell of *Augochlora pura* and *Lasioglossum albipes* (Duffield et al., 1981; Cane, 1981) and in the nest tunnels of *Halictus hesperus* (Brooks and Cane, 1984).

In our study, we demonstrated that the upper centimeter of the nest contained extraordinarily large amounts of Dufour's gland secretion. It is thus conceivable that the glandular secretion adheres to the resident bee bodies while moving in the nest, besides the possible emission of the secretion from the gland at will. The fact that the secretion composition is species specific, as well as individual, is another argument for the suggestion that the secretion also has a communicative function. We suggest, on the basis of the data obtained, that Dufour's gland secretion is at least in part responsible for nestmate recognition. We further postulate that although individuality exists even within sister bees, the variability in this case is not large enough to be detected by the bees and that it is considered by them to be the same odor. This is also in accordance with the findings of Greenberg (1979) that related bees are more readily accepted by a nest guard than nonkin bees. Similarily, although we do not exclude the possibility of learning, we think that the acceptance of nonkin nestmates is partially due to their acquisition of Dufour's gland components from the nest lining as well as olfactory habituation by the other nonkin residents. Confirmation of these assumptions must await further behavioral studies using Dufour's gland secretions as well as its synthetic components.

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ALLELOPATHIC SUPPRESSION OF WEED AND NITRIFICATION BY SELECTED CULTIVARS OF *Sorghum bicolor* (L.) MOENCH

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Abstract—Root exudates of 100 cultivars of *Sorghum bicolor* L. (Moench) were screened for their ability to inhibit seed germination and seedling growth of *Amaranthus retroflexus* L. Exudates of some of the test cultivars were found to inhibit significantly seed germination and/or seedling growth of *A. retroflexus*, and most of the inhibitory activity was found in neutral and acetone fractions of root exudates. Testing of aqueous extracts and decaying residues of four selected *Sorghum* cultivars (two toxic and two nontoxic through root exudation) revealed that all four cultivars significantly reduced *A. retroflexus* growth and nitrification processes with greater inhibition achieved by the toxic cultivars. This study suggests a potential biological control of *Amaranthus* and nitrification by some *S. bicolor* cultivars.

Key Words—Allelopathy, *Sorghum*, root exudates, crop residues, water extracts, *Amaranthus*, nitrification, biological control.

INTRODUCTION

Recently, the recognized importance of allelopathy in agricultural practice has been accelerated with the main goal of using this phenomenon in biological control (Rice, 1979; AlSaadawi and Rice 1983; Putnam, 1983; Leather, 1983). One approach for utilizing this phenomenon is to screen various accessions of allelopathic crops for their ability to reduce weedy species and only a few crops have been evaluated in this aspect (Putnam and Duke, 1974; Fay and Duke, 1977; Leather, 1983).

Recently, the allelopathic potentiality of *Sorghum bicolor* has been re-

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ported and well documented. Leon (1976) found that the reduction in *Sorghum* productivity under a continuous monoculturing regime was due to allelopathy. Putnam (1983) pointed out that mulches from the genus *Sorghum* provide great weed-killing capacity. Hussain and Gadoon (1981) suggested that *Sorghum bicolor* fields be rotated with other crops to maximize crop productivity. However, no work was done to test the possible differences in the allelopathic potentiality among *Sorghum* cultivars against weeds or to test the possible allelopathic effects of *Sorghum bicolor* on the nitrification process. Inhibition of nitrification is of great importance in agricultural ecosystems. Huber et al. (1977) concluded that inhibition of nitrification may markedly increase the efficiency of food production, reduce energy requirements for growing crops, decrease the incidence of plant disease, and reduce the pollution potential of nitrogen fertilizers. Inhibition of nitrification in natural ecosystems is well documented and discussed by several investigators (Rice and Pancholy, 1972, 1973, 1974; Jones and Richards, 1977; Lodhi, 1978, 1981).

With the above in mind, this project was developed to screen 100 cultivars of *Sorghum bicolor* introduced from the Institute of Forage, Plevin, Bulgaria, for their allelopathic ability against selected weeds through root exudation and to collect and characterize the allelopathic compounds in root exudates. Another aim of the study was to carry out more allelopathic studies on selected cultivars against weeds and the nitrification process.

METHODS AND MATERIALS

Collection and Biological Activities of Root Exudates. Five seeds from each *Sorghum bicolor* cultivar were soaked for 24 hr and planted in 8-cm-diameter plastic pots each containing 260 g of sterilized washed quartz sand. The pots of this and all subsequent experiments were placed in a growth room under 14 hr photoperiod (1900 ft-c) at 26 ± 2°C/24 ± 2°C day–night conditions. The pots were watered with distilled water for the first four days, followed by half strength Hoagland's solution (Hoagland and Arnon, 1950) for the remaining days. The amounts of water or Hoagland's solution were adjusted to prevent any losses of root exudates from the bottom of the pots. Pots with sand only were treated the same way and used as controls.

After 10 days, the bottom of each pot was fitted to a glass container connected to gentle vacuum to facilitate collection of root exudates. Thirteen milliliters from each collection were added to Petri dishes (10 cm diameter) containing 60 g of sterilized sand and 25 seeds of *Amaranthus retroflexus*. Seed germination and radicle and hypocotyl lengths were measured 7 days after seeding. The experiment was arranged in a randomized complete-block design with five replications.

Characterization of Biologically Active Compounds. The idea of the sys-

tem used for collection and characterization of root exudates was based on that of Tang and Young (1982) with slight modification. Five seeds of *Sorghum bicolor* cv. 219, one of the most allelopathic cultivars found from the previous experiment, were planted in sterilized cylindrical funnels containing sterilized acid-washed sand and watered with 0.2 strength sterlized Hoagland's solution. Funnel controls without sorghum plants were treated similarly at the same time.

XAD_4 resin, purchased from Fluka, cleaned with methanol, was used to pack columns of 20 cm ID × 24 cm long. The residual methanol was removed by washing the columns with distilled water several times. Each column was fixed to the cylindrical pot to receive the dripping solution. Nutrient solution was dripped from the upper reservoir to the cylindrical pot and from the latter to XAD_4 resin which then was collected by a reservoir at the bottom of the system. The nutrient solution was recycled twice a day.

Each column was detached after four days, washed with distilled water several times, and eluted with 200 ml methanol. Eluates from 18 columns were pooled, evaporated to dryness under vacuum at 40°C, and made up to 50 ml distilled water. The water extract (pH 6.8) was extracted three times with 100 ml chloroform. The chloroform fractions were pooled and concentrated to 15 ml. The water fraction was acidified to pH 2.0 with 1 N HCl and extracted three times with chloroform. The basic fraction was obtained by adjusting the water fraction to pH 12.0 and extracted three times with chloroform. The chloroform extracts at pH 12 were concentrated in vacuum separately to 15 ml. Distilled water control was run similarly to find out if toxins of microbial origin appeared in the control funnels during the experiment. The XAD_4 resin of 18 columns was removed from the columns, extracted three times with 100 ml acetone, and concentrated to 15 ml.

The biological activities of neutral, acidic, basic, and acetone fractions were determined using the Petri dish technique. From each fraction, 0.8 ml was applied separately on Whatman No. 3 filter paper (5 cm diameter). The solvent was evaporated and the filter paper was placed in a 5.5-cm Petri dish. Each Petri dish was seeded with 25 seeds of *A. retroflexus* and watered with one ml of distilled water. Seed germination and radicle and hypocotyl lengths were recorded six days after seeding. The experiment was conducted in a randomized complete-block design with three replications.

Evaluation of Allelopathic Potential of Aqueous Extracts of Selected Sorghum bicolor *Cultivars.* Aqueous extracts of *Sorghum bicolor* cultivars 219, 260, 264, and 177 were prepared following the method of Rice (1972). Ten grams of fresh weight from each cultivar were boiled separately in 100 ml distilled water for 10 min, ground in a homogenizer for 5 min, suction filtered through filter paper (15–40 μm pore size) and purified by centrifuge at 15000 rpm for 10 min. The purified solution of each cultivar was made up to 100 ml with distilled water and adjusted to pH 6.8 with 1 N HCl. Solutions for germination and growth tests were made by diluting the purified solution to 1 : 1

(v/v extract–Hoagland's nutrient solution). Control solutions were made similarly except that the extract was replaced by distilled water.

The biological activities of the extracts were determined using sand culture technique. Twenty-five seeds of *Amaranthus retroflexus* were planted per pot containing 330 g of washed, sterilized sand. Immediately after planting, 70 ml of the extract solution was added to each of the five pots making a total of 20 test pots. Controls were made similarly by adding 70 ml of the control solution to each of the five pots. After two weeks, seed germination was recorded, and the plants were thinned to the three largest per pot. Twice a month, each pot received 15 ml of appropriate solution, otherwise, they were watered when necessary with equal amounts of 0.5 strength Hoagland's solution alternated with distilled water, the amounts of solution or distilled water were adjusted to prevent any leaching from the bottom of the pots. The experiment was arranged in a randomized complete-block design with five replications.

Thirty-five days after planting, seedlings were carefully removed from each pot, washed with tap water, separated into tops and roots, and compared on the basis of oven-dry weight.

Evaluation of Allelopathic Potential of Decaying Plants from Selected Sorghum bicolor *Cultivars*. Three grams of air-dried powder form each cultivar were mixed with 500 g of loamy soil and placed in plastic pots. Twenty-five seeds of *A. retroflexus* were planted in each pot. The control was run similarly except that cultivar powders were replaced by equal amounts of peat moss. All pots were watered when necessary with tap water.

Seed germination was recorded two weeks after planting, then the seedlings were thinned to the three largest per pot, allowed to grow for another two weeks and harvested. Oven-dried biomass of roots and tops was determined. The experiment was arranged in a randomized complete-block design with five replications.

Biological Activity of Selected Sorghum bicolor *Cultivars Against Nitrification*. For the aqueous extract bioassay, the nitrification process was evaluated using a soil incubation method. A sample of a rich garden loam soil was collected to a depth of 10 cm on March 10, 1984. The soil was mixed thoroughly, air dried, and passed through a seive with 2-mm openings. One hundred grams of soil were placed into a 100-ml plastic beaker, and each plastic beaker received 12 ml aqueous solution containing 0.0944 g of $(NH_4)_2SO_4$ to make the concentration of NH_4 nitrogen added 200 ppm for the control. Treatments were run similarly except distilled water was replaced by 10% plant extracts of sorghum cultivars 219, 260, 261, and 177. The plant extracts were prepared in the same manner as mentioned previously. All beakers were incubated in the dark at 28°C. All treatments were arranged in a randomized complete-block design with five replications.

The progress of nitrification was followed every two days by measuring

the amounts of NH_4^+-nitrogen and NO_3^--nitrogen using MgO–Devarda alloy methods (Bremner, 1965).

For the residue bioassay, 1 g air-dried powder from each of the test cultivars was added to a plastic beaker containing 100 g of garden soil. An equal amount of peat moss was added to the control pot to keep the organic matter the same. Each pot received 12 ml aqueous solution which contained 0.0944 $(NH_4)_2SO_4$ the make the NH_4-nitrogen concentration 200 ppm and incubated in a growth room at 28°C under a dark condition. The experiment was arranged in a randomized complete-block design with five replications. Measurements of NH_4-nitrogen and NO_3-nitrogen were taken every two days using the same methods as mentioned previously.

RESULTS

Collection and Biological Activities of Root Exudates. All of the 100 cultivars of *Sorghum bicolor* tested differed greatly in their ability to alter seed germination and/or seedling growth of *Amaranthus retroflexus*. Seed germination was reduced by approximately 70% of control in 11 cultivars and 82% of control in 25 cultivars, the other cultivars caused no significant reduction in seed germination. The growth range was 77–113% of control. Ten cultivars, some of them economically important (cv. 219, 260) inhibited growth of *A. retroflexus* by more than 79% of control (Table 1) and 25 cultivars by approximately 85% of control. The others either did not show significant inhibition or caused no significant stimulation to seedling growth. Most of the inhibitory effects occurred in radicle growth.

Characterization of Biologically Active Substances in Root Exudates. Seed germination was slightly inhibited by the neutral fraction and slightly stimulated

TABLE 1. ALLELOPATHIC EFFECT OF ROOT EXUDATES OF SELECTED *Sorghum bicolor* CULTIVARS ON SEED GERMINATION AND SEEDLING GROWTH OF *Amaranthus retroflexus*

Cultivar	Mean length (mm)[a]			Seed germination (% of control)
	Radicle	Hypocotyle	Seedling	
Control	15.8a	20.6b	36.4b	100
219	10.8c	18.8c	29.6c	70
260	11.9c	17.5c	29.4c	72
177	13.8b	25.1a	38.9a	104
264	14.4b	25.4a	39.8a	98

[a] Average of at least 20 seedlings. Means within column followed by the same letter are not significantly different according to Duncan's multiple-range test.

TABLE 2. INHIBITORY EFFECTS OF DIFFERENT FRACTIONS OF ROOT EXUDATES FROM *Sorghum bicolor* CV. 219 ON SEEDLING GROWTH OF *Amaranthus retroflexus*

Extract fraction	Radicle length (mm)			Hypocotyl length (mm)			Seed germination (% of funnel control)
	Distilled water	Funnel control	Root exudates	Distilled water	Funnel control	Root exudates	
Ethanol							
Acidic, pH 2.0	22.35	21.64	10.87[b]	7.54	6.64[a]	6.96	104
Neutral, pH 6.8	22.35	15.65[a]	8.29[b]	7.54	5.50[a]	4.47[b]	81
Basic, pH 12.0	22.35	22.36	22.32	7.54	6.71[a]	6.13	110
Acetone	22.10	19.43[a]	19.12	8.76	7.04[a]	5.60[b]	96

[a] Significantly less than water control at 0.05 level by Student's *t* test.
[b] Significance compared to pot control at 0.05 level by Student's *t* test.

by the basic one. There was virtually no effect on germination by acidic and aceton fractions (Table 2).

All fractions, except the neutral, caused drastic inhibition to radicle growth of *A. retroflexus*. The hypocotyl length was significantly reduced by neutral and acetone fractions only. Both neutral and acetone fractions of the funnel control revealed inhibitory effects against radicle and hypocotyl growth when compared to the distilled-water control. Inhibition could be attributed to the toxins of microbial origin in the control funnels.

Bioassay of Aqueous Extracts of Selected Sorghum bicolor *Cultivars against* Amaranthus retroflexus. Aqueous extracts of all cultivars drastically reduced root, shoot, and seedling biomass of *A. retroflexus* (Table 3). However, cultivars 219 and 260 caused the maximum inhibition among all aqueous extracts of the test cultivars. No significant differences in growth were found between cultivars 219 and 260 and between cultivars 177 and 264.

Seed germination was reduced by the aqueous extracts of all test cultivars; however, the reduction is more in the aqueous extracts of cultivars 219 and 260 than in the aqueous extracts of the others.

Biological Activities of Decaying Residues from Selected Sorghum bicolor, *Cultivars against* A. retroflexus. Decaying materials of all test *Sorghum* cultivars significantly reduced growth of roots, tops, and seedlings of *A. retroflexus* (Table 4). Cultivar 219 exhibited greater inhibitory action among the test cultivars followed by the cultivar 260.

Seed germination was significantly reduced by decaying material of all test cultivars; however, no significant differences in germination percentage were recorded between the cultivars.

Biological Activity of Selected Sorghum bicolor *Cultivars against Nitrification.* In the aqueous extracts bioassay, aqueous extracts of all test cultivars significantly inhibited the nitrification process (Figure 1). The aqueous extract

TABLE 3. COMPARISON OF ALLELOPATHIC POTENTIAL OF AQUEOUS EXTRACTS OF SELECTED CULTIVARS OF *Sorghum bicolor* AGAINST *Amaranthus retroflexus*

Cultivar	Dry weight (mg)[a]			Seed germination (% of control)
	Root	Shoot	Whole plant	
Control	34.3a	114.0a	148.3a	100
219	4.8c	15.4c	20.2c	71
260	3.8c	13.7c	17.5c	71
177	8.1c	26.3b	34.4b	90
264	6.5b	26.4b	32.9b	86

[a]Average of at least 20 seedlings, means within column followed by the same letter are not significantly different at 0.05 level according to Duncan's multiple-range test.

TABLE 4. COMPARISON OF ALLELOPATHIC POTENTIAL OF DECAYING MATERIALS OF SELECTED CULTIVARS OF *Sorghum bicolor* AGAINST *Amaranthus retroflexus*.

Cultivars	Oven-dry weight (mg)[a]			Seed germination (% of control)
	Root	Shoot	All plant	
Control	52.5a	166.1a	218.6a	100
219	12.2c	79.2d	91.4d	40
260	19.5b	103.6c	133.1c	41
177	23.3b	134.6b	157.9b	45
264	20.9b	128.0b	148.9b	47

[a]Average of at least 20 seedlings, means within column followed by the same letter are not significantly different at 0.05 level according to Duncan's multiple-range test.

of cultivar 219 showed greater inhibitory action than those of the others. High correlation coefficients were recorded between the ammonia remaining and nitrate produced in all treatments.

In the residue bioassay, the presence of decaying materials of all test cultivars in soil significantly reduced the nitrification process (Figure 2). However, the inhibitory effects on nitrification were stronger in cultivars 260 and 219 than

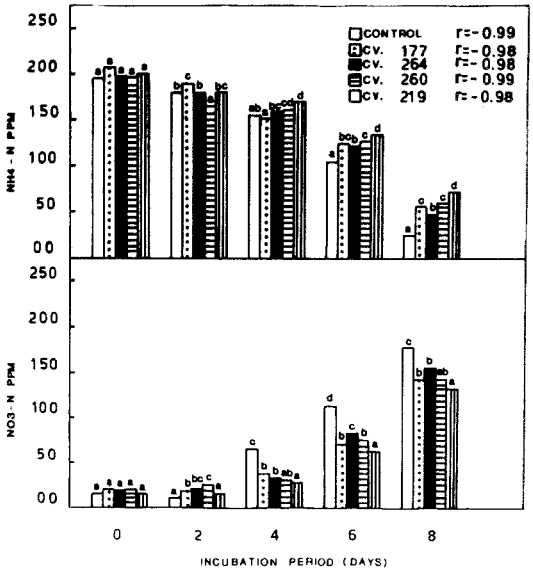

FIG. 1. Effect of aqueous extracts of selected cultivars of *Sorghum bicolor* on nitrification. Means within each sampling period sharing the same letter are not significantly different according to Duncan's multiple-range test.

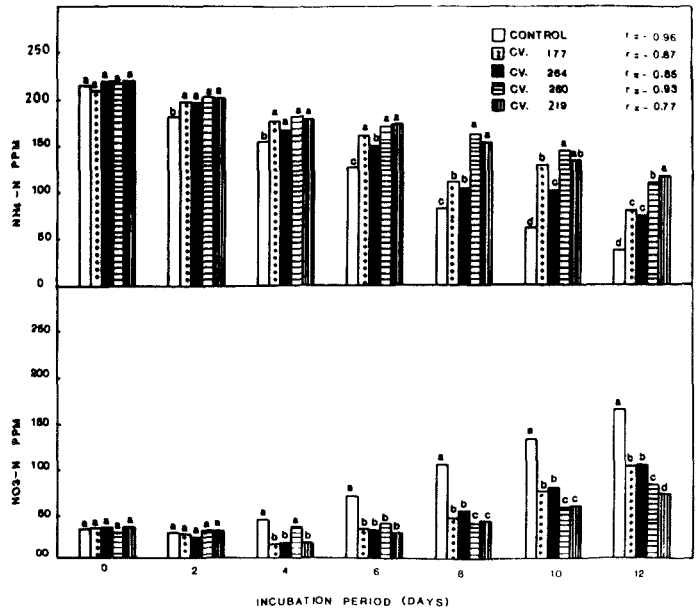

FIG. 2. Effect of decaying residues of selected cultivars of *Sorghum bicolor* on nitrification. Means within each sampling period sharing the same letter are not significantly different according to Duncan's multiple-range test.

in cultivars 177 and 264. Significant correlation coefficients between the ammonium remaining and nitrate produced were found in all treatments including the control.

DISCUSSION

The screening technique employed revealed considerable variations in the allelopathic potential of *Sorghum* cultivars. These variations could be attributed to the genetic differences between the test cultivars, since all cultivars received the same treatments during the experimental periods. Similar observations on the other crop varieties were also reported (Putnam and Duke, 1974; Fay and Duke, 1977). Moreover, the results showed that the neutral fraction of root exudates was more inhibitory than the other fractions. This result is particularly important since the pH used is similar to the pH of the soil in the fields. Tang and Young (1982) found that the neutral fraction of *Hemarthia altissima* has greater toxicity than the basic and acidic fractions.

The results also showed that the aqueous extracts of all test cultivars were inhibitory to seed germination and seedling growth of *A. retroflexus* and to the nitrification process. This suggests that the inhibition resulted from water-sol-

uble phytotoxins. This explanation was further substantiated by the *Sorghum* cultivar residue bioassay in which decaying material caused significant reduction in *A. retroflexus* growth and in the nitrification process. The results concerning the allelopathy of the test *Sorghum* cultivars against test plants agree with Guenzi et al. (1967), Abdul-Wahab and Rice (1967), Qureshi and Hussain (1980), and Lehle and Putnam (1982), who reported allelopathic effects of other *Sorghum bicolor* cultivars and other *Sorghum* species. However, the allelopathic effects of *Sorghum* against nitrification were not investigated elsewhere.

It is shown that selected cultivars, which were found to be nontoxic through root exudation, exhibited considerable toxicity through aqueous extracts and decaying materials. However, the degree of phytotoxicity is less than those which were found to be toxic through root exudation. Many reports indicated that allelopathic plant species can release phytotoxins by one or more sources of release (Rice, 1979; Horsley, 1977).

Our report indicates that some *Sorghum* cultivars reveal marked phytotoxicity, and these cultivars may prove useful in the control of some weeds and nitrification. It is premature to suggest an effective methodology; however, such developments could significantly reduce the requirements for commercial herbicides and nitrification inhibitors.

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EFFECTS OF THREE PHENOLIC ACIDS ON CHLOROPHYLL CONTENT AND IONS UPTAKE IN COWPEA SEEDLINGS

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Abstract—This study was conducted to test the hypothesis that interfering with chlorophyll metabolism and ion uptake may be mechanisms through which some phenolic acids inhibit the growth of cowpea seedlings. Three concentrations (10^{-4} M, 5×10^{-4} M, and 10^{-3} M) of each of syringic, caffeic, and protocatechuic acids were used to test their effects using sand-culture medium. It was found that seedling growth, chlorophyll a, total chlorophyll, chlorophyll a/b ratio, and the uptake of N, P, K, Fe, and Mo were significantly reduced by most of the test concentrations of the phenolic acids. However, chlorophyll b content and the Mg uptake were not significantly affected by all the phenolic acid concentrations. Calcium uptake was significantly inhibited by 5×10^{-4} M and 10^{-3} M of caffeic acid and 5×10^{-4} M of protocatechuic acid. In most cases, the reduction in dry weight was parallel to the reduction in chlorophyll content and ion uptake, and the reduction in chlorophyll was also parallel to the reduction in ion uptake. The relationships among the inhibition of dry weights, chlorophyll content, and ion uptake are briefly discussed.

Key Words—Syringic acid, caffeic acid, protocatechuic acid, chlorophyll content, ions.

INTRODUCTION

Inhibitory effects of various chemical compounds released from plants are well documented. Rice (1974) and, recently, Putnam (1983) categorized some of these compounds and determined their ecological role in manipulated and natural ecosystems. The mechanisms through which some of these compounds

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inhibit plant growth are also recognized. However, the efforts on this approach are still scarce and need further investigation (Einhellig and Rasmussen, 1979).

Allelopathic effects of *Sorghum bicolor* have been the subject of many investigators. Guenzi and McCalla (1966a,b) were able to isolate the phytotoxins ferulic, syringic, vanillic, and *o*-hydroxybenzoic acids from sorghum residues and soil from no-tillage sorghum plants. Leon (1976) demonstrated the autotoxicity of *Sorghum vulgare* in a monoculturing regime and isolated syringic, *o*-hydroxybenzoic, *m*-hydroxybenzoic, vanillic, protocatechuic, *p*-coumaric, and caffeic acids from sorghum roots. Putnam (1983) reported that sorghum residues provided significant weed-killing capacity under field conditions. In our laboratory, we screened 100 cultivars of *Sorghum bicolor* for their allelopathic potentiality and found that some of these cultivars exhibited significant inhibitory effects against *Amaranthus retroflexus* and the nitrification process (AlSaadawi *et al.*, 1986). Although the mechanisms of inhibition of some phenolic acids isolated from *Sorghum bicolor* are known, the mechanisms of some other phenolic acids such as syringic, caffeic, and protocatechuic acids were not investigated. These acids were reported to have considerable allelopathic activity and to be widely distributed in allelopathic plant species (Rice, 1974).

The aim of the present investigation, therefore, is to test the effect of the above-mentioned phenolic acids on chlorophyll content and ion uptake in plants. These two parameters are known to limit the total plant growth (Epstein, 1976; Colton and Einhellig, 1980; Einhellig and Rasmussen, 1979).

METHODS AND MATERIALS

Plant Material and Growing Conditions. Cowpea, (*Vigna sinensis* L.) was used as a test species to study the effects of phytotoxins on chlorophyll content and ion uptake. Five seeds were planted in each of 50 pots containing 650 g sterilized, washed sand. Five days after planting, the seedlings were thinned to the largest seedling per pot. The pots were divided into 10 groups with five pots each.

Three concentrations (10^{-4} M, 5×10^{-4} M, and 10^{-3} M) from each phytotoxin in Hoagland's solution (Hoagland and Arnon, 1950) were prepared making a total of nine treatments. The solution of each treatment was adjusted to pH 5.8 with N HCl and autoclaved for 15 min.

Immediately after thinning, 120 ml of a test solution was added to each pot of the appropriate group. An equal amount of sterilized Hoagland's solution at pH 5.8 was added to each pot of the control group. Two weeks later, the pots were watered again with 120 ml of appropriate solution. Between treatments and after the second treatment, the pots were watered with an equal amount of distilled water alternated with an equal amount of Hoagland's solution whenever necessary. The amounts of distilled water or nutrient solution

were adjusted to prevent any leaching from the bottom of the pots. The experiment was arranged in a randomized complete-block design and conducted in a growth room at 14 hr photoperiod (1500 ft-c) and 26 ± 2°C/24 ± 2°C day–night conditions.

Determination of Chlorophyll Content. Thirty-five days after planting, the plants were harvested and their roots washed with dilute HCl followed by deionized water. Immediately after harvesting, the first trifoliate leaf of each plant was taken for chlorophyll extraction, while the rest of the plant was oven dried for 24 hr at 105°C. The extraction procedure of chlorophyll was basically that of Knudson et al. (1977). Two grams fresh weight from the first trifoliate leaf of each plant were soaked in 30 ml of 95% ethanol for 24 hr. The ethanol–chlorophyll solution of each treatment was decanted in a container and kept at room temperature in dark conditions. The leaves were soaked again in a similar manner for two additional 24-hr periods in 30 ml of 95% ethanol. The chlorophyll–ethanol solutions of each plant were pooled, and the absorbance (A) was measured at 665 nm and 649 nm on a CE 292 Digital ultraviolet spectrophotometer. The quantities of chlorophyll a and b were measured using the following equations, respectively (Wintermans and Demots, 1965; Knudson et al., 1977).

$$\frac{\mu\text{g chloroplyll a}}{\text{ml solution}} = [(13.70)\ (\text{A } 665\text{ nm})] - [(5.76)\ (\text{A } 649\text{ nm})]$$

$$\frac{\mu\text{g chloroplyll b}}{\text{ml solution}} = [(25.80)\ (\text{A } 649\text{ nm})] - [(7.60)\ (\text{A } 665\text{ nm})]$$

Two grams of the first trifoliate leaf fresh weight were oven dried for 24 hr at 105°C and used to calculate micrograms chlorophyll per milligram dry weight.

Determination of Mineral Elements in Cowpea Plant. Oven-dried material of each plant was ground in an electrical grinder for plant analysis. Total nitrogen was determined by the macro-Kjeldahl method (Chapman and Pratt, 1961) and total phosphorus by the vanadomolybdophosphoric colorimetric method (Jackson, 1958). Calcium, Mg, K, Fe, and Mo were determined on a Perkin-Elmer model 305 atomic absorption spectrophotometer after digestion according to instructions in the analytical manual supplied with the instrument (Anonymous, 1976).

RESULTS

Effect of Phytotoxins on Growth and Chlorophyll Content. All test concentrations of all phenolic compounds, except 1×10^{-4} M syringic acid, significantly inhibited seedling growth of cowpea compared with the control (Table 1). Chlorophyll a, chlorophyll a/b ratio, and total chlorophyll were significantly

TABLE 1. EFFECTS OF SYRINGIC, CAFFEIC, AND PROTOCATECHUIC ACIDS ON SEEDLING GROWTH AND CHLOROPHYLL CONTENT OF COWPEA LEAVES ($N = 5$)

Treatment	Concentration (M)	Plant dry weight (g)	Chlorophyll (μg/mg dry weight)		Ratio (a/b)	Total chlorophyll (a + b)
			Chlorophyll a	Chlorophyll b		
Control		1.481	13.05	7.67	1.71	20.72
Syringic acid	1×10^{-4}	1.517	9.90*	8.43	1.17*	18.33
	5×10^{-4}	1.410*[a]	8.33*	8.60	0.95*	16.93*
	1×10^{-3}	1.357*	6.67*	7.57	0.88*	14.24*
Caffeic acid	1×10^{-4}	1.249*	12.85	8.43	1.52	21.28
	5×10^{-4}	1.278*	11.10*	7.79	1.42*	18.89
	1×10^{-3}	1.071*	10.20*	8.45	1.20*	18.65*
Protocatechuic acid	1×10^{-4}	1.347*	9.69*	7.96	1.21*	17.65*
	5×10^{-4}	1.303*	8.66*	7.86	1.10*	16.52*
	1×10^{-3}	1.223*	8.07*	7.33	1.10*	15.40*

[a] Values marked with asterisk are different from control at 0.05 level according to Student's t test.

reduced by all concentrations of all test phenolic acids except 1×10^{-4} M caffeic acid. However, chlorophyll b was not significantly affected by any of the concentrations of acids tested. Thus the significant reduction in the ratio of chlorophyll a to chlorophyll b was mainly due to the reduction of chlorophyll a.

Effect of Phenolic Acids on Mineral Uptake. The uptake of N was significantly reduced by all the test concentrations of phenolic acids except 1×10^{-4} M syringic acid (Table 2). All the test concentrations of phenolic acids significantly inhibited the uptake of P, Fe, and Mo in cowpea seedlings. Potassium uptake was significantly reduced by all the test concentrations of all phenolic acids except 1×10^{-4} M of syringic and protocatechuic acids. Concentrations of 5×10^{-4} M and 1×10^{-3} M caffeic acid and 1×10^{-4} M protocatechuic acid were the only concentrations that caused a significant reduction in Ca uptake. The uptake of Mg was not significantly affected by any of the concentrations of different phenolic acids.

DISCUSSION

The results revealed that growth is inhibited by all test compounds, and the degree of inhibition is correlated to the concentration of the test phenolic acids. Apparently, the concentration 5×10^{-4} M of syringic acid is the threshold level at which the growth of cowpea seedlings is significantly inhibited. However, the results could not show the threshold inhibitory level of the other phenolic acids since these acids revealed significant inhibition at the lower concentration tested (10^{-4} M).

The results also indicated that the reduction in growth is almost parallel to the reduction in chlorophyll a, total chlorophyll, ratio of chlorophyll a to chlorophyll b, and the uptake of N, P, K, Fe, and Mo. Since chlorophyll content and mineral uptake are known to limit the productivity of plants (Epstein, 1976; Buttery and Buzzell, 1977, Colton and Einhellig; 1980, Patterson, 1981), it is possible that the reduction in growth is a result due to the reduction in chlorophyll content and mineral uptake. Parks and Rice (1969) reported that several phenols caused significant reduction in chlorophyll content in blue-green algae and thus affect growth. Einhellig and Rasmussen (1978) demonstrated that the reduction in chlorophyll content is the allelopathic mechanism through which ferulic, *p*-coumaric, and vanillic acids affect soybean seedlings. Rice (1979) and Putnam (1983) reviewed the effect of several phenolic compounds on ion uptake by plants. Moreover, the results also suggest that the reduction in chlorophyll is due to the reduction in ion uptake since some of the ions tested are involved in the chlorophyll structure and/or in the metabolic pathway of chlorophyll biosynthesis.

It is noteworthy to mention that the inhibitory effects of the individual test

TABLE 2. EFFECT OF SYRINGIC, CAFFEIC, AND PROTOCATHECHUIC ACIDS ON NUTRIENT CONTENT OF COWPEA SEEDLINGS ($N = 5$)

Treatment	Concentration (M)	Nutrient content						
		Dry weight (mg/plant)					Dry weight (μg/plant)	
		N	P	K	Ca	Mg	Fe	Mo
Control		44.9	18.0	54.8	27.8	7.7	286.1	5.2
Syringic acid	1×10^{-4}	41.6	10.6*	54.3	25.4	6.7	200.2*	3.3*
	5×10^{-4}	39.2*[a]	10.2*	48.0*	24.3	7.8	190.8*	4.0*
	1×10^{-3}	38.5*	9.2*	44.9*	27.1	7.9	172.9*	2.4*
Caffeic acid	1×10^{-4}	36.2*	8.2*	50.9*	28.1	7.4	192.1*	3.2*
	5×10^{-4}	36.2*	9.8*	49.9*	20.8*	7.7	169.8*	2.9*
	1×10^{-3}	37.2*	7.8*	45.9*	21.6*	6.4	163.2*	2.9*
Protocatechuic acid	1×10^{-4}	38.6*	11.2*	52.6	26.9	7.8	167.8*	3.4*
	5×10^{-4}	39.7*	14.3*	50.6*	22.5*	7.7	173.8*	2.9*
	1×10^{-3}	38.1*	9.9*	49.3*	24.1	7.8	167.8*	3.0*

[a] Values marked with asterisk are different from control at 0.05 level according to Student's t test.

phenolic acids reported here are at low concentrations. The additive or synergistic inhibitory effects of such allelopathic compounds may become more detrimental than the effects of individual phenolic acids (Rasmussen and Einhellig, 1978; Einhellig and Rasmussen, 1978). Moreover, combined applications of these phenolic acids may cause significant inhibitory effects at a concentration lower than the minimum concentration used in this investigation.

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FEMALE SEX PHEROMONE OF THE MELONWORM, *Diaphania hyalinata* (LEPIDOPTERA: PYRALIDAE), AND ANALYSIS OF MALE RESPONSES TO PHEROMONE IN A FLIGHT TUNNEL[1]

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Abstract—Ten C_{16} chain-length compounds were identified from heptane extracts of ovipositors of female melonworm, *Diaphania hyalinata* (L.). The major constituents of the extracts were (*E*)-11-hexadecenal and (*E,E*)-10,12-hexadecadienal [(*E,E*)-10,12-16:Ald] and the alcohols and acetates of these olefins were found in trace amounts (<2%). Extracts also contained traces of (*E,Z*)- and (*Z,Z*)-10,12-16:Ald, hexadecanal, and 1-hexadecanol. Analysis of the behavioral responses of males to synthetic mixtures of these compounds and responses to ovipositor extracts in a flight tunnel showed that a synthetic mixture of the 10 compounds elicited a behavioral repertoire from males that was indistinguishable from that elicited by ovipositor extract. Flight tunnel studies also indicated that six of the 10 compounds probably represent the essential components of the female's sex pheromone.

Key Words—*Diaphania hyalinata, D. nitidalis*, melonworm, pickleworm, Lepidoptera, Pyralidae, sex pheromone, insect behavior, flight tunnel, 10,12-hexadecadienal, 11-hexadecenal.

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INTRODUCTION

Larvae of the melonworm, *Diaphania hyalinata* (L.), feed almost exclusively upon the foliage of pumpkin and other cucurbits, and it is rarely considered a pestiferous insect. The impetus for studying the sex pheromone of *D. hyalinata* was an interest in the comparative sex pheromone chemistry of *D. hyalinata* and *D. nitidalis* (Stoll). The two species occur sympatrically and exhibit similar life cycles and habits; however, *D. nitidalis* is considered a serious pest because it feeds upon and damages the fruits of cucurbits. The results reported here define the chemistry of pheromone-like compounds from the melonworm female and male behavioral responses to synthetic mixtures of the female-borne compounds. These findings were pivotal to elucidation of the *D. nitidalis* sex pheromone (Klun et al., 1986) because compounds discovered here aided in the detection of minute amounts of similar compounds in *D. nitidalis* and the combined findings provide a basis for comparative insight into the pheromone communication systems of the two species.

METHODS AND MATERIALS

Insects. Insects used in this study were reared on pinto bean diet (Robinson et al., 1979) at the Vegetable Insects Laboratory in Charleston, South Carolina. Pupae were shipped to Beltsville, Maryland, and pupae and adults of each sex were kept in separate enviornmental chambers that were maintained under a reverse photoperiod of 16:8 light–dark, 26°C light, 20°C dark; 60% relative humidity. Adults were provided a 10% sucrose solution.

The ovipositors (terminal abdominal segments containing the pheromone gland) of 2- to 3-day-old females at 5–6 hr of scotophase (the time of their sexual activity in nature: K.D. Elsey, personal communication) were excised and transferred to heptane (ca. 5 μl/ovipositor). The tissue was soaked for ca. 30 min, and then the solvent was drawn away from the tissue using a 50-μl syringe. The behavioral response of males to the ovipositor extracts signaled the presence of female sex pheromone, and these extracts served as a resource for chemical analyses.

Chemicals and Analytical Methods. The combined extracts of several hundred melonworm ovipositors were analyzed by open-tubular capillary chromatography (OTCC) using 60 m × 0.25 mm (ID) polar and nonpolar (SE-30) columns and by OTCC–mass spectrometry using instruments and operating conditions described by Klun et at.(1982). The ovipositor extracts were derivatized by successive treatment with $NaBH_4$ and acetic anhydride–pyridine (9:1) (Klun et at., 1982), fractionated by preparative gas–liquid chromatography (Schwarz et al., 1983), and the chromatographic fractions were ozonized and

the ozonolysis products analyzed by OTCC (Klun et al., 1982) to establish sites of unsaturation in the compounds.

The monounsaturated and saturated C_{16}-chain-length compounds used in this study were purchased. All 10,12-hexadecadienes were stereoselectively synthesized in our laboratory using the methods described below.

(Z,Z)-10,12-hexadecadienal. Synthesis of this compound was patterned after the preparation of (Z,Z)-9,11-hexadecadien-1-ol reported by Svirskaya et al. (1980). Thus, 10-undecyn-1-ol was coupled in a Cadiot-Chodkiewicz reation with 1-pentyne to produce 10,12-hexadecadiyn-1-ol. The latter compound was reduced stereospecifically to (Z,Z)-10,12-hexadecadien-1-ol with dicyclohexylborane. Oxidation of the alcohol with pyridinium chlorochromate (Corey and Suggs, 1975) afforded the desired (Z,Z)-10,12-hexadecadienal.

(E,Z)-10,12-Hexadecadienal. This compound was prepared by the reaction scheme reported for the same compound by Hall et al. (1980).

(E,E)-10,12-Hexadecadienal. Pure (E,E)-10,12-hexadecadien-1-ol was obtained by low-temperature recrystallization from pentane of the equilibrium mixture of all four isomers of this alcohol obtained after treatment of (E,Z)-10,12-hexadecadien-1-ol with 0.5% thiophenol at 100°C (Henrick et al., 1975). Pyridinium chlorochromate oxidation (Corey and Suggs, 1975) then afforded the desired aldehyde.

Each of the compounds used in bioassays was purified by argentation high-pressure liquid chromatography (Klun et al., 1982), and they were >95% pure based upon OTCC.

Flight Tunnel, Test Procedures, and Experimental Design. The flight tunnel was 3 m × 60 cm, horseshoe-shaped in cross-section, and made from Plexiglas. Two air conditioners, with a combined capacity of 42,000 BTU, provided the source of air that was conditioned in a chamber (12 m^3 provided with supplemental heating and humidification) at the head of the flight tunnel. The air was blown from the chamber by a fan, driven by a 0.75 HP variable-speed motor, through a Varicel filter and cheesecloth screen into the tunnel. the downwind end of the tunnel was fitted with an 8-mesh stainless-steel screen having a 7-cm-diam. circular hole at the center of the tunnel's cross-section 16 cm from the tunnel floor. The opening was fitted with a collar that held a Plexiglas tube (20 cm long × 6.5 cm diam.) that was used for release of individual males. The release tube was fitted with a spring-mounted lid on the upwind end and a cheesecloth cover on the other. Air flow from the tunnel was directed into a hood and exhausted to the outside of the building. Three sliding windows, located along the side of the tunnel, provided access to the interior of the tunnel across its length. Overhead illumination was provided by red incandescent bulbs that were rheostat-regulated to 2.5 lux. A laminar air flow was maintained through the tunnel at 50 cm/sec and 20.5 ± 1.5°C. The position of the stimulus-plume generated in the tunnel was determined by observing the path of

smoke generated from a burning incense stick. The observation indicated that the plume traversed the length of the tunnel in a narrow band and exited the tunnel through the male-release tube at the downwind end of the tunnel.

Males, 2–3 days old, were used in the bioassays after they had been conditioned for ca. 30 min in the flight-tunnel room. Males were used once and discarded. Tests were carried out at 5–6 hr of scotophase. Each male was allowed to settle on the cheesecloth at the downwind end of the release tube, and observations were commenced when the lid was removed from the tube.

Based on previous observations of male response to the extracts of female glands and virgin females in the tunnel, six behavioral categories were chosen for quantitative analysis. These were (1) Activation (a dichotomous variable), characterized by onset of wing fanning and rapid walking, each male was allowed a maximum of 2 min to express the activation response; (2) time to activation, represented elapsed time from the removal of the lid to start of wing fanning; (3) upwind flight in plume, a dichotomous variable, represented the percentage of insects that traversed the length of the tunnel and arrived at the stimulus source; (4) flight time, the elapsed time from start of flight to arrival at the source; (5) time near source, the time a male spent hovering near the source with extended claspers and making frequent antennal and tarsal contacts with the pheromonal stimulus; (6) return to source (dichotomous), represented those males that hovered at the source, flew away, reentered the plume, and returned to the source. A male was allowed a maximum of 2 min to return to the source. The test chemicals and ovipositor extracts were applied to a 4 × 0.5-cm filter paper strip that was suspended 16 cm above the tunnel floor from a narrow clip 20 cm from the upwind end of the tunnel. The quantity of ovipositor extracts applied to the paper was adjusted so that the quantity of female-derived compounds and the quantity of synthetic compounds applied to the paper were equivalent (according to OTCC).

The experimental design for the behavioral tests was a randomized complete-block design replicated 24 times over 24 days. All the variables involving time measurements in test I were analyzed by the nonparametric M-rank procedure because the data did not meet the assumptions for homogeneities of variances. The M-rank procedure is a generalization of the Friedman's test, which can be used for unbalanced data and a randomized complete-block design (Benard and Van Elteren, 1953). The computer program used was a supplemental procedure of the Statistical Analysis System statistic programs (Sarle, 1981). The dichotomous response variables were analyzed by the analysis of variance (Cochran, 1950). Significance was tested by χ^2 analysis, and the means were ranked using Duncan's multiple-range test.

In test II, three 2-day-old, virgin females or filter paper strips treated with synthetic pheromonal components were placed in a 5-cm × 3-cm-diam. stainless-steel screen cage that was suspended by the clip at the upwind end of the

TABLE 1. COMPOUNDS IDENTIFIED FROM OVIPOSITOR EXTRACTS OF FEMALE MELONWORM[a]

Compound number	Composition (%)	Compound (abbreviation)
1	50.4	(*E*)-11-Hexadecenal (*E*-11-16:Ald)
2	5.2	Hexadecanal (16:Ald)
3	0.3	(*E,Z*)-10,12-Hexadecadienal (*E,Z*-10,12-16:Ald)
4	0.5	(*Z,Z*)-10,12-Hexadecadienal (*Z,Z*-10,12-16:Ald)
5	39.1	(*E,E*)-10,12-Hexadecadienal (*E,E*-10,12-16:Ald)
6	1.8	(*E*)-11-Hexadecen-1-ol (*E*-11-16:OH)
7	0.4	1-Hexadecanol (16:OH)
8	2.0	(*E,E*)-10,12-Hexadecadien-1-ol (*E,E*-10,12-16:OH)
9	0.1	(*E*)-11-Hexadecen-1-ol acetate (*E*-11-16:OAc)
10	0.1	(*E,E*)-10,12-Hexadecadien-1-ol acetate (*E,E*-10,12-16:OAc)

[a] Percentage composition values are averages derived from analyses of the combined extracts of 62 females on polar and nonpolar capillary columns.

tunnel. Male responses to these treatments were compared by scoring male responses in the six behavioral categories. The amount of (*E*)-11-hexadecenal in each treatment of tests I and II was fixed at 252 ng, and the amounts of other compounds in the various treatments were proportional to the percentage compositions found in the female extracts (Table 1). The test was replicated 36 times over 36 days. Data were analyzed statistically using the same procedures described for test I.

Studies of the responses of feral melonworm males to pheromonal stimuli were attempted, but field populations of the insect were too low to allow acquisition of any meaningful data. Therefore, we had to rely on the flight-tunnel assay to assess the impact of the female compounds on male sexual behavior.

RESULTS AND DISCUSSION

Chemistry. Analyses of the ovipositor extracts of the females revealed the presence of 10 pheromone-like compounds (Table 1). The identities of these compounds were deduced from the coincidence of retention times with authentic reference compounds that were fully resolved on the capillary columns and by comparison of mass spectral fragmentation patterns with the reference compounds. Reduction of the ovipositor extract components with $NaBH_4$ followed by acetylation of the resulting alcohols yielded a mixture of five C_{16} acetates. These acetates had OTCC retention times coincident with (*E*)-11-16:OAc;

16:OAc; (*E,Z*)-, (*Z,Z*)-, and (*E,E*)-10,12–16:OAc, respectively. The (*E*)-11–16:OAc and (*E,E*)-10,12–16:OAc were major components of the mixture. This result was consistent with the analyses of the underivatized extract which showed that the corresponding C_{16} aldehydes were major components of the extract (Table 1). The mixture of acetates was fractionated by preparative chromatography; *E*-11–16:OAc and 16:OAc (fraction 1) and the 10,12–16:OAc isomers (fraction 2) were collected and ozonized. OTCC analysis of ozonized fraction 1 on polar and nonpolar columns showed the presence of two compounds: unreacted 16:OAc and 11-acetoxyundecanal. Ozonized fraction 2 yielded a single compound having OTCC retention times coincident with 10-acetoxydecanal on polar and nonpolar OTCC. These results confirmed that the site of unsaturation in the monoenes of the extract was 11 and that the site of conjugation in the dienes was 10,12.

Flight Tunnel Tests. Replicated observations (24/treatment) of responses of individual males to the ovipositor extracts in the flight tunnel (Table 2, treatment M) revealed the following: Males became active within 8 sec after exposure to the pheromone and subsequently took flight. Plume-oriented upwind flight was completed by 83% of the males tested. The average flight time from the release-tube to the stimulus source was 21.3 sec. During the upwind flight, males displayed a helical-like flight pattern as they repeatedly crossed the pheromone plume over 2/3 of the flight path. The last 1/3 of the flight was most often a straight-line dash in the pheromone plume to the stimulus source. After reaching the source, the males hovered with their claspers fully extended and made repeated antennal and tarsal contacts with the paper strip. Males also landed on the strip and made copulatory strikes (abdominal curving with claspers extended) at the paper strip. The average time spent by the males near the stimulus source was 33 sec, after which they flew away downwind and then began upwind flight as they casted widely across the width of the tunnel. Seventy-five percent of the males that exhibited this behavior reentered the plume and returned to the source.

Data in Table 2 (test I) show that treatment B, which lacked 16:Ald and 16:OH, was not significantly different from treatment A in any of the behavioral categories and elicited significantly better response in time to activation category compared to female extracts. Treatments C, D, E, G, and L (blank) were significantly less effective than female extracts in two or more of the behavioral categories of the test. These treatments lacked (*E*)-11–16:Ald (*compound* 1, Table 1), (*E,E*)-10,12–16:Ald (5), (*E,Z*)-10,12–16:Ald (3), and (*E,Z*)- and (*Z,Z*)-10,12–16:Ald (4), respectively. Results showed that (*E*)-11–16:Ald (1) and (*E,E*)-10,12–16:Ald (5) were absolutely essential for pheromonal activity; male responses to treatments (C and D) lacking either of these compounds were statistically equivalent to the blank treatment. Thus, these compounds are essential pheromonal components, but they must be sensed in context with other compounds to elicit optimal behavioral reactions from the males.

TABLE 2. RESPONSES OF ADULT MELONWORM MALES IN A FLIGHT TUNNEL TO PERMUTATIONS OF COMPOUNDS, EXTRACTS OF FEMALE, AND VIRGIN FEMALES

Treatment and compounds deleted from the mixture of ten[a]	Behavioral category [least-squares mean percentage response or time (sec)]					
	Act. (%)	Time to act.	Flt. upw. in plume (%)	Flt. time to source	Time at source	Return to source (%)
Test I (N = 24)[b]						
A. None	100 a	6 ab	96 a	22 ab	31 bc	70 a
B. 2,7	100 a	3 a	100 a	19 a	42 a	63 a
C. 1,2,7	33 b	45 d	0 e	—	—	—
D. 2,5,7	13 c	50 d	0 e	—	—	—
E. 2,3,7	88 a	13 cd	63 c	25 b	19 c	21 b
F. 2,4,7	88 a	10 bc	67 c	22 ab	32 ab	60 a
G. 2,3,4,7	88 a	7 ab	29 d	25 b	7 c	52 ab
H. 2,6,7	92 a	11 cd	71 bc	20 ab	34 ab	64 a
I. 2,7,8	100 a	3 a	96 a	19 a	49 a	79 a
J. 2,7,9	100 a	12 cd	67 c	24 ab	30 bc	74 a
K. 2,7,10	100 a	7 ab	88 ab	20 ab	37 ab	74 a
L. Blank	17 c	38 d	0 e	—	—	—
M. Female extracts	92 a	9 bc	83 abc	22 ab	36 ab	75 a
Test II (N = 36)						
B. 2,7	100	3 b	100	25 a	28 a	67 b
N. 2,7,8,10	100	2 a	100	23 a	45 a	83 a
M. Female extracts	100	2 a	100	23 a	40 a	89 a
O. 3 females	100	2 a	100	25 a	44 a	97 a

[a] Compounds, designated by number, are defined in Table I. Means followed by a common letter within each test in each column are not statistically different at 0.05% level of probability. Abbreviations: Act. = activation, flt. upw. = flight upwind.
[b] N = Number of males tested against each treatment.

(*E,Z*)-10,12-16:Ald (3) proved important to maintenance of male behavioral activity in the vicinity of the pheromonal stimulus; when it was deleted from the stimulus (E) males spent less time at the source and they returned to it less frequently (E vs. A, B, or M). Deletion of (*Z,Z*)-10,12-16:Ald (4) from the stimulus (F) had a detrimental effect on percent flight upwind category in comparison to A and B, but not M. When the *Z,Z* and *E,Z* isomers were simultaneously deleted from the stimulus (G), the expected negative effects attendant with deletion of either isomer were greater than anticipated. Response scores were impacted negatively in the percent flight upwind category as well as in the time at source category. This result indicates that the two dienes have an interactive effect on male behavior and that male perception of them involves a contextual relationship.

Although results of test I indicated that compounds 1, 3, 4, and 5, (Table 1) were behaviorally important components of the female's sex pheromone, a replicated ($N = 6$) test of the four-component mixture in the flight tunnel showed that it would not elicit upwind flight in the stimulus plume. Test I data showed that treatments containing compounds 1, 3, 4, and 5 plus at least three other compounds (6, 8, 9) always produced response scores that were either equivalent to or greater than those generated by female ovipositor extracts. Inspection of data for treatments H, I, J, and K and inference led us to the conclusion that a combination of compounds 1, 3, 4, 5, 6, and 9 represented the minimum set of compounds required for elicitation of a complete repertoire of male response in all behavioral categories of the assay. Test II (Table 2) confirmed the validity of this inference. The assay showed that the 1, 3, 4, 5, 6, and 9 combination of compounds elicited responses from males that were statistically equivalent to the responses generated by female extracts or virgin females. The mixtures of the eight olefins (treatment B) identified from the female extracts was slightly less effective than all other treatments in the time to activation and percent return to source categories. This result may reflect the limits of precision for the assay, since treatment B did not differ from female extracts in Test I.

Thus, the chemical data and the behavioral studies in the flight tunnel indicate that the melonworm female sex pheromone may comprise a complex set of six compounds:(*E*)-11-16:Ald, (*E,Z*)-10,12-16:Ald, (*Z,Z*)-10,12-16:Ald, (*E,E*)-10,12-16:Ald, (*E*)-11-16:OH, and (*E*)-11-16:OAc. The copious amounts of (*E,E*)-10,12-16:Ald found in the melonworm female alerted us to the possibility that a similar compound might be found congenerically, and this cognizance proved indispensable in identification of the pickleworm female sex pheromone. The four other compounds (2, 7, 8, and 10; Table 1) identified in the melonworm female extracts did not have any detectable behavioral significance in the assay; however, they appear to be related to the pheromonally active set in a biosynthetic sense.

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FEMALE SEX PHEROMONE OF THE PICKLEWORM, *Diaphania nitidalis* (LEPIDOPTERA: PYRALIDAE)

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Abstract—Heptane extracts of the ovipositors from pickleworm adults (*Diaphania nitidalis*) were found to contain (*E*)-11-hexadecenal along with proportionally smaller amounts of (*Z*)-11-hexadecenal, (*E*)- and (*Z*)-11-hexadecen-1-ol, hexadecanol, hexadecanal, and a trace amount of (*E,Z*)-10,12-hexadecadienal. Assays conducted in a flight tunnel and in the field showed that a synthetic mixture of the five unsaturated compounds elicited behavioral responses from pickleworm males that were indistinguishable from those elicited by extracts of the female or by mate-calling females. When any component was deleted from the set of five unsaturated compounds, the intensity and extent of male responses to the resulting mixtures were significantly attenuated. The female sex pheromone of the pickleworm resembles the pheromone of a congeneric species, *D. hyalinata*, but bioassays indicated that (*E,E*)-10,12-hexadecadienal, produced by *D. hyalinata* but not by the pickleworm, plays a role in pheromonal specificity.

Key Words—*Diaphania nitidalis, D. hyalinata*, pickleworm, melonworm, Lepidoptera, Pyralidae, (*E*)-11-hexadecenal, (*E,Z*)-10,12-hexadecadienal, (*E,E*)-10,12-hexadecadienal, sex pheromone, pheromonal specificity, flight-tunnel bioassays.

INTRODUCTION

The pickleworm [*Diaphania nitidalis*, (Stoll)] is distributed from Canada to South America and is particularly destructive to plantings of cantaloupe, cu-

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cumber, muskmelon, and squash in the southeastern and Gulf states of the United States. Larvae of this moth bore into the ripening fruits of these cucurbits and make them unsuitable for marketing (Elsey, 1981).

Pickleworm adults are nocturnal flyers, and Elsey (1982) indicated that mating in the species was mediated by a female sex pheromone. Monitoring the migrations and population fluctuations of this pest has proven difficult because the adults are not attracted to light traps. The need of a pickleworm attractant for monitoring purposes and our interest in the comparative female sex pheromone chemistry of the pickleworm and melonworm [*D. hyalinata* (Linnaeus)] constituted the impetus for this study.

METHODS AND MATERIALS

Insects used in the research were obtained from a laboratory culture (Robinson et al., 1979) that was maintained at Charleston, South Carolina. Pupae from the culture were mailed weekly to Beltsville, Maryland, and pupae and adults of each sex were held in separate environmental chambers at the conditions described by Raina et al. (1986). The ovipositors of 2- to 3-day-old pickleworm virgin females were exised and extracted with heptane at 3–4 hr of scotophase, a time when sexual activity is maximal in the species (Esley, 1982). The extracts were analyzed (Klun et al., 1982) by open tubular capillary chromatography (OTCC) using polar (Carbowax 20 M) and nonpolar (DB-1) columns and by combined OTCC–mass spectrometry (MS). All columns were 60 m × 0.025 mm (ID). The retention times of components in extracts of the pickleworm female were compared to authentic reference compounds. All synthetic compounds used in this study were purchased or prepared and purified as described by Raina et al. (1986). The percentage composition of pheromonal components in the ovipositor extracts was estimated by replicated OTCC analyses on Carbowax 20 M of extracts from individual females and by analysis of the pooled extracts from 87 females. Field tests and flight-tunnel studies utilized synthetic mixtures of the pheromonal compounds in proportions similar to those found in the extracts.

The ovipositor extracts of the females contained several alcohols in trace amounts and the mass spectrometric detection of these was enhanced by treating the extract from 20 ovipositors with a silylation reagent. The concentrated extracts of females (10 μl) were treated with 2 μl pyridine–heptane (1 : 9, v/v) and 1 μl Sylon BTZ (Supelco, Inc., Bellefonte, Pennsylvania 16823)–heptane (1 : 9, v/v). The reaction mixture was held at room temperature (ca. 22°C) for 3 hr, treated with 2 μ1 H_2O, and the heptane layer was analyzed by OTCC-MS. Other extracts of the female ovipositors wre reduced and acetylated by successive treatment with $NaBH_4$ and acetic anhydride (Klun et al., 1982). The acetate derivatives were fractionated by preparative gas chromatography (GC) on SE30

(Schwarz et al., 1983). The acetates were analyzed by OTCC, the fraction containing unsaturated acetates was ozonized, and the ozonolysis products were analyzed by OTCC to establish the site of unsaturation. Ovipositor extracts were also fractionated by preparative GC using the SE30 column and a polar column [10% DEGS (Supelco, Inc.) on 80–100 mesh Gas Chrom Q, 2 m × 4 mm, ID] operated at 160°C using the same instrumentation and gas flows described by Schwarz et al. (1983). The chromatographic fractions from the polar and nonpolar columns were bioassayed, individually and in various combinations, to determine which sets of components in the ovipositor extracts were responsible for pheromonal activity.

Laboratory assays and quantitative analyses of male behavioral responses to compounds identified from the pickleworm female were conducted in a flight tunnel. The repertoire of behavioral responses exhibited by the pickleworm males to calling virgin females or extracts of female ovipositors in the tunnel were identical to those displayed by melonworm males in response to its female sex pheromone. Therefore, the materials, methods, experimental design, and statistical methods related to the flight tunnel assay were similar to those described by Raina et al. (1986). Three tests were conducted in the flight tunnel and five to six behavioral categories were observed and scored (see Table 2). Test I was replicated 24 times, and it involved permutations of compounds that were identified from the females. Compounds were applied to a paper strip and positioned at the upwind end of the flight tunnel. (*E*)-11-Hexadecenal (750 ng) was applied to the paper strip, and the amounts of all other compounds applied to the strip in each treatment were proportional to the percentage composition of each compound found in the pooled extracts of 87 females (Table 1). The amount of (*E*)-11-hexadecenal in each treatment was equal to the amount of (*E*)-11-hexadecenal in the female extracts according to OTCC analyses of the female extracts. Tests II and III were replicated 12 times, and they involved a study of pickleworm male responses to pickleworm synthetic female sex pheromone to which 580-, 58-, 5.8-, and 0.58-ng doses of(*E*,*E*)-10,12-hexadecadienal were added. The (*E*,*E*)-aldehyde is a pheromonal component of the melonworm female sex pheromone (Raina et al., 1986) that is not produced by the pickleworm female. Data from test III were analyzed statistically by the chi-square method; the test III data were amenable to this statistical analysis whereas test I required the M-rank procedure because of the large number of treatments in it and the variability attendant to treatments that elicited no response from males in certain behavioral categories.

Field tests using insect traps were conducted in the vicinity of Charleston, South Carolina, during August and September 1983. The insect trap was a cubical wood-framed (1 m/side) screened (6 × 16 mesh) cage that was open at the bottom. The cage opening was horizontally supported 30 cm from the ground by legs at each corner of the cage bottom. A cylindrical screened cage 8 × 3 cm, OD) containing two to five virgin females (2-day-old, reared and main-

TABLE 1. COMPOUNDS IDENTIFIED FROM PICKLEWORM FEMALE AND PERCENTAGE COMPOSITION OF OVIPOSITOR EXTRACTS

Compound number	Chemical	Extract composition (%)	
		5 individual	87 pooled
1	(*E*)-11-Hexadecenal	71.8 (67–77)[a]	75.0
2	(*Z*)-11-Hexadecenal	2.6 (2–3)	3.3
3	Hexadecanal	20.2 (17–24)	17.5
4	(*E,Z*)-10,12-Hexadecadienal	0.6 (0.4–0.7)	0.2
5	(*E*)-11-Hexadecen-1-ol	1.4 (1–2)	2.1
6	(*Z*)-11-Hexadecen-1-ol	2.6 (1–6)	0.9
7	Hexadecan-1-ol	0.9 (0.4–2.0)	0.8

[a]Mean and range of values. The quantity of pheromone per female was not determined.

tained in environmental chambers that were programmed in synchrony with the diurnal cycle of the field) or a rubber septum (Arthur H. Thomas, Co., Swedesboro, New Jersey, Cat. No. 8753-D22) that had been treated with 10 μl of a heptane solution containing test chemicals was positioned at the top of a wooden stake in the plane and at the center of the trap opening. Synthetic lures and females were renewed after three nights. The cubical, screened trap, baited with virgin females, proved effective in trapping pickleworm males in preliminary studies while sticky traps (Pherocon 1C, etc.) proved to be ineffective against this species (unpublished results).

Three field tests were conducted by using baited traps in a randomized complete block design with replication over nights. Traps were positioned in fields of cantaloupe with at least 30 m between traps. Test I (12 replicates) involved two virgin pickleworm females and permutations of pickleworm-female compounds that were tested in flight-tunnel assays; however, the dose of (*E*)-11-hexadecenal (75 μg) and proportional amounts of other pheromonal components (a total load of 100 μg) on the rubber septa was 100 times greater than the amount used in the flight tunnel. Test II (32 replicates) evaluated the response of males to traps baited with 1-, 0.1-, and 0.01-mg doses of the mixture of seven compounds identified from the pickleworm female (treatment A) on rubber septa versus their response to traps baited with five virgin pickleworm females. Test III (12 replicates) assayed the response of males in the field to 1 mg (A) and a 0.818-mg mixture of only the five unsaturated compounds identified from the females.

RESULTS AND DISCUSSION

Sex Pheromone Chemistry. OTCC analysis of the pickleworm ovipositor extracts initially indicated the presence of six compounds. These had retention

times, on the polar and nonpolar capillary columns, that were coincident with standard samples of (*E*)-11-hexadecenal [(*E*)-11-16:Ald], (*Z*)-11-16:Ald, hexadecanal (16:Ald), (*E*)-11-hexadecen-1-ol [(*E*)-11-16:OH)], (*Z*)-11-16:OH, and 1-hexadecanol (16:OH). Extracts that were reduced with $NaBH_4$ and then acetylated with acetic anhydride yielded three acetates that had OTCC retention times coincident with hexadecan-1-ol acetate and (*E*)- and (*Z*)-11-hexadecen-1-ol acetate. The unsaturated acetates were isolated by preparative GC and ozonized. Ozonolysis yielded a single compound having an OTCC retention time that was coincident with 11-acetoxyundecanal. This result fixed the olefinic site in the unsaturated compounds at the 11 position. Direct OTCC-MS analysis of the extracts and analysis of silanized ovipositor extracts provided confirming evidence for identity of each of the six compounds. Bioassay of a synthetic mixture of the six compounds, using proportions of compounds (Table 1) and in the amounts found in the female extracts, showed that the mixture would elicit wing fanning and rapid walking behavior from males within the release-tube of the flight tunnel, but males did not fly to the stimulus source or exhibit other behavioral responses that were elicited by extracts of the female. This result clearly indicated that the six compounds we had identified did not represent an accurate synthetic replica of the female's sex pheromone.

When the ovipositor extracts were fractionated by preparative GC on SE30, pheromonal activity was recovered only when the hexadecenal and hexadecenol chromatographic regions were collected and combined. Preparative GC fractionation of the extracts on DEGS showed that pheromonal activity could be regenerated when chromatographic regions corresponding to hexadecenal, hexadecenol, and a region preceding hexadecenol were collected and combined. A combination of the hexadecenal and hexadecenol regions alone from DEGS did not regenerate biological activity. These chromatography–bioassay results indicated that a substance responsible for biological activity in the female extracts was not a hexadecenol, although it eluted after hexadecanal and coeluted in the hexadecenol fraction on the SE30-preparative GC.

Inspection of chromatograms obtained by OTCC of the extracts on the DB-1 (a liquid phase that is chromatographically equivalent to SE30) revealed the presence of a minute component that eluted between hexadecanal and hexadecenol. The mass spectrum of this trace component was obtained by injection of the concentrated extracts from ca. 60 female ovipositors into the OTCC-MS system. Its mass spectrum was identical to the 10, 12-hexadecadienal that had previously been identified from the melonworm (Raina et al., 1986). OTCC of a synthetic mixture of the four isomers of the diene (fully resolved on both columns) showed that the trace amount of diene in the female extracts had chromatographic properties identical to (*E*,*Z*)-10,12-hexadecadienal [(*E*,*Z*)-10,12-16:Ald]. Replicated OTCC analyses of the extracts of individual female ovipositors showed that the compound comprised 0.4–0.7% of the pheromonal components in the extract and 0.2% of the pooled extracts from 87 females (Table 1). A synthetic mixture of the seven compounds was prepared with a

percentage composition identical to the pooled female extracts shown in Table 1. Preliminary assays indicated that the biological potency of the seven-component mixture was equivalent to the extracts of the pickleworm female. This result represented biological proof that the minute amount of diene in the extracts was (*E*,*Z*)-10,12–16:Ald, and it set the stage for an in-depth quantitative analysis of the behavioral responses of males to various sets of the pickleworm compounds.

Flight-Tunnel Assays. Test I results obtained with permutation of the seven pickleworm compounds (Table 2) showed that only two treatments, A and B, elicited behavioral responses from males that were indistinguishable from the responses elicited by female extracts in all behavioral categories of the assay. Treatment A represented all compounds identified from the female extracts and 16:Ald and 16:OH were omitted in B. The assay results indicated that the saturated compounds did not have any detectable communicative function in the flight-tunnel behavioral assay. Male responses to treatments E, F, and H were not statistically different from those elicited by female extracts in five behavioral categories. However, the percentages of male moths that successfully flew upwind to these treatments, lacking (*E*)-11–16:OH and/or (*Z*)-11–16:OH, were significantly smaller than those recorded for the female extracts or treatment B. Therefore, the assay results indicated that the geometrical isomers of 11–16:OH were important to maintenance of male upwind flight in the pheromone plume. Test results showed that upwind flight in the plume was even more dependent upon male perception of a trace amount (ca. 3%) of (*Z*)-11–16:Ald in combination with the four other unsaturated compounds; only 29% of the males tested flew upwind in a plume lacking (*Z*)11–16:Ald (treatment D), and the flight time to this stimulus was significantly greater than the flight time to the female extracts or the five-component mixture of unsaturated compounds (treatment B). In addition, none of the males returned to treatment D after making initial contact with it. The flight-tunnel assay results also showed that (*E*)-11–16:Ald and (*E*,*Z*)-10,12–16:Ald were each essential to elicitation of the activation behavior and upwind flight in the plume; treatments C and G, each lacking (*E*)-11–16:Ald or the trace component (*E*,*Z*)-10,12–16:Ald, were significantly different from female extracts or the five-component mixture in nearly every behavioral category of the assay.

Overall, the flight tunnel assay showed that males must sense a complete set of the five unsaturated compounds listed in Table 1 in order to exhibit a repertoire of behavioral reactions comparable to the repertoire elicited by extracts of the female. These five compounds ostensibly comprised the essence of the female's sex pheromone.

Test II (Table 2) showed that when (*E*,*E*)-10,12–16:Ald was added to a synthetic mixture of pickleworm compounds (treatment A), in a proportion equivalent to that found in the melonworm (1.3:1, (*E*)-11–16:Ald–(*E*,*E*)-10,12–16:Ald) (Raina et al., 1986), the resulting mixture was ineffective in

TABLE 2. RESPONSES OF ADULT PICKLEWORM MALES IN FLIGHT TUNNEL TO PERMUTATIONS OF COMPOUNDS, EXTRACTS OF FEMALE, AND MELONWORM PHEROMONAL COMPONENT ADDED TO PICKLEWORM PHEROMONE

Treatment[a]	Behavioral category [least squares mean percentage response or time (sec)]					
	Act. (%)	Time to act.	Flt. upw. in plume (%)	Flt. time to source	Time at source	Return to source (%)
Test I (N = 24)[b]						
A. 1,2,3,4,5,6,7	100 a	7 b	96 a	25 ab	61 a	69 a
B. 1,2,4,5,6	100 a	12 b	92 a	23 b	34 b	66 a
C. 2,4,5,6	46 cd	19 ab	0 d	—	—	—
D. 1,4,5,6	96 a	13 ab	29 c	36 a	37 ab	0 b
E. 1,2,4,6	92 a	8 b	58 b	22 b	50 ab	88 a
F. 1,2,4,5	92 a	12 b	54 b	24 ab	48 ab	83 a
G. 1,2,5,6	63 bc	19 ab	0 d	—	—	—
H. 1,2,4	88 ab	5 b	67 b	26 ab	36 b	62 a
Blank	29 d	54 a	0 d	—	—	—
Female extracts	100 a	4 b	92 a	22 b	45 ab	93 a
Test II (N = 12)						
A.	100	2	100	23	34	N.M.
A. + 580 ng (*E*,*E*)-10,12-16:Ald	25	24	0	—	—	"
Female extracts	100	1	100	19	52	"
Test III (N = 12)						
A.	100 a	12 a	83 a	22 a	32 a	"
A. + 58 ng (*E*,*E*)-10,12-16:Ald	67 b	22 a	0 b	—	—	"
A. + 5.8 ng (*E*,*E*)-10,12-16:Ald	92 ab	11 a	42 a	19 a	5 b	"
A. + 0.58 ng (*E*,*E*)-10,12-16:Ald	100 a	4 a	75 a	19 a	38 a	"

[a] Compounds, designated by number, are defined in Table 1. Means within each test in each column that are followed by the same letter are not statistically different. Abbreviations: Act. = activation, flt. upw. = flight upwind, N.M. = not measured.

[b] N = number of males tested against each treatment.

elicitation of behavioral responses from pickleworm males in all of the behavioral categories of the assay. Test III showed that the (*E*,*E*)-10,12-16:Ald suppressed male pickleworm responses even when the proportional quantity of the compound was reduced by two orders of magnitude from the 1.3:1 proportion found in the melonworm. These results indicate that (*E*,*E*)-10,12-16:Ald had sufficiently detrimental impact on the response of pickleworm males to its female's sex pheromone to identify it as a key substance in the specificity of pheromonal signals for the two *Diaphania* spp.

Field Studies. The results obtained in the field with permutations of the seven pickleworm compounds (Table 3, test I) were similar to the results obtained in the flight-tunnel assay inasmuch as treatments A and B proved to be as effective as the virgin females in luring males into the field traps, and all other treatments except treatment E were significantly less effective than the females. In contradiction of the flight-tunnel assay, the field-trapping results indicated that treatment E was as effective as the virgin females in causing

TABLE 3. RATE OF MALE PICKLEWORM CAPTURES IN FIELD TRAPS BAITED WITH VIRGIN FEMALES, PERMUTATIONS OF COMPOUNDS, AND DOSES OF MIXTURES OF COMPOUNDS IDENTIFIED FROM FEMALE PICKLEWORM

Treatment[a]	$\bar{X}$ Males/trap/night
Test I (N = 12)	
A. 1,2,3,4,5,6,7	1.3 abc
B. 1,2,4,5,6	1.3 abc
C. 2,3,5,6	0 e
D. 1,4,5,6	0.8 bcde
E. 1,2,4,6	1.4 ab
F. 1,2,4,5	0.3 cde
G. 1,2,5,6	0.2 e
H. 1,2,4	1.1 bcd
Blank	0 e
2 Virgin	2.2 a
Test II (N = 12)	
1 mg A	4.5 a
0.1 mg A	1.2 b
0.01 mg A	0.2 b
5 Virgin	4.1 a
Test III (N = 12)	
1 mg A	1.8 a
0.818 mg B	2.5 a
5 Virgin	1.8 a

[a] Compounds, designated by number, are defined in Table 1. Means with a test in each column that are followed by the same letter are not significantly different according to Duncan's new multiple-range test (P = 0.05).

capture of pickleworm males. This result was somewhat perplexing because tests in the wind tunnel showed that deletion of (*E*)-11-16:OH from the mixture of five unsaturated compounds (treatment E) was detrimental to sustained upwind flight in the pheromone plume. Despite this instance of inconsistency, indications are that the five unsaturated compounds represent the minimum set of compounds required for release of a full repertoire of sexual behavior from the males. It is apparent that the flight-tunnel assay had significant utility in ethological analysis of the roles of compounds in the complex pheromonal stimulus of the pickleworm, and assay results obtained in the flight tunnel were largely in agreement with the field trial results.

Although field test I showed that 0.1 mg treatment A/septum was equivalent to the efficacy of two females in bringing males into traps, test II (Table 3) indicated that this dose or a 0.01-mg dose of A was not equivalent to five females in a trap, but a 1-mg dose of treatment A was as effective as the five females. Test III reaffirmed the comparative efficacy of 1 mg A to five virgin females and demonstrated that 0.818-mg mixture of treatment B (a mixture of the five unsaturated pickleworm compounds) was equivalently effective as a lure source when compared to either treatment A or five virgin pickleworm females. We therefore conclude: a set of five compounds, (*E*)-11-16:Ald (91.6%), (*Z*)-11-16:Ald (4.0%), (*E,Z*)-10,12-16:Ald (0.7%), (*E*)-11-16:OH (2.6%), and (*Z*)-11-16:OH (1.0%), constitutes the female sex pheromone of the pickleworm.

Table 4 shows that four of the five pheromonal components produced by the pickleworm are also components of the melonworm female sex pheromone. The chemical theme expressed by females of both species involves C_{16} chain-length compounds with sites of unsaturation at 11 and 10,12 positions. The geometry of Δ-11 double bond in the melonworm is exclusively *E*, while the pickleworm female produces both isomers. On the other hand, the pickleworm female produces only a trace amount of the *E,Z*-conjugated diene aldehyde while the melonworm produces three of the four possible geometrical isomers of the aldehyde and the *E,E* isomer of the C_{16} alcohol and acetate. Assays in the flight tunnel showed that (*E,E*)-10,12-16:Ald is an essential component of the melonworm pheromone (Raina et al., 1986); however, when it was added to the pickleworm pheromone, the response of pickleworm males was inhibited. These results provide evidence that the *E,E* aldehyde serves as a key substance in the specificity of pheromonal signals between the two species.

Table 4 also shows that both species produce 16:Ald and 16:OH; however, these compounds did not have any detectable behavior-eliciting roles for either species. Similarly for the melonworm, no behavior-eliciting roles were observed for (*E,E*)-10,12 alcohol or acetate.

Heretofore, pheromones having a 10,12-olefinic site have never been reported in the Pyralidae, and it is interesting to note that, among all the moth pheromones that have been identified, the 10,12-diene moiety is rare. Such

TABLE 4. COMPARATIVE FEMALE SEX PHEROMONE CHEMISTRY FOR PICKLEWORM AND MELONWORM MOTHS

Compound	Ovipositor extracts composition (%)[a]	
	Pickleworm	Melonworm[b]
E-11-16:Ald	75.0*	50.4*
Z-11-16:Ald	3.3*	—
16:Ald	17.5	5.2
E,Z-10,12-16:Ald	0.2*	0.3*
Z,Z-10,12-16:Ald	—	0.5*
E,E-10,12-16:Ald	—	39.1*
E-11-16:OH	2.1*	1.8*
Z-11-16:OH	0.9*	—
16:OH	0.8	0.4
E,E-10,12-16:OH	—	2.0
E-11-16:OAc	—	0.1*
E,E-10,12-16:OAc	—	0.1

[a] Asterisks identify compounds that are essential to pheromonal activity as determined by behavioral assays.
[b] From Raina et al. (1986).

unsaturation has been previously detected in only four species: *Bombyx mori* (Kasang et al., 1978; Kaissling et al., 1978), *Manduca sexta* (Starratt et al., 1979), *Earias insulana* (Hall et al., 1980), and *Amorbia cuneana* (McDonough et al., 1982). It seems, therefore, that the biosynthetic processes responsible for generation of 10,12-unsaturation may be narrowly restricted to comparatively few species in the order.

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SYNTHESIS OF PUNGENIN, A FOLIAR CONSTITUENT OF SOME SPRUCE SPECIES, AND INVESTIGATION OF ITS EFFICACY AS A FEEDING DETERRENT FOR SPRUCE BUDWORM [*Choristoneura fumiferana* (CLEM.)]

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Abstract—Pungenin was synthesized from 3,4-dihydroxyacetophenone by a short sequence involving manipulation of protecting groups on the 3 and 4 hydroxyl functions. Bioassays indicated that the glucoside is a modest feeding deterrent for sixth-instar spruce budworm larvae, but it does not appear to retard the development of small larvae or lead to increased mortality.

Key Words—*Picea pungens*, *Picea glauca*, feeding deterrent, spruce budworm, *Choristoneura fumiferana* (Clem.), Lepidoptera, Tortricidae, pungenin, protection of OH groups, glucoside, phenolic glucoside.

INTRODUCTION

Almost 30 years ago, during an investigation of the water-soluble constituents of foliage of Colorado spruce, *Picea pungens* Engelm., Neish (1957, 1958) isolated the glucoside pungenin, in yields up to 5% of the dry weight depending on the age of the foliage. Pungenin, which was also isolated from white spruce (*P. glauca* (Moench) Voss) was characterized as 3-(β-D-glucopyranosyloxy)-4-hydroxyacetophenone (I, Scheme 1) by Neish (1957, 1958) who, in the course of the structure elucidation, synthesized its 4-monomethyl, as well as its pentamethyl ether, but not the natural product itself. The same compound, designated pungenoside was later isolated from *P. pungens*, in an independent investigation by Takahashi et al. (1960).

In a pioneering study on chemical aspects of feeding by the spruce budworm, *Choristoneura fumiferana* (Clem.), on white spruce, Heron (1965) de-

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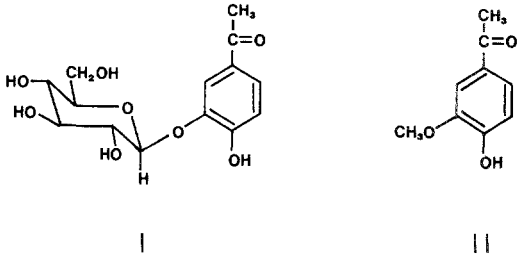

I II

SCHEME 1.

scribed laboratory assays which indicated that pungenin behaves as a feeding deterrent for the spruce budworm. In feeding tests where fifth-instar larvae were offered a choice between disks of Japanese elder pith impregnated with a solution of the phagostimulant sucrose and identical disks also treated with the glucoside, he reported that "pungenin had a significant deterrent effect on response to 0.02 M and 0.05 M sucrose at concentrations of 0.8 and 1%, respectively." (To estimate the amount of glucoside actually present in Heron's diet disks, the concentrations of the pungenin solutions used must be multiplied by a factor reflecting the uptake of solution by the pith disks.) Noting that the new vegetative shoots of spruce, virtually devoid of pungenin, are highly acceptable to budworm larvae, while mature needles containing (in winter) up to 5% of the glucoside are not, Heron suggested that the observed feeding preference might be due, in part, to the deterrent effect of pungenin.

There appears to have been little subsequent research on pungenin, although the suggestion that it may be an important feeding deterrent for spruce budworm is still debated by entomologists (Mattson et al., 1983).

This paper reports the synthesis of pungenin, and the results of some bioassays designed to shed more light on its role in the budworm–white spruce interaction.

METHODS AND MATERIALS

Melting points were determined on a hot-stage apparatus and are uncorrected. Infrared spectra were recorded on a Beckman Acculab 2 spectrophotometer. Nuclear magnetic resonance spectra were run on Varian T-60 or XL-200 instruments. Optical rotations were measured using a Perkin Elmer 241 polarimeter. High-resolution and chemical-ionization mass spectra were provided by Dr. A. Hogg, University of Alberta. Microanalyses were conducted by Spang Microanalytical Laboratory, Eagle Harbor, Michigan.

3,4-Dihydroxyacetophenone

This compound [mp 116–120°, lit (Weast, 1976) 115–116°] was prepared from commercially available acetovanillone, (II), using the procedure of Lange (1962).

3-Benzoxy-4-hydroxyacetophenone

Procedure 1. A solution of 3,4-dihydroxyacetophenone (316 mg, 2.08 mmol) in pyridine (20 ml) was cooled in an ice bath, and benzoyl chloride (0.27 ml, 327 mg, 2.33 mmol) was added slowly with stirring during 5 min. The ice bath was removed, and the mixture was set aside with stirring at ambient temperature in an atmosphere of dry nitrogen for 1 hr. After addition of 4-dimethylaminopyridine (22 mg), the mixture was heated under reflux for 40 hr, cooled, and evaporated to dryness on a rotary vacuum evaporator. The residue was subjected to flash chromatography (Still et al., 1978) on a column of silica gel with chloroform–ethyl acetate (87:13) as eluent, which yielded 3-benzoxy-4-hydroxyacetophenone (378 mg, 71%), as well as the 3,4-dibenzoate (51 mg). The crystalline monobenzoate thus obtained was sufficiently pure to be used directly in the next step. A sample, recrystallized from toluene–chloroform melted at 143–156°, IR (KBr) (inter alia) 3320 (br), 1738, 1660, 1610, and 1590 cm^{-1}; NMR [60 MHz, $(CD_3)_2CO$] δ: 2.52 (3H,s), 7.00–8.27 (8H, m); mass spectrum, *m/z* 256 (M^+), 105 (base peak) ($PhCO^+$). Anal. calcd. for $C_{15}H_{12}O_4$: C 70.30, H 4.72; found C 70.11, H 4.72.

Procedure 2. Benzoyl chloride (0.78 ml, 944 mg, 6.72 mmol) was added slowly to a stirred ice-cold solution of 3,4-dihydroxyacetophenone (442 mg, 2.91 mmol) in dry pyridine (15 ml). The mixture was allowed to attain ambient temperature and was then set aside with stirring under nitrogen for 24 hr. After conventional work-up and flash chromatography on silica gel with carbon tetrachloride–ethyl acetate (92:8) as eluent, crystalline 3,4-dibenzoxyacetophenone was obtained in 96% yield. A sample, recrystallized from carbon tetrachloride had mp 118–121°, IR (KBr) (inter alia) 1750, 1735, 1687, and 1605 cm^{-1}, NMR (60 MHz, $CDCl_3$) δ: 2.61 (3H, s), 7.15–8.15 (13 H, m), mass spectrum, *m/z* 360 (M^+), 105 (base peak) ($PhCO^+$). A solution of 3,4-dihydroxyacetophenone (76 mg, 0.5 mmol) and its dibenzoate (180 mg, 0.5 mmol) in dry pyridine (12.5 ml) containing 4-dimethylaminopyridine (5 mg) was heated at reflux in a nitrogen atmosphere for 18 hr. The mixture was evaporated to dryness in vacuo, and the residue was subjected to flash chromatography on silica gel with chloroform–ethyl acetate as eluent, affording crystalline 3-benzoxy-4-hydroxyacetophenone (197 mg, 77%) and unreacted 3,4-dibenzoate (31 mg).

3-Hydroxy-4-(2-methoxyethoxy)methoxyacetophenone

To a solution of 3-benzoxy-4-hydroxyacetophenone (378 mg, 1.48 mmol) in dry methylene chloride (4 ml) containing diisopropylethylamine (0.39 ml, 289 mg, 2.24 mmol) was added slowly 2-methoxyethoxymethyl chloride (0.25 ml, 273 mg, 2.19 mmol). The reaction mixture was stirred under nitrogen at ambient temperature for 15 hr. Chloroform and brine were added and, after separation of the organic layer, the aqueous phase was extracted with chloroform. The extracts were dried and evaporated in vacuo to give crude 3-benzoxy-4-(2-methoxyethoxy)methoxy-acetophenone, sufficiently pure to be used directly for the next step.

The product, in dry tetrahydrofuran (30 ml), was stirred at ambient temperature with sodium methoxide (280 mg, 5.18 mmol) in a nitrogen atmosphere for 30 min. An aqueous solution of KH_2PO_4 was added until the pH was close to 7, and most of the tetrahydrofuran was then removed on a rotary vacuum evaporator. After addition of more water, the mixture was extracted with chloroform and the extracts were dried and evaporated to dryness in vacuo. The residue was purified by preparative layer chromatography on silica gel with ether as eluent to yield 327 mg (92%) of 3-hydroxy-4-MEM-acetophenone as a colorless oil, IR ($CHCl_3$) (inter alia) 3550, 1678, 1612, and 1590 cm^{-1}, NMR (60 MHz, $CDCl_3$) δ: 2.54 (3H, s), 3.40 (~3H, s) 3.43–3.97 (~4H, m), 5.34 (2H, s), 6.60 (1H, br s), 7.02–7.53 (3H, m), mass spectrum, *m/z* 240 (M^+).

3-(β-tetraacetylglucopyranosyloxy)-4-MEM-acetophenone

A mixture of 3-hydroxy-4-MEM-acetophenone (327 mg, 1.36 mmol), acetobromo-α-D-glucose (1.12 g, 2.72 mmol), and silver oxide (736 mg, 3.18 mmol) in dry quinoline (3.7 ml) was set aside with stirring at ambient temperature in a nitrogen atmosphere for 6 hr. The mixture was diluted with ethyl acetate and filtered through sintered glass. The filtrate was washed with 2 M HCl (3× 15 ml), water (1× 10 ml), saturated $NaHCO_3$ (2× 15 ml), dried, and evaporated in vacuo. The residue, still containing some quinoline, was subjected to flash chromatography on silica gel. The eluent was ether–hexane (4:1), ether (100%), and ether–acetone (95:5) in a stepwise gradient. The MEM-tetraacetylglucoside (770 mg) was obtained as a colorless oil, sufficiently pure to be used directly in the next step, IR ($CHCl_3$) 1756, 1748 (infl), and 1681 cm^{-1}, NMR (60 MHz), $CDCl_3$) δ: 2.05–2.08 (12 H, m) 2.54 (3H, s), 3.37 (3H, s), 3.43–4.30 (7H, m), 4.88–5.42 (6H, m), 7.08–7.78 (3H, m), mass spectrum, (CI, NH_3) m/z 588 $(M+NH_4)^+$.

3-(β-Tetraacetylglucopyranosyloxy)-4-hydroxyacetophenone

A mixture of the 4-MEM-3-tetraacetylglucoside (770 mg, 1.35 mmol) and zinc bromide (1.52 g, 6.75 mmol) in dry methylene chloride (3.0 ml) was stirred at ambient temperature under nitrogen for 24 hr. The mixture was poured into

saturated sodium bicarbonate solution (20 ml) and ethyl acetate (20 ml). The mixture was filtered through sintered glass, the aqueous phase was separated, and the organic layer was washed twice with saturated sodium bicarbonate solution and twice with brine. The ethyl acetate solution was dried and the solvent was removed under reduced pressure. The residue was subjected to preparative layer chromatography on silica gel with ether as eluent, which afforded the 4-hydroxy-3-tetraacetyl glucoside (tetraacetyl pungenin) (495 mg, 76%) as an oil, which gave crystals from ether–cyclohexane, mp 106–112°, IR ($CHCl_3$) 3525, 1755, 1675, and 1605 cm^{-1}, NMR (200 MHz, $CDCl_3$) δ: 2.06 (3H, s, $-OCOCH_3$), 2.07 (3H, s, $-OCOCH_3$), 2.12 (3H, s, $-OCOCH_3$), 2.14 (3H, s, $-OCOCH_3$), 2.55 (3H, s, $ArCOCH_3$), 3.89–3.98 (1H, 8 line m, C-5′H), 4.17–4.34 (2H, 8 line m, C-6′H_2), 5.04–5.40 (4H, m, H on C-1′, C-2′, C-3′, C-4′) 6.42 (1H, s, OH), 7.00 (1H, d, J = 8.9 Hz, C-5H), 7.66 (1H, dd, J = 2.0 Hz, 8.9 Hz, C-6H), 7.65 (1H, d, J = 1.9 Hz, C-2H), mass spectrum (CI, NH_3) m/z 500 $(M+NH_4)^+$, mass spectrum (EI) m/z 331.1027 (13%) ($C_{14}H_{19}O_9$) [cleavage at C-1′/O(C-3)], 169.0499 (base peak) ($C_8H_9O_4$), 109.0293 (96% ($C_6H_5O_2$).

Pungenin

Dry ammonia was bubbled into an ice-cold solution of the 4-hydroxy-3-tetraacetylglucoside (248 mg, 0.514 mmol) in dry methanol (10 ml) for 40 min. The solution was allowed to attain ambient temperature and was set aside with stirring for 15 hr, after which it was evaporated to dryness in vacuo. The residue crystallized from water to give pungenin (72%), first crop (65 mg) mp 190–195°, second crop (51 mg) mp 188–195° [lit. Neish (1957) 198–199°, Takahashi et al. (1960) 190–191°], mp undepressed on admixture with a sample isolated from *P. glauca* according to Neish (1957), $[\alpha]_D$-88.9° (c 0.53, water) [lit. Neish (1957) $[\alpha]_D^{25}$ − 96.8 (c 0.53 water), Takahashi et al. (1960) $[\alpha]_D^{30}$ − 85.47 (c 1.17 water)], IR (KBr) 3300 (br), 2930, 2885, 1664, 1590, 1521, 1425, 1360, 1295, 1257, 1212, 1193, 1123, 1108, 1093, 1053, 1042, and 1022 cm^{-1}, NMR [200 MHz, $(CD_3)_2CO$] δ: 2.48 (3H, s, $-COCH_3$) 2.83 H_2O ?), 3.52–3.50 (4H, m) (masked on addition of D_2O), 3.96–3.66 (4H, m) (modified on D exchange), 4.31 (1H, d, J = 3.8 Hz) (exch), 4.42 (1H, m) (exch), 4.86 (1H, d, J = 7.3 Hz, anomeric H), 5.14 (1H, br) (exch), 6.94 (1H, d, J = 8.4 Hz), 7.66 (1H, dd, J = 8.4 Hz, 2.1 Hz), 7.81 (1H, d, J = 2.1 Hz), mass spectrum (CI, NH_3) m/z 332 $(M+NH_4)^+$, mass spectrum (EI) m/z 314.0960 (0.44%) $C_{14}H_{18}O_8$, M^+), 152.0477 (100%) ($C_8H_8O_3$) 137.0242 (57%) ($C_7H_5O_3$). Anal. calcd. for $C_{14}H_{18}O_8$: C 53.50, H 5.77; found C 53.46, H 5.76.

Bioassays

Sixth Instar: Paper. Spruce budworm larvae were reared on modified McMorran diet (McMorran, 1965; Grisdale 1970), from the second instar (beginning of feeding) to the end of the fifth instar.

The bioassay procedure of Bentley et al. (1982, 1984) was followed with a few modifications. Assay disks were 1.3 cm diameter analytical paper circles (mean weight 44 mg) (Schleicher and Schuell) treated with the appropriate solution(s) of pungenin and/or phagostimulant. Pungenin was tested at levels of 1, 2, 4.3 and 5% (based on dry weight of disk plus applied phagostimulant), using 10 larvae, five of each sex, for each concentration and for the corresponding controls.

Differences in frass counts between larvae of the same sex on test and control disks were not significant at the 5% level of probability. (In this experiment male larvae produced significantly more frass pellets than females, reflecting different ages within the sixth instar.)

Sixth Instar: Diet. The meridic diet used for the major bioassay was a modification of the standard McMorran formula (McMorran, 1965), with nutrient quantities adjusted so that the weight of diet ingested would approximate more closely the weight of foliage normally consumed by spruce budworm larvae (cf. Miller, 1977). Thus, the caseine, sucrose, and salt mixture were reduced to 25% of their levels in the standard diet and the Alphacel content was increased 13-fold. The hot diet was poured into Petri dishes and allowed to cool. Disks of the diet (2 cm diam.) were cut out with a cork borer and were freeze-dried and weighed. In this manner, porous diet disks of suitable weight ($\sim$250 mg) could readily be prepared. About 1 ml of a freshly prepared aqueous solution of natural pungenin and potassium sorbate (fungicide) was added slowly to each test disk to give disks containing 4.76–4.78% pungenin and 0.19% sorbate (both dry weight), and 79% water content. Control disks received only the water and potassium sorbate.

Newly moulted, unfed, sixth-instar spruce budworm larvae were placed into separate 35 $\times$ 10-mm Petri dishes, each containing a test or control disk. The experiment involved 15 male and 15 female larvae on the test diet, and 15 larvae of each sex on the control food, all maintained at 22°C, with a 17-hr photoperiod. After the moult, pupae were removed and placed in separate vials until emergence of adults, which were killed 24 hr later.

Residual diet, frass produced, and moths were oven-dried at 60°C, and weights of diet consumed, frass produced, and adult moths were used as indices of budworm performance.

Second to Sixth Instars: Diet. Powdered lyophilized McMorran-Grisdale diet (Grisdale, 1970) (850 mg) was treated with a solution containing natural pungenin (45 mg) and potassium sorbate (1.8 mg) in distilled water (3.4 ml). Vigorous mixing produced a paste containing pungenin (5%) (dry weight) and sorbate (0.2%) which was transferred in approximately 100 mg portions to 4 ml vials. Control diet lacked the pungenin.

Newly emerged (from hibernacula) second instar larvae were placed in separate vials (32 test, 40 controls) and reared at 25°C, with a 17-hr photoper-

iod. Because the amount of pungenin available was insufficient to prepare enough diet for complete development of larvae to pupae, the experiment was terminated when the larvae moulted to the sixth instar.

RESULTS AND DISCUSSION

The aglucone of pungenin, 3,4-dihydroxyacetophenone, can be prepared in some 70% yield from commercially available acetovanillone, (II) by aluminum chloride-catalyzed cleavage of the methyl ether (Lange, 1962). It was anticipated that the greater acidity conferred on the C-4 hydroxyl group by the *para* acetyl function would enable the phenolic hydroxyl groups of 3,4-dihydroxyacetophenone to be differentiated readily. In fact, although attempts at direct protection of the C-4 hydroxyl group by a variety of procedures led predominantly to the desired products, the degree of selectivity of the process left much to be desired. This problem was overcome by the development of an indirect procedure, which allowed the 4-MEM ether of 3,4-dihydroxyacetophenone to be prepared selectively in satisfactory yield.

The ease with which acyl migration can occur in catechol monoesters (Fischer et al., 1918), and the stability of the 4-acylphenolate anion suggested that base-catalyzed acylation of 3,4-dihydroxyacetophenone under equilibrating conditions should result in derivatization of the 3-hydroxyl group in a highly selective manner. This expectation was largely realized on treatment of the diol with an equivalent of benzoyl chloride in pyridine containing a catalytic amount of dimethylaminopyridine (Steglich and Höfle, 1969). The reaction mixture, heated under reflux for 40 hr, afforded a 71% yield of 3-benzoxy-4-hydroxyacetophenone. The dibenzoate was recovered as a minor biproduct ($\sim$7%).

An alternative procedure, which gave a slightly improved yield (overall 74%), consisted in refluxing 3,4-dihydroxyacetophenone with one equivalent of its dibenzoate for 18 hr in the pyridine–dimethylaminopyridine system.

Derivatization of the C-4 phenolic hydroxyl group by means of 2-methoxyethoxymethyl chloride (Corey et al., 1976), followed by methoxide-catalyzed cleavage of the benzoate, afforded the desired 4-MEM ether of 3,4-dihydroxyacetophenone in 68% overall yield from the dihydroxy precursor.

The location of the MEM protecting group was confirmed by remethylation of the C-3 hydroxyl function with methyl iodide-potassium carbonate and zinc bromide-catalyzed cleavage of the MEM ether (Corey et al., 1976), which afforded acetovanillone identical with the starting material.

Glycosidation of the free 3-hydroxyl group was effected in excellent yield with acetobromoglucose and silver oxide in dry quinoline (Kubinyi et al., 1973). The MEM protecting group was removed by zinc bromide treatment (76%) and, finally pungenin was generated by treatment of the tetraacetyl glucoside with methanolic ammonia solution. The overall yield of pungenin from 3,4-dihydroxyacetophenone was 36.7%.

In preliminary bioassays using the method of Bentley et al. (1982, 1984), we were unable to observe significant reduction of feeding by sixth-instar spruce budworm larvae as a result of treating paper diet disks with synthetic pungenin at levels up to 5% (calculated on dry weight of paper test disk plus phagostimulant). The apparent disparity between these findings and Heron's observations undoubtedly stems from differences in the bioassay conditions used (Schoonhoven, 1982).

In a more sensitive test, sixth-instar larvae were offered a meridic diet (cf. McMorran, 1965) containing natural pungenin at a concentration of 4.76%. While in the observed sample the performance of male larvae on test and control diets, as reflected in weights of diet consumed, frass produced, and adult moths, did not differ significantly ($P > 0.05$; Student's t test) female larvae, which ate more than the males, were affected more noticeably, and the corresponding weights were some 30% lower for test insects than for controls (Table 1).

Linear relationships, with similar slopes (1.20–1.29) and intercepts, were observed on plotting dry weight of diet consumed against dry weight of frass produced for all larvae studied (test, control, male, female). The approximate digestibilities (AD) (Waldbauer, 1968) were also similar (Table 1). Both of these characteristics indicate that pungenin did not affect food digestion. Digested food is the difference between the amount of food ingested and the amount of frass egested. The ratio of food digested to moth weight gives the amount of digested food required to produce unit weight of moth. Figures for the males, 3.83 (controls) and 3.90 (test), were not significantly different. The difference for the females, 3.21 (controls) and 4.09 (test), was significant and indicates that pungenin caused conversion of the digested food to be less efficient for these larvae. The presence of pungenin in the diet did not cause any increase in mortality, and other than the reduction in size of the test females, both test and control insects appeared to develop normally to the adult stage. It is likely

TABLE 1.

Larvae	N	Diet consumed (mg, mean ± SE)	Frass produced (mg, mean ± SE)	A.D[a]	Moth weight (mg mean ± SE)
♂ ♂ Control	15	146 ± 7.4a[b]	111 ± 5.7b	23.8	9.2 ± 0.6c
♂ ♂ Test	11[c]	133 ± 8.6a	102 ± 6.9b	23.3	8.2 ± 0.7c
♀ ♀ Control	14[d]	243 ± 7.2	184 ± 5.8	24.3	18.5 ± 0.9
♀ ♀ Test	12[c]	177 ± 16.5	131 ± 12.6	26.1	12.4 ± 1.7

[a] AD = approximate digestibility (16) = 100 [(diet consumed − frass produced)/diet consumed].
[b] Within-sex means with same letter in a column are not significantly different ($P > 0.05$); Student's t test.
[c] Missing samples discarded because of fungal contamination.
[d] One pupa died ($N = 14$).

that female fecundity, which correlates with moth size, would have been affected, but this was not investigated.

To examine the effects of pungenin on smaller larvae, insects were reared from second to sixth instar on a diet containing 5% of the glucoside. Because the amount of pungenin available was insufficient to prepare enough diet for complete development of larvae to pupae, the experiment was terminated when the larvae moulted to the sixth instar.

The first moult to sixth-instar occurred on day 9 of the experiment. By day 21, 15 of the control larvae (37.5%) and 11 of the test larvae (34.4%) had died. Of the 25 control larvae alive on day 21, 15 (60%) had moulted to sixth instar, and 19 (90%) of the 21 living test insects had reached the same stage. Although the mortality in the control was higher than expected, we have found no evidence that pungenin (5%) caused increased mortality or retarded development up to the beginning of the sixth instar.

Heron's bioassays, based on choice situations, led to the suggestion that the presence of pungenin may be a contributing factor in the preference of large budworm larvae for new vegetative shoots over mature needles (Heron, 1965), and the present results are not at variance with this hypothesis. It is clear, however, that the compound does not prevent mature needles from being mined by second-instar larvae in the spring.

The present work demonstrates that spruce budworm larvae are capable of developing successfully on a diet containing pungenin at concentrations close to the highest natural levels reported, but that the resulting female insects are smaller than normal.

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CHEMICAL ATTRACTANTS TESTED AGAINST THE AUSTRALIAN BUSH FLY *Musca vetustissima* (DIPTERA: MUSCIDAE)

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Abstract—A number of chemical compositions known to attract several synanthropic and pest fly species were tested against the Australian bush fly *Musca vetustissima*, a severe and persistent pest of man and domesticated and wild mammals. A standard composition containing small quantities of trimethylamine and indole, blended with large amounts of ammonium sulfate and anchovy meal, showed good attractancy against this fly. Deletion of ammonium sulfate did not significantly alter attractancy. Similarly, incorporation of *n*-butyric acid and linoleic acid into the formulation did not alter attractancy, nor did addition of several carbonates and bicarbonates. Skatole, a closely related compound to indole, decreased attractancy significantly. In all the tests, the attractant compositions lured greater number of females than males. The attractancy of the standard composition was studied and compared with equal amount of fresh cattle dung. Chemical attractants lured bush flies and not dung beetles, while dung attracted both flies and beetles. The dung resource was 4.6× more attractive to flies than the standard attractant composition. This suggests that there are other chemical attractants emanating from dung that warrant isolation and identification work. Nevertheless, the chemical attractants studied here provide a starting point for further investigations. The ratio of female to male flies attracted to humans was greater than the ratio of flies attracted to chemicals. The sex ratio of flies netted from human host was 3.0, while the sex ratio of flies attracted to chemical attractants was 1.8. The preponderance of females attracted to both sources was probably due to physiological requirements of the sexes.

Key Words—Bush fly, Australian bush fly, *Musca vetustissima*, Diptera, Muscidae, chemical attractants.

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INTRODUCTION

Early explorers in Australia noted the pestiferous nature of the Australian bush fly *Musca vetustissima* Walker, some three centuries ago (Cleland, 1913). One of the explorers, John Lort Stokes, in 1838 related that "the flies are at you all day, crawling into your eyes, up your nostrils and down your throat" (Hooper, 1982). The nature and scope of annoyance brought about by these flies remains the same today.

This pest is widely spread over most of the continent (Hughes et al., 1972) and is especially abundant in cattle-raising areas. They breed in dung, preferring freshly deposited dung of cattle (Hughes, 1981; Hughes et al., 1972; Norris, 1966). Adult bush flies are attracted to large animals, including man, females outnumbering the males attacking or landing on hosts (Hughes, 1977, 1981; Hughes et al., 1972; Norris, 1966). We have noted that the flies can find the host in a few seconds in the field and that flies at some distance orient and move upwind toward the hosts. Chemical odors emanating from hosts or breeding medium (dung) are some of the factors providing cues for directed response by the flies. However, no definitive studies on the role of chemical attractants influencing bush fly behavior have been accomplished on this important synanthropic and synzootic fly.

The hovering and feeding behavior of bush flies approaching and attacking hosts is quite similar to that of the tiny flies known as eye gnats (*Hippelates* species, family Chloropidae) prevailing in the Western hemisphere (Mulla and Stains, 1977). Chemical attractants inducing positive responses in female *H. collusor* (Townsend) were isolated and identified from putrified chicken eggs (Hwang and Mulla, 1973; Hwang et al., 1975, 1976) and field tested by Mulla and Axelrod (1974) and Mulla et al. (1974, 1976). Some of the principal chemicals attracting *H. collusor* were also found to manifest a high level of attractancy against the common house fly *Musca domestica* (Mulla et al., 1977), and the coastal lesser house fly *Fannia femoralis* on poultry ranches (Mulla et al., 1984).

It is well known that many species of synanthropic and synzootic flies are attracted by odors emanating from putrefying proteins. *Musca sorbens*, a closely related species to *M. vetustissima*, was found to be attracted to putrefying egg and fish meat (Legner et al., 1974), and baiting of an insular population with these baits reduced populations of *M. sorbens* markedly. *M. vetustissima* has been found to be attracted to liver baits with sodium sulfide (Hughes, 1977; Norris, 1966; Vogt et al., 1981). Most recently Vogt et al. (1985) have used a mixture of putrefying liver and dung with live blow fly maggots to stir the bait for the purpose of trapping bush flies.

In view of these findings and the extensive research on the isolation and identification of chemicals showing field attractancy to a variety of pest flies, the present studies were initiated. Compositions of chemical attractants with

proven attractancy to *Hippelates*, *Musca*, and *Fannia* species were evaluated against the Australian bush fly. A simple method for field evaluation of attractant compositions was developed, where attractancy of various chemical compositions and natural attractants emanating from fresh dung was tested against field populations of *M. vetustissima*.

METHODS AND MATERIALS

Compositions of chemical attractants found attractive to the eye gnat *Hippelates collusor* (Hwang et al., 1975, 1976; Mulla et al., 1976), *Musca domestica* (Mulla et al., 1977), and *Fannia femoralis* (Mulla et al., 1984) consisted of mixtures of a carrier (anchovy meal) with one or more of the following chemicals: trimethylamine (from trimethylamine hydrochloride), ammonia (from ammonium sulfate), indole, linoleic acid, and a few lower aliphatic acids. Against the bush fly, a composition containing anchovy meal carrier (57.25%), trimethylamine hydrochloride (2.5%), ammonium sulfate (40%), and indole (0.25%) by weight was used as a standard mix with which other compositions with fewer or more chemicals were compared for luring the flies. When additional chemicals were included or deleted, the content of carrier was varied in the composition accordingly.

The composition was mixed in 1:1 ratio with Improved Golden Malrin (IGM) (Zoecon Corporation, Dallas, Texas) toxic bait. The ingredients of this bait are: 1% methomyl, 0.025% (*Z*)-9-tricosene, and 98.97% inert ingredients. Fine sand (780 g) forming a 2-cm layer was placed in a plastic bowl (18 cm diam. and 8 cm deep) and wetted with 140 ml of tapwater, bringing the moisture content initially to its field capacity level. For testing, a 5-g quantity of each mix was placed on the damp sand. In some tests, especially in testing attractancy of dung, the IGM was not mixed with the attractants, but rather it was spread on the top and around the dung or other attractant compositions.

The bowls (replicated five to six times in each experiment) containing various test attractant compositions were placed on the ground 5 m apart in paddocks infested with bush flies. They were distributed in randomized-block design in a line at right angles to the wind direction or in a circular pattern. The tests were run in two areas near Perth, Western Australia. The Serpentine area, with extensive livestock pastures, is located in the coastal plain 100 km south of Perth, while Cunderdin, a predominantly wheat growing and pasturing area, is located on the Darling plateau 150 km east of Perth.

Flies attracted to the compositions oriented toward the attractant source and crawled or flew into the bowls, walking and milling around the attractant–toxicant compositions. Because of the action of the quick-acting toxicant methomyl, the flies on contact with the bait died in the bowl within a few seconds after arrival. All flies killed in the bowls were counted with no loss due to wind

or transportation into the laboratory. The compositions were exposed in the field for a period of 4–6 hr for each experiment, depending on the activity of bush flies. After termination of each experiment, the bowls were covered with lids and brought into the laboratory where the flies were identified, sexed, and counted under a dissecting microscope.

Analysis of data was carried out using standard computer program design for randomized-block analysis. The data were transformed to log $(n + 1)$ when fly numbers were high and variable. Transformed means were compared for significant differences using Duncans's multiple-range test, letter designations for denoting significant differences are shown on untransformed means for ease of interpretation. Where there were two treatments in a given test, the transformed data were analyzed by means of t test. Significant differences between or among means are expressed in the tables.

RESULTS AND DISCUSSION

Since the attractant composition utilized fish meal as carrier and Improved Golden Malrin (IGM) as a toxicant, it was necessary to test the attractancy of these two components. Fish meal and IGM (1:1) and IGM alone were compared in the first test. The fish meal showed a low level of attractancy which was significantly higher than that of IGM alone (Table 1). When the standard composition plus IGM was compared with fish meal plus IGM, the standard composition caught significantly higher numbers than the carrier (Table 1, test II). In a third test, this comparison was repeated, and again the standard attracted significantly higher numbers than the carrier (Table 1, test II-A). On the basis of these data, it was concluded that a composition containing three chemicals, carrier, and IGM was more attractive than the carrier plus IGM to field popu-

TABLE 1. ATTRACTION OF AUSTRALIAN BUSH FLY *Musca vetustissima* TO CHEMICAL ATTRACTANTS AND TOXICANT BAIT, IMPROVED GOLDEN MALRIN (IGM)

Experiment and treatment	Ingredients and % composition				Mean No. flies killed/unit[a]
	TMA · HCl	Indole	NH_4SO_4	Anchovy meal	
I A	0	0	0	100	5.6*[b]
B	0	0	0	0[c]	1.0
II A	0	0	0	100	45.5
B (std.)	2.5	0.25	40.00	57.25	78.4***
II-A A	0	0	0	100	18.4
B (std.)	2.5	0.25	40.00	57.25	39.6**

[a] $N = 5$. Significant differences by t-test for each pair in each test.
[b] *significant at 0.10 level;**, significant at 0.05 level;***, significant at 0.01 level.
[c] 100% IGM toxic bait.

TABLE 2. ATTRACTANCY OF VARIOUS COMPOSITIONS OF CHEMICAL AGENTS TO AUSTRALIAN BUSH FLY *M. vetustissima* (EXPERIMENT IV)

Treatment	Ingredients and % composition				Mean No. flies killed/unit[a]
	TMA · HCl	Indole	NH_4SO_4	Anchovy meal	
A	2.5	0.25		97.25	26.0 ab
B	2.5		40.0	57.50	15.3 bc
C		0.25	40.0	59.75	14.5 c
D (std.)	2.5	0.25	40.0	57.25	30.0 a

[a] $N = 6$. Means followed by same letters not significantly different from each other at 0.05 level.

lations of the Australian bush fly, but the carrier itself did show some attractancy also.

Compositions were prepared in which one of the three chemicals in the standard was deleted to see if deletion of each chemical would result in increased or decreased attractancy. The standard composition attracted significantly higher numbers than the compositions lacking trimethylamine hydrochloride or indole (Table 2). The composition lacking ammonium sulfate was not significantly different from the standard.

In studies on *Hippelates* eye gnats and house flies, *n*-butyric acid was found to increase the attractancy of chemical compositions (Hwang et al., 1975; Mulla et al., 1977). We deemed it desirable to study attractancy of this chemical, ammonium carbonate, and sodium carbonate in the standard composition (Table 3). *n*-Butyric acid and ammonium carbonate did not enhance attractancy. Addition of sodium carbonate, however, increased attractancy of the composition. Additionally, calcium carbonate and sodium bicarbonate were studied for enhancing attractancy, but neither one significantly increased attractancy (data not presented).

TABLE 3. ATTRACTANCY OF VARIOUS COMPOSITIONS OF ATTRACTANT CHEMICALS TO AUSTRALIAN BUSH FLY *M. vetustissima* (EXPERIMENT V)

Treatment	Ingredients and % composition				Mean No. flies killed/unit[a]
	TMA · HCl	Indole	NH_4SO_4	Anchovy meal	
E (*n*-butyric acid 5%)	2.5	0.25	40.0	52.25	21.6 b
F (ammonium carbonate 10%)	2.5	0.25	40.0	47.25	36.0 ab
G (sodium carbonate 10%)	2.5	0.25	40.0	47.25	54.7 a
D (standard)	2.5	0.25	40.0	57.25	27.2 b

[a] $N = 6$. Means followed by same letters are not significantly different from each other at 0.05 level.

Skatole, a chemical closely related to indole, has been reported to be attractive to certain fly species and to *H. collusor* in particular (Hwang et al., 1976). This material at 0.25% concentration was added to the standard formulation. The standard composition lacking skatole attracted a mean number of 66.5 ± 18.5 flies per unit compared to 9.3 ± 2.0 flies per unit in the composition containing skatole. The means were significantly different from each other at 0.05 level. It is possible that the combined concentration of indole and skatole was too high or that skatole is acting as a repellent against the flies.

One further test was designed to evaluate the addition of *n*-butyric acid and linoleic acid to the standard composition and to delete ammonium sulfate, replacing it with *n*-butyric acid (Table 4). Addition of both linoleic and *n*-butyric acid did not significantly alter response of flies from that of the standard. However, deleting ammonium sulfate and replacing it with *n*-butyric acid significantly decreased attractancy as compared to that of the standard. Ammonium sulfate provides bulk and improves flowability and handling of the formulation. Therefore its inclusion aside from attractancy is desirable. The IGM alone attracted very few flies.

Attraction of Bush Flies and Dung Beetles to Chemicals and Dung. Bush flies and dung beetles are attracted to cattle dung. Dung beetles are employed as biological control agents to disperse cattle dung for improving pastures and to reduce bush fly production in dung pads (Ridsdill-Smith, 1981; Ridsdill-Smith and Matthiessen, 1984). Bush flies are highly attracted to freshly dropped cattle dung pads or older pads when the surface is scraped (Figure 1). During the course of our studies, we noted that large numbers of bush flies were attracted to fresh dung pads for a short period and dung attractancy declined in time as the pads crusted.

To test and compare the attractancy of dung and chemical compositions tested, fresh dung and toxicant were tested in the experimental system designed.

TABLE 4. ATTRACTION OF BUSH FLIES TO VARIOUS CHEMICAL ATTRACTANT COMPOSITIONS WITH AND WITHOUT CARBOXYLIC ACIDS (EXPERIMENT VI)

	Ingredients and % composition						
Treatment[a]	TMA · HCl	Indole	NH_4SO_4	*n*-Butyric acid	Linoleic acid	Anchovy meal	Mean No. flies killed/unit[b]
A (std)	2.5	0.25	40			57.25	285.4 a
B	2.5	0.25	40	2	2	53.25	209.2 a
C	2.5	0.25		2		95.5	63.0 b
Check (IGM)							3.8 c

[a] One teaspoon of each composition and one teaspoon of IGM put in each treatment except in check when IGM alone was used.

[b] $N = 5$. Means followed by same letter are not significantly different from each other at 0.01 level.

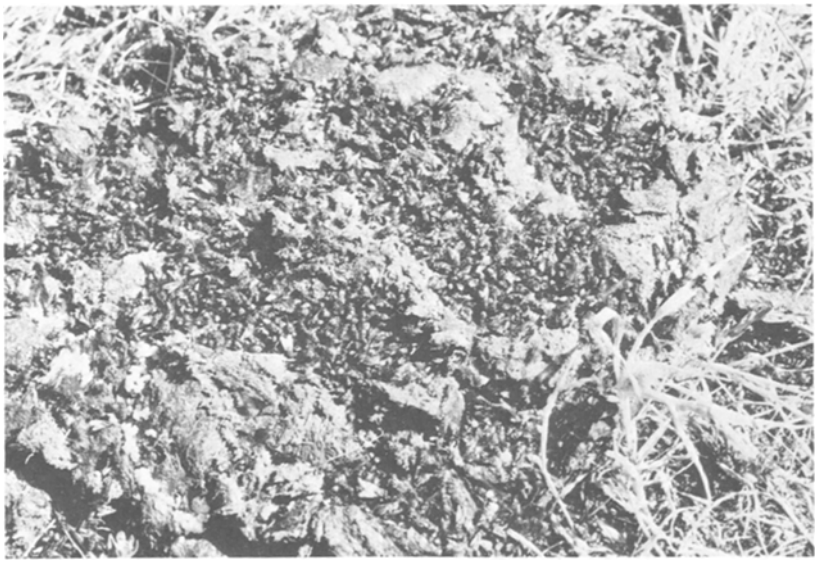

FIG. 1. Attraction of *M. vetustissima* to 1-day-old dung pad when its surface was scraped off. Flies are not attracted to such old pads; attraction ensues when the surface crust is removed. This indicates release of trapped volatiles in the pad covered with a hard crust.

Almost five times more flies were attracted to equal amounts of the dung than the chemical attractants (Table 5). The magnitude of attractancy of dung was significantly different from the chemical attractants. Dung beetles, *Onthophagus binodis* Thunberg and *Euoniticellus pallipes* (Fabricius), prevalent during the test, were not attracted to the chemical attractants but were lured to the dung in large numbers. It thus seems that bush flies and dung beetles utilize, in part, different cues for oviposition and feeding on the dung pads. Formulations of toxic chemicals and attractant compositions, if used in bush fly control programs, would not attract and harm the beneficial beetle fauna inhabiting this resource.

Relative Attraction to Chemicals and Humans and Fly Sex Ratios. Activity of bush flies is influenced by many factors, among which temperature and wind velocity play an important part. Due to variation in environmental conditions, activity patterns of bush flies are quite variable. The extent of population activity can be roughly measured by sweeping in a standard manner from human hosts (Hughes, 1970) and the numbers sampled corrected for influence of temperature.

On each day where chemical attractant compositions were exposed in the field, flies coming to a human host were netted in a 20-min sample, sexed, and counted. The data in Table 6 clearly indicate a great deal of variation in the numbers of flies either attracted to chemicals or human hosts in the different

TABLE 5. COMPARISON OF ATTRACTANCY OF STANDARD CHEMICAL COMPOSITION AND FRESH CATTLE DUNG TO AUSTRALIAN BUSH FLY *M. vetustissima* AND DUNG BEETLES (EXPERIMENT VIII)[a]

Treatment	Mean No. of insects killed/unit[b]	
	Bush flies	Dung beetles[c]
A (std.)	41.3	0
B (dung)	191.3	21

[a] A teaspoonful of the attractant and toxicant mixture, and a teaspoonful of dung plus toxicant were placed in each unit.
[b] Means significantly different for each insects group at 0.01 level
[c] Dung beetles (Scarabaeinae): *Onthophagus binodis* 55%, *Euoniticellus pallipes* 45%.

experiments. In general, as the extent of attractancy to humans increased, so did attractancy to compositions of chemical attractants. However, there were instances where trends of attractions to the two sources did not follow the same trend.

The sex ratio of bush flies coming to human hosts was more in favor of females. The sex ratio of female to male was 3.0 for flies attracted to human

TABLE 6. SEX RATIO OF BUSH FLIES ATTRACTED TO STANDARD CHEMICAL ATTRACTANT COMPOSITIONS AND HUMAN HOSTS (TAKEN BY NETTING) MEASURED ON SAME DAY

Experiment	Mean No. of flies collected			
	Chemical compositions[a]		Human net samples[b]	
	Female	Male	Female	Male
I	23	10	105	16
IA	5	5	315	117
II	508	111	105	16
II-A	32	23	315	117
III	293	231	655	204
IV	531	306	655	204
V	239	209	232	77
VI	234	221	263	90
VII	1949	858	385	168
VIII	101[c]	147	263	90
Total	3914	2121	3293	1099
Ratio	1.8		3.0	

[a] 4-6 hr trapping.
[b] 20-min sample.
[c] At dung, 756:392.

hosts, while the same ratio in flies attracted to the standard chemical attractant composition was 1.8. Since the sex ratio of flies emerging from pads is usually 1:1 (Hughes et al., 1972), the bias in sex ratio of flies attracted to hosts or attractant compositions is probably due to the physiological status of females requiring food for oogenic development and may vary with the age of flies, presence of other sources of protein, time of year, and composition of the bait (Hughes, 1981).

Measurement of quantitative responses of bush flies to chemical attractant compositions and to natural attractants emanating from fresh dung offers some interesting possibilities for further study and development of chemical attractants in bush fly control programs. It is apparent that the attractant compositions studied here are lacking one or more chemical attractants present in the dung, and isolation, identification, and evaluation of chemical attractants from dung sources warrant further studies. Nevertheless, this is the first attempt to test and evaluate chemical attractants against the widely distributed pest fly *M. vetustissima*.

It should be pointed out that the behavior of *M. vetustissima* is quite complex and variable, and is influenced by many environmental and physiological factors. Chemicals showing very low levels of attractancy will not provide a distinct separation in the behavioral response of this fly under different conditions. Further studies are warranted to work out concentration–attractancy relationships for these chemicals under various field conditions.

It is equally evident (Figure 1) that, due to the very high potency of chemical attractants emanating from dung pads, the dung pads can be used for attracting natural populations of flies and killing them on the pads with the application of suitable toxicant formulations. Addition of toxic bait to dung pads resulted in the kill of at least 1000 flies on one treated dung pad in 15–30min. In view of the low absolute density of bush flies, found to be 9000 flies/acre in one study (Norris, 1966) and less than 100 flies per acre during March in another study (Vogt et al., 1981), marked local reduction of flies could be achieved by treating dung pads in infested areas. Before such a program can be implemented, detailed studies on population dynamics and behavioral aspects of the pest are in order.

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CHEMICAL DETECTION OF "SELF" AND CONSPECIFICS BY CRAYFISH

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Abstract—Stimulus waters were used to examine behaviorally chemical communication in female crayfish *Procambarus clarkii*. Animals detected conspecifics. Response to stimulus water drawn from the animal's own tank, "self" water, mimicked either response to distilled water or response to water drawn from the tank of another animal. The response to "self" water depended on the relative concentration of substance(s) in "self" water stimuli to that of the same substance(s) in the test animal's tank.

Key Words—Chemical detection, chemical communication, self, recognition, *Procambarus clarkii*, crayfish.

INTRODUCTION

Chemical communication by crayfish has been shown in a number of species (e.g., Ameyaw-Akumfi and Hazlett, 1975; Hazlett, 1985; Little, 1975; Tierney and Dunham, 1982; Tierney et al., 1984) and has spawned at least one controversy concerning sexual recognition pheromones in *Procambarus clarkii* and the methodology used to assess such pheromones' existence and extent (Itagaki and Thorp, 1981; Thorp and Itagaki, 1982; Thorp, 1984; Rose, 1982, 1984; Hazlett, 1984). Detection and recognition of "self" have been largely ignored on the behavioral level (but see Teague and Friou, 1964; McCumber and Clem, 1983, concerning recognition of immunological "self"). Indeed, "self" has recently been used as a control in studying responses to "nonself" (Hazlett,

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1985, p. 187). The present study identifies one complication of using "self" as a control (or comparison) for chemical communication studies, i.e., that "self" can appear as either "self" or "nonself" depending on experimental conditions. Portions of this work have been reported previously in abstract form (Rose and Casper, 1980).

METHODS AND MATERIALS

Mature nongravid female intermolt crayfish (*Procambarus clarkii*; 40–60 mm carapace length; mean wet weight 31.1 g ± 7.4 SD) were obtained from commercial suppliers in Louisiana. They were housed individually in glass aquaria (20 × 40 × 25 cm high) for five days prior to experimentation. Each aquarium contained ~2 liters distilled H_2O and was aerated strongly via an airstone. Each animal was fed frozen fish daily, the uneaten tissue being removed after 30 min. If the water clouded, it was changed and the acclimation procedure started over. Temperature was maintained at 20–25° C (see Thorp, 1978; Itagaki and Thorp, 1981, concerning the effects of temperature on activity). Fluorescent overhead lighting was maintained 24 hr/day; the room was also sunlit. Experiments were performed at night (2100–0200 hr) because pilot studies indicated increased nocturnal activity. The presence of light did not appear to inhibit the activity of the test animals. In no tests were the subject animals handled or their aquaria moved or disturbed immediately prior to testing.

Behavioral variables were quantified temporally (with stopwatch and a system of alphabetic codes) with respect to resting, locomotion, and nonspecific nonlocomotory movement behaviors such as feeding and grooming. Locomotion was further subdivided into near, within 1 cm, and distant, elsewhere, with respect to the stimulus source (see below, see also Ameyaw-Akumfi and Hazlett, 1975).

Stimulus waters were obtained by gently scooping 50 ml from the source aquarium 1 hr before testing (except distilled H_2O). Stimulus waters were dripped from a burette (~0.3 drops/sec) onto the airstone during tests. This procedure precludes any response which could be due to mechanical agitation of the surface of the water. In pilot studies, both normal and blinded crayfish oriented to very slight agitations of the surface of previously calm water in their tanks.

Four treatments (described below) were used: (1) water collected from the animal's own tank ("self" water), (2) distilled H_2O, (3) "self" water–diluted test tank, and (4) water from the tank of another isolated nongravid female ("nonself" water). While treatments 2 and 4 are self-explanatory, treatments 1 and 3 are quite similar: the difference between these treatments is in the solute concentration of the environment rather than the stimulus. For treatment 1, 50 ml tank water was drawn 1 hr before testing. This water *was not* replaced.

Treatment 3 was accomplished as follows: 50 ml tank water was drawn from the test animal's tank 1 hr before testing. This water *was* replaced with 50 ml distilled H_2O which diluted the test tank by $<3\%$. This meant that the stimulus water was effectively at a higher concentration than the water currently in the tank. It is assumed that the test animal does not replenish all substances within the hour.

The rationale for each treatment is as follows: (1) "self" water represents no change in substance concentration, (2) distilled water represents an infinite dilution of virtually all substances, (3) "self" water–diluted tank represents the same stimulus as (1) but is presented in a relatively more concentrated form, and (4) "nonself" water represents the presence of a conspecific.

Test order and animals used for each test were randomized. Animals were used in a given treatment only once and only once as source or test animal on a given day. Animals were observed through a one-way screen. Test duration was 1 hr. Data were collected and analyzed in seconds. There were five to six replicates of each experiment.

RESULTS

Results are shown in Figure 1. ANOVAs of each variable show overall differences (at least $P < 0.05$). Note that female *P. clarkii* can detect conspecifics chemically (comparison of treatments 1 and 2 with 4), but appear unable

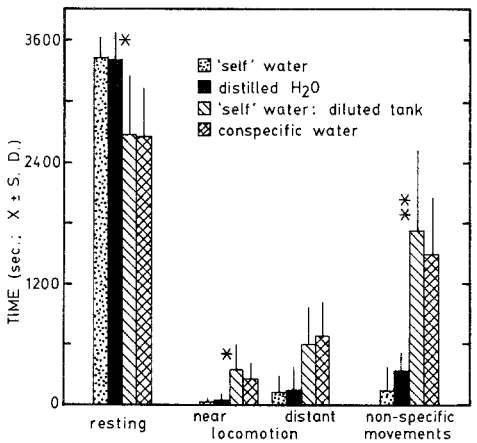

FIG. 1. Behavior of female *P. clarkii* in response to stimulus waters. Stars indicate *t*-test significance levels of the middle two means when means are rank ordered, e.g., in "locomotion, near" the means compared would be those of treatments 2 and 4. Single star, $P < 0.05$; double star, $P < 0.01$. In "locomotion, distant" significance level between 2 and 3 is $P = 0.06$; other apparent differences, e.g., 1 and 3 or 2 and 4 are $P < 0.05$.

to distinguish other individuals from themselves (i.e., the results from "non-self" and "self-diluted tank" are virtually identical, cf. treatments 3 and 4). Also, similar "self" stimuli (treatments 1 and 3) evoked significantly different responses when presented to crayfish in only slightly different environments.

DISCUSSION

Chemical recognition of "self" probably occurs widely throughout the animal kingdom. However, there is little direct evidence concerning this phenomenon. Limpets are believed to lay chemical trails when foraging that allow the individual to find its former resting position (home scar) on a rock (Cook et al., 1969; Cook, 1969). Yet I know of no evidence indicating whether these animals can distinguish their own trails from those of other limpets. Many male mammals chemically mark territories (e.g., canids, Kleiman, 1966; lorisids, Ilse, 1955). One would expect such animals to distinguish their own marks from those of conspecifics (e.g., lorisids, Seitz, 1969). Honeybees, *Apis mellifera*, a species where many consider the hive to be the individual, use "hive atmosphere" (LeComte, 1956) to chemically identify individual hives which are readily recognized by their members. Recently, two systems (salmon, Quinn and Busack, 1985; shrimp, Caldwell, 1985) have been described which should readily lend themselves to studies of chemical recognition of "self." Theoretical considerations of animal communication, in particular the biological necessity of the concept of "self" and self-recognition, are discussed in the (thoroughly entertaining) review of Markl (1985).

The behavioral variables monitored in the present study were chosen for historical reasons (see for example Ameyaw-Akumfi and Hazlett, 1975) and ease of categorization. They are not necessarily independent (see Hazlett, 1985, p. 184). The one resting and two locomotion categories are mutually exclusive and account for the entire test duration (cf. Tierney and Dunham 1982). The nonspecific nonlocomotory movement category is a subset of resting; the other subset being motionless (cf., Tierney and Dunham 1982). Present interpretations do not require such constraints as independence and exclusivity. Furthermore, Dunham's (1978) constraints for the use of novel stimuli, etc., do not apply, as this study is not concerned with the nature of the substance(s) but rather the reaction of animals to the same substances(s) under slightly different conditions (maximum possible concentration change $<3\%$ by the end of the tests).

Substance concentration in treatment 1 and 2 stimulus waters is unlikely to be greater than that in the test animal's tank. Since behavior in these two treatments was similar, and different from that in tests where substances are believed to be at a relatively higher concentration (i.e., 3, see also below), it seems that detection may be via recognition of substance at concentrations

greater than that previously in the locale. Furthermore, results from "self" water–dilute tank tests (3) and those from "nonself" water tests (4) suggest that substance(s) released may be similar for all females and again suggest that detection is somehow dependent on concentration. These results do not imply individual recognition and allow no inferences about sexual recognition. Indeed, female *P. clarkii* appear unable to distinguish "self" from "nonself" (cf., treatments 1 and 3, both "self" in reality, with 4, "nonself").

It is acknowledged that aging the stimulus may alter (e.g., deplete) the substance, but this seems unlikely (see Ameyaw-Akumfi and Hazlett, 1975; Hazlett, 1985). It is also possible that dilution of the tank somehow sensitizes the animals and that this in turn is responsible for the results observed.

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URINARY VOLATILE CONSTITUENTS OF THE HOUSE MOUSE, *Mus musculus*, AND THEIR ENDOCRINE DEPENDENCY

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Abstract—Mouse urine contains a great number of volatile constituents that may be used in chemical communication. Some of these volatiles, identified in this study by combined gas chromatography–mass spectrometry and gas chromatography–Fourier-transform infrared spectroscopy, appear unique to the mouse. Certain urinary volatiles exhibit strong dependence on the sex and endocrine status of the animals, as shown through castration, treatment with an antiandrogen, and hormone supplementation.

Key Words—Urinary volatile signals, endocrinological manipulations, capillary gas chromatography–mass spectrometry.

INTRODUCTION

The common house mouse, *Mus musculus*, is widely recognized as the mammalian species most studied and best documented regarding its use of chemical signals. Several primer pheromones and a large number of releaser factors can be traced to the urine of both male and female animals. Table 1 lists the best known cases of chemical communication in *Mus musculus*; some of these are also paralleled in other rodents. The nocturnal behavior of rodents creates a strong advantage for use of these chemical signals. As discussed by Bronson and Vandenbergh, pheromones may be particularly important (Bronson, 1971,

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TABLE 1. PHEROMONAL PHENOMENA IN THE MOUSE

Category	Effect	Description	Reference
Primer	Lee-Boot	Female grouping supresses estrus cycle in absence of male	Vander Lee and Boot, 1955
	Vandenbergh	Female puberty accelerated by male urine	Vandenbergh, 1969; Colby and Vandenbergh, 1974
	Bruce	Pregnancy block induced by presence of strange male	Bruce, 1959, 1965
	Whitten	Estrus acceleration and synchronization induced by male urine	Whitten, 1956
	Puberty delay	Puberty delay in young females caused by urine from grouped females	Drickamer, 1977
Releaser	Female attractivity	Estrous/pregnant urine more attractive to male	Davies and Bellamy, 1972
	Male attractivity	Male urine more attractive to estrous female	Scott and Pfaff, 1970
	Male aggression	Male urine elicits attacks between males	Chanel and Vernet-Maury, 1963
	Male fear	Urine voided under stress elicits avoidance response	Carr et al. 1970
	Male aversion	Male urine territorial marking	Jones and Nowell, 1973
	Female protection	Female urine decreases attacks by aggressive male	Mugford and Lowell, 1970; Dixon and Mackintosh, 1971
	Male ultrasound eliciting signal	Female urine elicits 70 KHz ultrasonic courtship whistle from experienced males.	Whitney et al., 1974; Nyby et al., 1979

1979; Vandenbergh, 1983) in various ecological considerations regarding *Mus musculus*, one of the most "successful" mammals on the earth.

Although many chemical signals in the mouse are now characterized biologically, chemical studies have been rare. This unsatisfactory state of affairs is partly due to the apparent complexity of the signals involved in mammalian communication, and partly due to the plasticity of response to releaser signals (Bronson, 1971). Nevertheless, chemical identification of these signals may eventually lead to an understanding of their biochemical origin, means of regulation of expression, influence of genetics, etc.

While the question of volatility of certain mouse primer pheromones remains unresolved to this date, the known releaser pheromones appear to act over a distance. Moreover, from the controversies surrounding the nature of primer pheromones (Whitten et al., 1968; Wilson et al., 1980; Bronson, 1979), it is reasonable to expect that volatile components are at least partially responsible for some of these observed effects. For example, individual recognition on the basis of airborne information appears to be an essential component of the pregnancy-block effect (Bruce, 1965) in which a strange male, or the urine of a strange male, can prevent the implantation of fertilized ova; participation of volatiles in conveying the "signature" of an individual is distinctly possible.

Mouse urine is known to possess an odor whose intensity and quality are distinctly different from those of other animals (e.g., rats), and these factors are sex-related. For the above-mentioned reasons, a significant rationale exists for the chemical identification of those urinary constituents which are unique to the mouse. In general, a search for the unusual chemicals in the urine and glandular secretions of mammals can reveal candidates for various pheromonal activities. Drawing parallels from the research on insect pheromones, it is clear that even though a pheromonal activity is not always associated with unusual compounds, the converse is very often true—structurally unique compounds are frequently associated with biological activity.

Systematic studies into the chemical nature of mouse urinary signals are, in our opinion, long overdue. The identification and subsequent synthesis of the mouse-unique constituents should permit their use in a variety of established bioassays for both primer and releaser effects. Such considerations have led us to design several experiments, employing established headspace sampling procedures (Novotny et al., 1974), capillary gas chromatography (GC), and gas chromatography–mass spectrometry (GC-MS). The complexity of mouse urinary signals and their dependency on genetic and endocrinological factors have been tentatively indicated in our previous publication (Novotny et al., 1980).

The present report describes a more complete chemical characterization of the urinary volatile fractions of male and female mice. Certain "mouse-specific" compounds have been identified here, together with the urinary constituents that show a profound endocrinological dependency, i.e., they are clearly

dependent on hormonal levels. Within the constraints of contemporary identification methodology, additional urinary volatile components are also reported. Although structurally less unusual, many of these constituents could also contribute to the overall complexity of olfactory perception in mice.

METHODS AND MATERIALS

Sample Collection and Analysis. Test animals were housed in groups of four or five in cages provided with hardwood chip bedding. Food (Purina Rat Chow, St. Louis, Missouri) and water were provided ad libitum. To collect urine, the animals were transferred to plastic metabolism cages with wire-mesh floors. The urine was collected in a polycarbonate vessel placed on Dry Ice, so that the collected samples were immediately frozen. Collection was performed in either 24- or 48-hr periods to minimize diurnal fluctuations in urine composition. At the end of a collection period, the frozen samples were thawed, filtered through a glass-wool to remove particulate matter, and refrozen in acid-washed glass vials provided with Teflon-lined screw-caps for storage prior to analysis.

Urinary volatiles were analyzed using the headspace technique referred to earlier, which employs a porous polymer (Tenax-GC, Applied Science Laboratories, State College, Pennsylvania) as a collection medium. The volatiles are sparged from 1-ml urinary samples at room temperature with purified helium gas at a flow-rate of 100 ml/min, and adsorbed onto a precolumn packed with Tenax-GC. The sample is subsequently desorbed in the heated injection port (220–240°C) of a gas chromatograph and retrapped into a cooled section of a glass capillary column. The analytical columns were glass capillaries (0.25 mm, ID) of a soda-lime type and coated statically with UCON 50-HB-2000, with benzyltriphenylphosphonium chloride added as a column deactivation agent (Franken et al., 1976).

The flame ionization detector was used for quantitative comparisons of various urinary samples. The urinary volatile constituents were identified through capillary GC-MS (Hewlett-Packard 5981 dodecapole mass spectrometer), using electron impact ionization at 70 eV. Whenever feasible, authentic samples were synthesized to verify agreement of spectral information and GC retention times. In addition, sulfur- and nitrogen-containing compounds were detected with the aid of selective flame-photometric and thermionic detectors on an instrument fitted with a three-way effluent splitter. This procedure significantly complemented the information acquired through GC-MS.

Investigations of Testosterone-Dependent Volatiles in Male Mice. Eight BALB/CWt male mice (Jackson Laboratory, Bar Harbor, Maine) were castrated at 8 weeks of age and permitted four weeks for recovery. Four males were then

implanted (Maruniak et al., 1977) with 5-mm pellets containing pure testosterone, while the other four were implanted with empty silastic tubes. Another group of males (neither castrated nor implanted) of the same age provided control urine samples.

Urines were collected daily from the individual males of the testosterone-implanted group for a total period of 20 days. The samples from blank-implanted castrates and normal males were collected at variable intervals and pooled to provide samples for comparison.

Quantitative comparisons were based on peak areas in the chromatograms obtained from the three groups. The mean integrated peak areas were plotted for different times after testosterone implantation.

Possible effects of cyproterone acetate, a well-known antiandrogen, on male urinary volatiles were also investigated. Osmotic minipumps (model No. 2002, Alzet Osmotic Minipump, Alza Corporation, Palo Alto, California) were filled with a solution of cyproterone acetate (CA) in propylene glycol (10.5 mg/ml). At an infusion rate of 0.5 μl/hr, the pumps supplied a continuous infusion of 5.3 μg/hr for two weeks in implanted animals. The 48-hr collections were started two days after implantation; in total, four samples were collected for GC analyses.

Investigation of Estrogen-Dependent Volatiles in Female Mice. In order to determine which of the mouse urinary constituents are sex-specific, female mouse urine samples were collected and analyzed. In order to control the endocrine status of these animals, mature females of three different strains (C57BL, BALB/C, and ICR) were implanted with 5-mm silastic tubes containing 17β-estradiol. Immature females (3–4 weeks old) served as controls. Occurrence of estrus in the implanted animals was verified by inspection of vaginal smears (Vandenbergh, 1967) before the urine samples were collected. Implantation induced a continuous state of estrus throughout the collection period in all investigated animals.

RESULTS

Male Urines. Figure 1 shows a typical chromatogram obtained with normal male urine. Peak numbers refer to the list of identified components in Table 2. Some of these compound identifications remain tentative at this time.

A visual comparison of the three chromatograms in Figure 2 clearly demonstrates the effects of castration and testosterone supplementation. In general, castration decreases the concentration of most volatile constituents, while androgenization of castrates restores a number of components to normal levels.

A very substantial endocrine dependency in males has been indicated for several compounds through the plots of mean peak areas during the testosterone

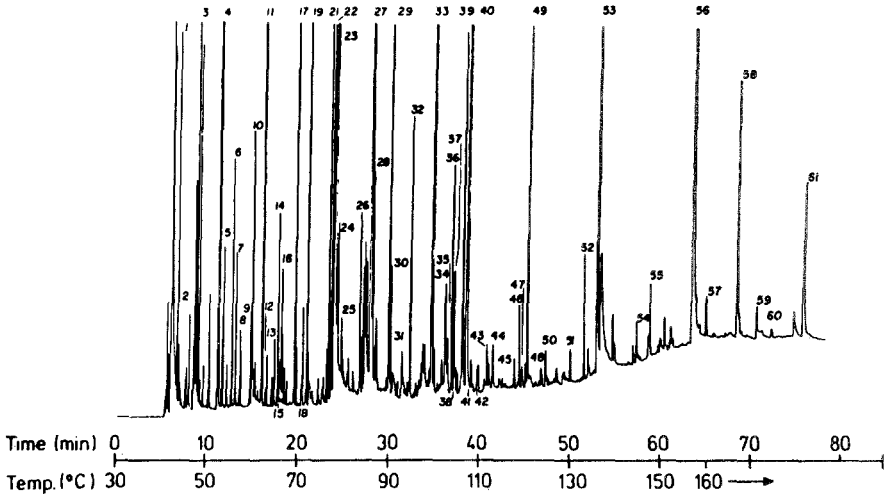

FIG. 1. Chromatogram of male mouse (BALB/CWt) urinary volatiles. Numbers refer to compound identifications in Table 2.

treatment period. For example, peak number 33 identified positively (Novotny et al., 1984) as 7-*exo*-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]-3-octene (trivially named as 3,4-dehydro-*exo*-brevicomin), is a large urinary component in normal males which is drastically decreased following castration. Testosterone treatment restores it to the original value. A similar dependency is shown by *p*-toluidine (peak 52).

Two heterocyclic compounds, 2-isopropyl-4,5-dihydrothiazole and 2-*sec*-butyl-4,5-dihydrothiazole (peaks 31 and 39) are present in normal male urine but disappear completely with castration (Liebich et al., 1977). Our observations indicated that testosterone supplementation does not readily restore these heterocyclic constituents (thiazolines) to their original concentrations. However, the distinctive odor of these unusual compounds alludes to their possible involvement in some signaling phenomena related to males.

Our recent results of behavioral testing for intermale aggression (Novotny et al., 1985) have established that 3,4-dehydro-*exo*-brevicomin and *sec*-butylthiazoline are essential components for that communication. Moreover, their attractiveness to females (Jemiolo et al., 1985a) and estrus synchronization effect (Jemiolo et al., 1986) were also verified.

While the remaining urinary constituents, as identified in Table 2, may fluctuate under different conditions, none appears to give the same pronounced endocrine dependency as the thiazolines, 3,4-dehydro-*exo*-brevicomin, and *p*-toluidine. Our previous experience with the urine samples of a number of mam-

TABLE 2. MASS SPECTRAL DATA AND IDENTIFICATIONS OF MOUSE URINARY VOLATILES

Peak No.	Compound	Authenticated	Significant *m/e* (relative intensity)
1	acetone	[a]	43(100), 58(34)
2	3-buten-2-one		70(51), 55(100), 43(79)
3	benzene	[a]	78(100), 77(36)
4	126 mol wt	[c]	126(34), 111(22), 97(57), 70(11)
5	a cyclic vinyl ether, $C_7H_{12}O$		112(56), 97(13), 83(91), 67(47), 57(100), 55(67)
6	toluene	[a]	92(59), 91(100)
7	dimethyl disulfide		94(99), 45(100), 79(74)
8	2-ethyl-5-methylfuran		110(26), 95(100), 79(11), 81(8), 67(18), 65(15), 43(34)
9	2,5-diethylfuran		124(80), 109(100), 97(53), 91(96), 81(54), 57(73), 55(88)
10	octamethylcyclotetrasiloxane (contaminant)		281(100), 282(31), 283(21), 265(13), 73(10)
11	126 mol wt	[c]	126(96), 111(52), 97(52), 93(25)
12	a cyclic vinyl ether of mol wt 112		112(58), 97(23), 83(38), 70(25), 67(29), 56(100)
13	a cyclic doubly unsaturated ether of mol wt 138		138(49), 109(77), 91(52), 81(100), 79(94), 57(100)
14	126 mol wt		126(62), 111(65), 97(45), 85(59), 69(15), 67(14), 57(48), 55(100), 43(27)
15	*m*-xylene	[a]	106(40), 91(85), 71(83)
16	*p*-xylene	[a]	106(58), 91(100)
17	126 mol wt	[c]	126(53), 111(32), 97(39), 85(7), 83(26), 70(35), 69(57), 57(100), 55(85)

TABLE 2. Continued

Peak No.	Compound	Authenticated	Significant *m/e* (relative intensity)
18	3-methylcyclopentanone a cyclic doubly unsaturated vinyl ether of mol wt 138		98(39), 69(100), 55(79), 138(20), 123(23)
*19	2-heptanone	[b]	114(9), 99(7), 85(7), 71(26), 58(80), 55(9), 43(100)
*20	*n*-pentyl acetate	[b]	
*21	*cis*-2-penten-1-yl acetate	[b]	128(3), 113(3), 99(6), 86(30), 85(13), 68(46), 67(45), 57(23), 53(20), 43(100), 41(30), 39(23)
*22	*trans*-5-hepten-2-one	[b]	112(9), 97(8), 94(12), 69(13), 43(100), 41(29), 39(15)
*23	*trans*-4-hepten-2-one	[b]	112(4), 97(6), 94(8), 69(9), 43(100), 41(26), 39(18)
24	bis (methylthio)methane		108(75), 61(100)
25	3-methyl-2-buten-1-yl acetate		86(25), 69(27), 68(35), 67(32), 57(24), 53(25), 43(100)
*26	3-hepten-2-one		112(13), 97(37), 69(16), 55(100), 53(10), 43(54), 39(24)
27	6-methyl-6-hepten-3-one	[b]	126(4), 111(5), 97(18), 69(52), 57(100), 55(24), 41(42)
28	5-methyl-2-hexenal		112(56), 97(47), 83(82), 57(86), 55(100), 43(77), 41(82)
29	6-methyl-5-hepten-3-one		126(13), 111(4), 97(14), 69(38), 57(100), 55(6), 53(9), 41(36)
30	6-methyl-5-hepten-2-one	[a]	126(29), 111(34), 97(38), 69(81), 57(100), 43(76), 39(49)
31	6-octen-3-one		126(19), 97(28), 69(51), 67(51), 57(85)

32	2-isopropyl-4,5-dihydrothiazole	[a]	129(28), 114(27), 95(12), 71(17), 60(100), 48(38)
33	7-*exo*-ethyl-5-methyl-6,8-dioxabicyclo [3.2.1]-3-octene	[b]	154(7), 136(13), 125(26), 121(18), 111(56), 97(24), 95(63), 83(39), 81(40), 71(15), 57(42), 43(100)
34	3,5-heptadien-2-one		110(32), 95(100), 67(74), 43(40), 41(42), 39(31)
35	benzaldehyde	[a]	106(100), 105(98), 77(98)
36	methylvinylpyrazine		120(100), 119(39), 105(10), 66(14), 54(30), 51(28), 50(20)
37	4,6-octadien-3-one		124(25), 109(8), 96(13), 95(85), 77(11), 67(100), 65(26), 57(12), 55(11), 43(12), 41(35), 39(24)
38	methanethiol butyrate		118(47), 95(30), 75(47), 43(100)
39	2-cyclohexenone		124(16), 96(28), 95(34), 91(28), 68(27), 57(43), 57(43), 43(51)
40	2-*sec*-butyl-4,5-dihydrothiazole	[b]	143(3), 128(10), 115(52), 60(100), 57(13), 45(15), 41(18)
41	unknown structure, mol wt 154		154(26), 95(22), 82(93), 72(53), 67(80), 43(100)
42	5-methyl-4-nonanone		156(19), 113(29), 71(38), 43(100)
43	4-hydroxy-4-methylpentanoic acid lactone		100(17), 99(61), 85(30), 70(49), 56(73), 55(69), 43(100)
44	4-octen-3-ol		128(5), 99(100), 57(76), 43(82)
45	2-isopropyl-4-methyl-3-cyclopentenone		138(31), 123(16), 109(20), 96(39), 81(45), 67(41)
46	1-acetyl-2-methylcyclopentene		124(20), 109(71), 98(34), 81(49), 43(100)
47	linalool		121(44), 93(81), 71(97), 55(92), 43(100)
48	2-acetoxy-1-propanol		118(12), 97(19), 87(33), 69(19), 58(28), 55(30), 43(100)
49	acetophenone	[a]	120(34), 105(100), 77(82), 51(30), 43(21)

TABLE 2. Continued

Peak No.	Compound	Authenticated	Significant *m/e* (relative intensity)
50	4-hydroxyhexanoic acid lactone		85(100), 70(30), 57(43), 43(36)
51	phenylacetone		134(20), 119(3), 92(31), 91(76), 65(38), 43(100)
52	a lactone, $C_6H_{10}O_2$		70(67), 55(42), 42
*53	*p*-toluidine	[b]	107(80), 106(100), 79(28), 77(32), 51(20), 39(16)
54	C_7OHN_{13} (a pyrrolidone ?)		127(31), 126(31), 98(45), 84(51), 43(100), 42(85)
55	caryophyllene	[a]	161(8), 148(4), 133(14), 120(14), 93(45), 79(29), 69(100), 41(83)
56	phenol	[a]	94(100), 66(74), 65(52), 39(38)
57	heptadecane		99(22), 85(42), 71(72), 57(100), 43(78), 41(52)
58	*m*-cresol		108(81), 107(100), 90(15), 79(43), 77(57), 53(27), 51(30)
59	$C_{15}H_{19}NO$ (unknown structure		243(8), 159(12), 108(19), 107(24), 71(100), 43(42), 41(18)
60	formanilide	[a]	121(62), 93(100), 66(87), 65(72)
61	ethylphenol		122(31), 107(100), 91(12), 77(35)

[a] Identification by comparison to literature spectra.
[b] Verified by coelution and mass spectra with authentic sample.
[c] Coelution and mass-spectral match with decomposition product of independently synthesized precursor.

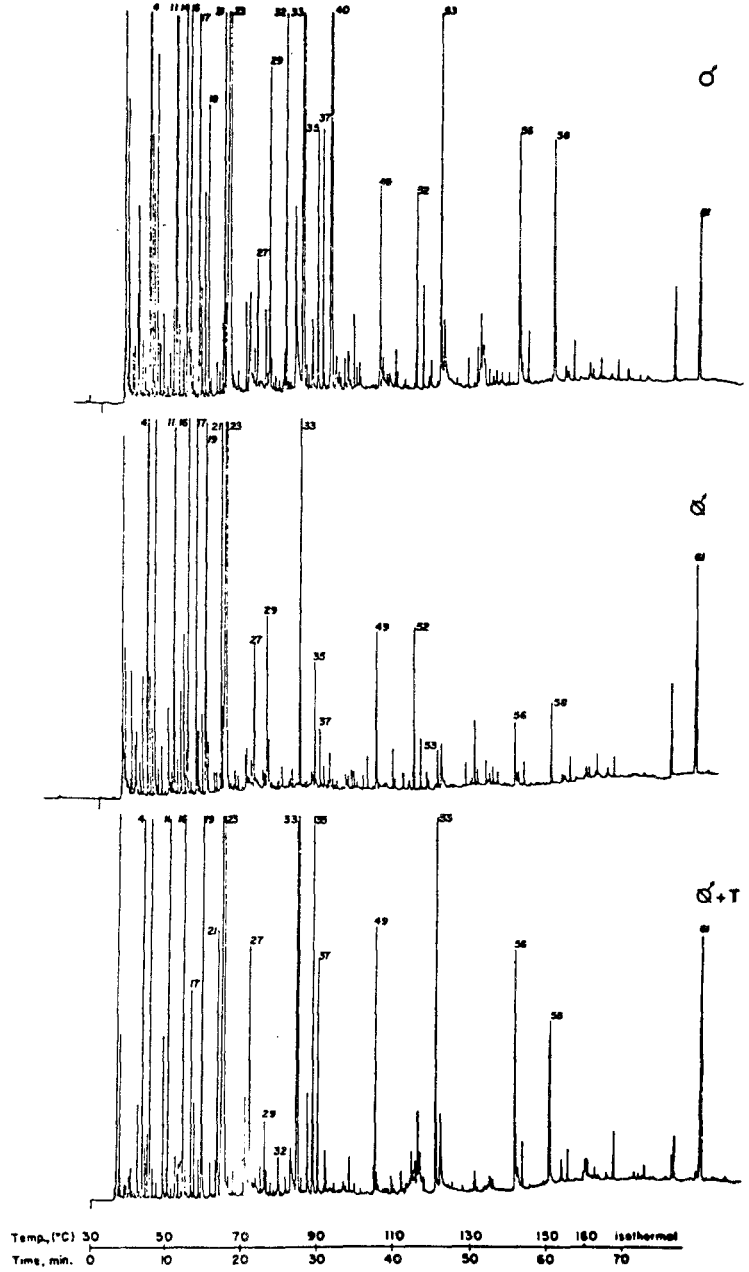

FIG. 2. Comparison of the urinary volatile chromatograms from normal (top), castrated (middle), and castrated plus testosterone-treated (bottom) male mice (strain BALB/CWt). Peak numbers refer to Table 2.

mals strongly suggests that the above-mentioned compounds are unique to the house mouse. For example, we find no such compounds in the urine of male rats, foxes, wolves, dogs, or primates.

The GC-MS results revealed four additional compounds unique to the mouse (peaks 4, 11, 14, and 17). Mass spectra at nominal resolution (see Table 2) yield few clues to their identity other than a molecular weight of 126 and a strong structural resemblance to one another. The notion that they might be cyclic vinyl ethers (e.g., dihydrofurans or dihydrapyrans) was suggested by their early appearance in the chromatogram and the similarity of their mass spectra to those of simpler cyclic vinyl ethers found in published collections of mass spectra. This notion was reinforced by high-resolution mass spectrometry (Table 3) and Fourier-transform IR spectroscopy (FT-IR) (Figure 3). The high-resolution data were helpful in that they narrowed further consideration to molecules possessing a single oxygen atom (formula $C_8H_{14}O$), while the FT-IR data, obtained only for the compound represented by GC peak number 11, pointed to an alkyl-substituted dihydrofuran. By analogy with published spectra, it was believed that a 4,5-dihydro-2-ethylfuran moiety should account for six of the carbon atoms, with the positions of the other two carbon atoms unclear.

Synthesis of a number of such dihydrofurans was accordingly undertaken. The strategy consisted of synthesizing a γ-hydroxyketone, expected to be in equilibrium with a lactol, and subjecting that lactol to dehydrating conditions. After a number of unfruitful syntheses of γ-hydroxyketones, 6-hydroxy-6-

TABLE 3. HIGH-RESOLUTION MS DATA FOR SUSPECTED VINYL ETHERS

Peak number	Significant *m/e*	Chemical formula
4	126.1044	$C_8H_{14}O$
	111.0830	$C_7H_{11}O$
	97.0646	C_6H_9O
	69.0332	C_4H_5O
11	126.1069	$C_8H_{14}O$
	111.0825	$C_7H_{11}O$
	97.0649	C_6H_9O
	69.0711	C_5H_9
	69.0338	C_4H_5O
	67.0555	C_5H_7
14	126.1048	$C_8H_{14}O$
	111.0840	$C_7H_{11}O$
	97.0624	C_6H_9O
	85.0657	C_5H_9O
17	126.1076	$C_8H_{14}O$
	97.0656	C_6H_9O

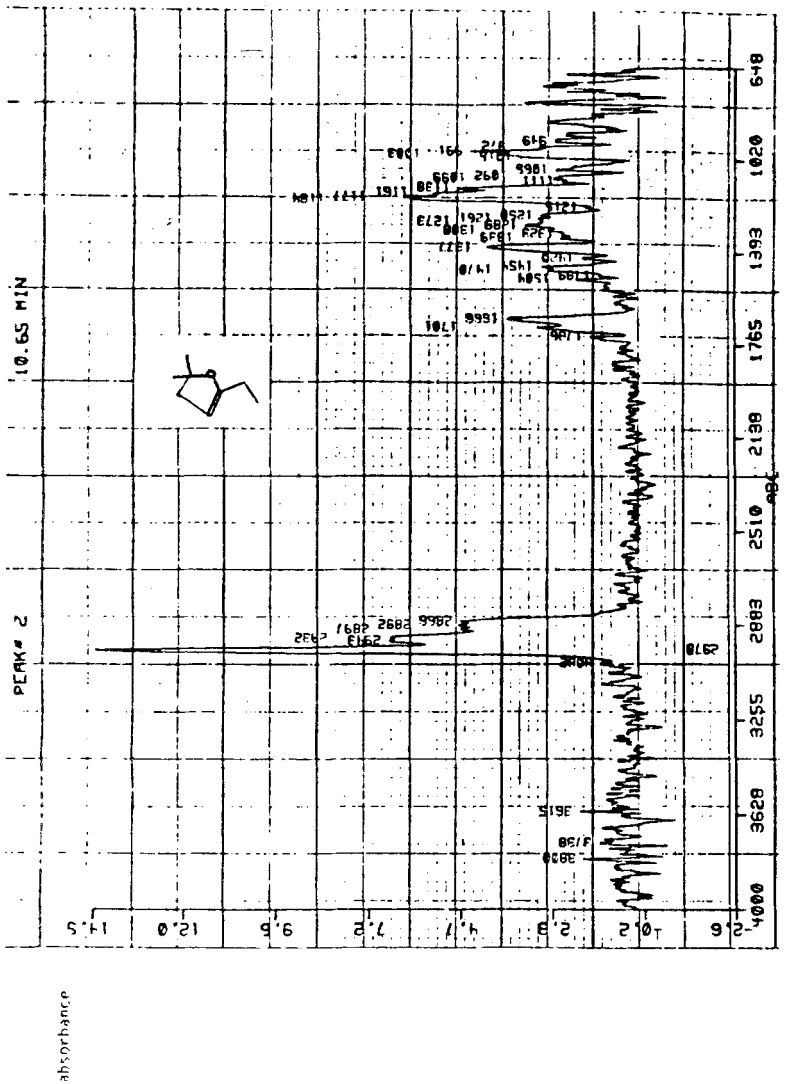

FIG. 3. IR spectrum of peak number 11, a 126 mol/wt isomer.

methyl-3-heptanone was prepared, and subsequently subjected to dehydrating conditions in the injection port of a gas chromatograph. Earlier indications, supported by the report of DiVincenzo et al., (1976), had led us to believe that the high temperature of a GC injection port should suffice to effect the desired dehydration of the lactol. Indeed, when the above hydroxy ketone was injected, three peaks appeared which agreed in both retention times and mass spectra with peaks 4, 11, and 17. Although unequivocal structure assignments have not been performed, we believe these peaks to represent 5,5,-dimethyl-2-ethyl-4,5-dihydrofuran, 5,5-dimethyl-2-(*Z*)-ethylidenetetrahydrofuran, and 5,5-dimethyl-2-(*E*)-ethylidenetetrahydrofuran. The compound represented by peak 14 remains unidentified.

It is not clear whether the urine contains the observed dehydrofurans or the γ-hydroxyketone/lactol precursor, although in light of the ease which vinyl ethers are known to undergo hydrolysis, the second possibility seems more reasonable.

The synthetic route to 6-hydroxy-6-methyl-3-heptanone was chosen partly because it includes 6-methyl-6-hepten-3-one as an intermediate, which was suspected of being a later-eluting component (peak 27), also of apparent molecular weight 126, and exhibiting a fragmentation pattern suggestive of an unsaturated aliphatic ketone. This suspicion was confirmed by a perfect match of a mass spectrum and retention time. This unsaturated ketone is surely present in the urine; it was not found upon injection of the synthetic hydroxy ketone.

A series of chromatograms shown in Figure 4 demonstrates the effect of cyproterone acetate on the excretion of mouse urinary volatiles. As the time of treatment is prolonged, the antiandrogen appears to depress the levels of all volatiles in males. This effect is similar to surgical castration.

Female Urine. Typical chromatograms of urinary volatiles for immature animals and the female with induced estrus are shown in Figure 5, while peak numbers refer again to the list of compounds in Table 2. Estrogenization of mature females produces a dramatic increase in a number of volatile compounds, indicated by an asterisk in Table 2, and causes the appearance of a compound unique to the estrus state, *n*-pentyl acetate (peak 20).

Quantitative aspects of this experiment are reported elsewhere (Schwende et al., 1984b). We have previously established that females of three different strains reacted similarly to estrogenization, while quantitative proportions of the compounds marked by an asterisk (Table 2) were somewhat different. Figure 5 shows an example of the urinary excretion by C57BL/10 females. These results are communicated here in order to contrast the major differences between male and female unique urinary constituents. None of the thiazoline compounds were observed in female urines, confirming the sex-specific nature of these substances. Likewise, only trace amounts of 3,4-dehydro-*exo*-brevicomin were encountered in female mice.

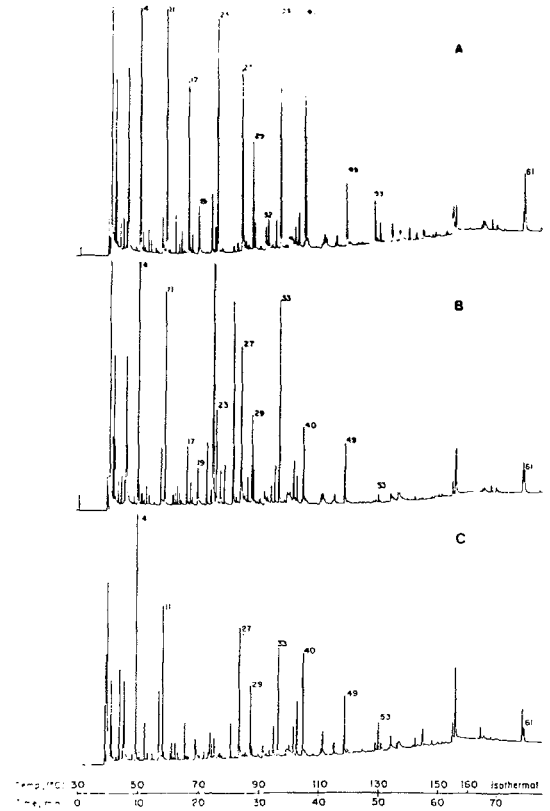

FIG. 4. Effect of cyproterone acetate on male urinary volatile profiles. Chromatograms: (A) 3 days; (B) 9 days; and (C) 13 days after implantation with continuous infusion device. Peak numbers refer to Table 2.

DISCUSSION

It is generally recognized that mammalian odors are due to complex mixtures of organic compounds. While certain specific components of those mixtures (loosely referred to as "pheromones") undoubtedly exist, less specific mixture components may also have importance for the overall chemical message perceived by the mammalian olfactory organs and interpreted by the brain. Müller-Schwarze et al. (1974) speculated that the alcohols and esters accompanying isovaleric acid in the subauricular gland of the male pronghorn antelope may act as diluents and releasing agents for the more volatile active signal components; they could also convey information regarding the individual identity. It could be reasoned that a series of structurally similar compounds, or

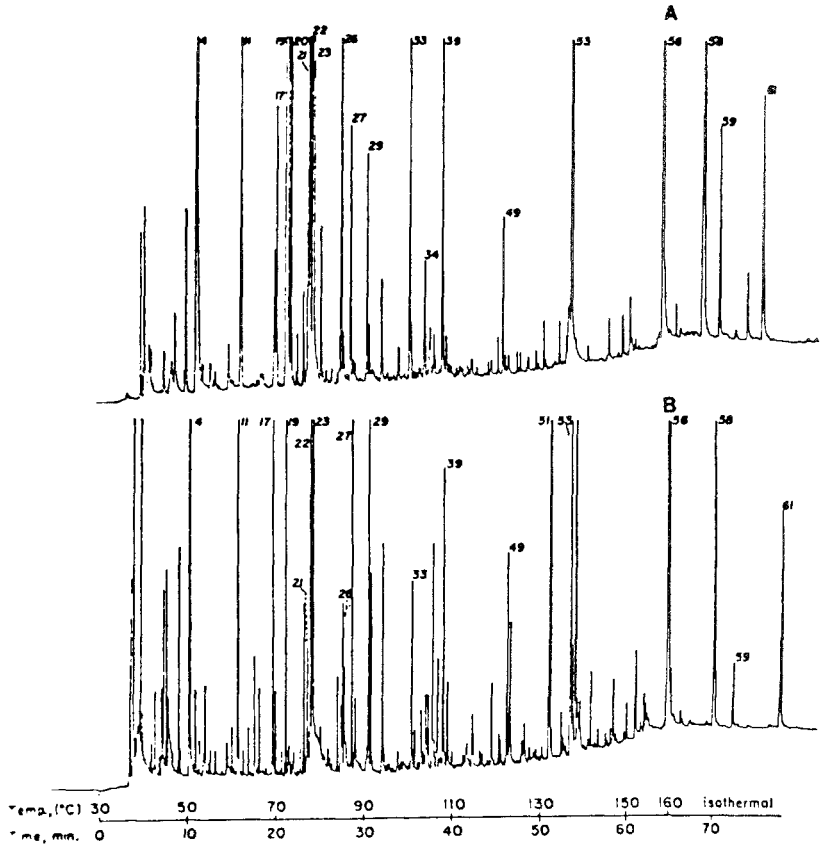

FIG. 5. Comparison of urinary chromatographic profiles for (A) mature, estrogenized female; and (B) immature female. All animals were of C57BL/10 strain. Peak numbers refer to Table 2.

members of a homologous series of odorous compounds, are likely to participate in such individual recognition of mammals. Similar functions could be operative in a number of mammals including the mouse.

Some experimental evidence indicates that the individual recognition and genetic background (including major histocompatibility genes) in the house mouse are conveyed by urinary odors. It has been shown, in restricted laboratory experiments, that mice are able to distinguish between genetically identical individuals in Y-maze tests where only volatile cues were operative (Bowers and Alexander, 1967). Although not necessarily restricted to volatile compounds, individual recognition appears to be an essential component of the pregnancy blocking effect (Bruce, 1965).

In agreement with the above biological rationale, the chemical investigations of our laboratory have demonstrated that genetic background has an observable influence over the relative concentrations of mouse urinary volatiles. While the urinary volatile profiles obtained under different circumstances are qualitatively similar (Novotny et al., 1980), strain differences can be distinguished from quantitative data. As observed recently (Schwende et al., 1984a), this holds true even for congenic mice which differ only in a small section of the major histocompatibility gene complex. Here, the influence of the major histocompatibility complex over the excretion of secondary metabolites makes the quantitative deviations discernible in spite of a great "dilution" caused by the remaining genome. A tentative molecular basis for the observations by other workers (Yamazaki et al., 1976, 1979) regarding the possible influence of the major histocompatibility complex over mating preferences is thus provided.

It is conceivable that complex mixtures of volatiles could serve as integral components of various mouse pheromones. While an active compound (or a limited number of components) may be primarily responsible for a given biological phenomenon, other components may also be essential to provide a suitable olfactory context in order to elicit a typical response. Alternatively, a chemical message may be encoded into the relative proportions and overall intensity of the entire volatile mixture. It is not unreasonable to speculate that the highly developed and integrative mammalian brain could respond in specific ways to the multivariate stimuli presented by relatively complex mixtures of organic compounds such as odors. Thus, it may not be a mere coincidence that the loss of male primer pheromone and certain releaser pheromone activities upon castration or cyproterone acetate treatment is accompanied by an overall decrease of urinary volatiles, as demonstrated in this work.

Similarly, the estrogen-dependent volatiles of female mouse urine may all be required, in specific proportions, to elicit male investigative behavior. The presence of some estrogen-independent volatiles may further be required to produce a typical male response. These possibilities are currently under investigation.

The potential significance of mouse urinary volatiles in chemical communication has led us to pursue their structural characterization. A number of unusual volatile compounds, unique to the mouse urine, have been identified here and reported for the first time. The series of related dihydrofurans is found in both male and female mice, but not in any other species observed to date. Males and females share many of the same volatile components, but their excreted amounts in different sexes vary significantly.

3,4-Dehydro-*exo*-brevicomin has shown a distinct dependency on testosterone levels in the male, suggesting its potential role as a pheromone or a pheromone adjuvant. Structurally similar compounds have sex pheromone activities in the insect world (Silverstein et al., 1968). The two thiazoline com-

pounds are also specific to male mice. We have recently verified biological activities of these substances (Novotny et al., 1985; Jemiolo et al., 1985a, 1986.)

Estrus-related compounds (Schwende et al., 1984b), except for *n*-pentyl acetate, are present in both sexes, but increase dramatically upon the estrogen treatment of females. *n*-Pentyl acetate is clearly associated with females in estrus. Our recent experiments (Novotny et al., 1985; Jemiolo et al., 1986) indicate that certain of these compounds may play an important role in puberty delay of young female mice. Their excretion seems primarily controlled by the adrenal glands, although some participation from the ovaries cannot be ruled out.

Within the scope and limitation of the techniques employed, the major volatile constituents of male and female mouse urine have been chemically characterized. Several mouse-specific compounds have been identified, including 3,4-dehydro-*exo*-brevicomin, and a series of dihydrofurans, the latter of which may all be related to a common precursor keto alcohol, 6-methyl-6-hydroxy-3-heptanone. In addition, the testosterone dependency of 3,4-dehydro-*exo*-brevicomin, the two thiazolines, and *p*-toluidine, as well as the estrogen dependencies of 2-heptanone, *n*-pentyl acetate, *cis*-2-penten-1-ol acetate, *p*-toluidine, and a series of hepten-2-ones, suggest their possible roles as important components of chemical signals.

The quantitative relationships within the complex mixture of volatile compounds in mouse urine (producing a volatile profile in our headspace sampling and chromatographic procedure) may reflect information on the individual identity and genetic background. A detailed chemical knowledge of mouse urine odor may eventually allow investigations of the signaling potential of these compounds, alone as well as in mixtures.

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CHEMICAL SCENT CONSTITUENTS IN URINE OF WOLF (*Canis lupus*) AND THEIR DEPENDENCE ON REPRODUCTIVE HORMONES

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Abstract—The volatile components of castrated male and ovariectomized female wolf urine were investigated and correlated with the administration of testosterone or estradiol and progesterone. The results indicate that testosterone induces in the castrated male the formation of some compounds typically associated with the intact male, while reducing the levels of some compounds associated with castrated male and female. The production of some of the "male" compounds was also induced in the ovariectomized female, although at lower levels. Changes in hormone levels during treatment of females are reflected in the composition of the urinary volatiles. Consequently, many of these compounds could be used to communicate gender as well as reproductive status.

Key Words—Wolf, *Canis lupus*, urinary volatiles, hormone treatment, chemoolfactory communication, testosterone, estradiol, progesterone.

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INTRODUCTION

Scent-marking is a well-recognized and important aspect of mammalian communication (Johnson, 1973; Ralls, 1971). This behavior has been observed in a variety of mammals, ranging from small rodents (Bronson, 1979) to rabbits (Goodrich and Mykytowycz, 1972; Goodrich et al., 1978), ungulates (Moore and Marchinton, 1974), felids (Wemmer and Scow, 1977), and canids (Kleiman, 1966; Fox and Cohen, 1977; Peters and Mech, 1975; Rothman and Mech, 1979). All of these animals use both glandular secretions and urine as the principal means of chemical communication.

The display of marking behavior is often related to the endocrine status or seasonal changes in the hormone levels of marking mammals. Mykytowycz (1974), working with European rabbits (*Oryctolagus cuniculus*), and Thiessen (1973), studying Mongolian gerbils (*Meriones unguiculatus*), both learned that the development and use of scent glands are related to sexual maturity and the presence of gonadal hormones. The main pheromone effects observed in both male and female mice are clearly related to the endocrine status of these animals (Bronson, 1979).

Hormonal influence upon the concentrations of certain excreted chemical scent constituents can often be used to indicate those chemical substances likely to be important in communication; i.e., putative pheromones can be sought in complex biological matrices following endocrinological manipulations of the mammals. In turn, once these pheromone candidates are identified and synthesized in the laboratory, their biological testing becomes feasible. Specifically, this investigational sequence has been applied in the identification and chemical synthesis (Wiesler et al., 1984) of testosterone-dependent volatile constituents of male mouse urine (Novotny et al., 1984) and their testing in promoting intermale aggression (Novotny et al., 1985) as well as behavioral and endocrinological changes in females (Jemiolo et al., 1985). Canids have been similarly studied; characteristic red fox (*Vulpes vulpes*) urinary constituents identified by Jorgenson et al. (1978) were later shown to be attractive to the foxes in their natural habitat (Whitten et al., 1980).

We have also studied the volatile constituents of wolf (*Canis lupus*) urine during the breeding season (Raymer et al., 1984). The present study investigates the hormonal dependencies of these compounds in castrated males and ovariectomized (OVX) female wolves. Changes in the urinary profiles of volatile substances were followed after treating the animals with testosterone, estradiol, and progesterone.

METHODS AND MATERIALS

Tested Animals. For the testosterone supplementation experiment, castrated males 143 and 103 as well as ovariectomized (OVX) females 104 and

147 were used. The males were surgically altered on July 19, 1983 and April 13, 1982, respectively, while both females were altered on July 26, 1983. The experiment in which the animals were treated with female hormones utilized males 39 and 103 along with females 108 and 116. Castration dates for these animals were September 26, 1979, April 13, 1982, January 16, 1980, and January 14, 1981, respectively. All animals were postpubertal at the time of surgery.

Testosterone Treatment. Two castrated male (CM) wolves (103, 143) and two OVX females (104, 147) were given two 200-mg intramuscular injections of testosterone cypionate in cottonseed oil (Depo-Testosterone, Upjohn, Kalamazoo, Michigan), two weeks apart. Each injection resulted in a slow release of the hormone for a period of two to three weeks, based on manufacturer's information and physiological observations. Urine samples from these animals were collected weekly during the following few weeks. The collection procedure involved anesthetizing the animals with sufficient ketamine hydrochloride (Ketaset, Bristol Labs, Syracuse, New York) and promazine hydrochloride (Sparine, Wyeth Labs, Philadelphia, Pennsylvania) to provide adequate relaxation for urine withdrawal by catheter. The urines were placed into acid-washed vials with Teflon-lined caps, immediately frozen, and shipped to Indiana University for chemical analysis. Each animal served as its own control. It was obvious from the physical changes in these animals that sufficient testosterone had been administered to simulate the seasonal testosterone increase. These changes included dramatically increased secretions from the preputial area in the treated castrated males and a secretion from the clitoral fossa in the treated OVX females. These secretions apparently correlated with elevated testosterone levels (Ebling, 1977).

Clitoral enlargement was also noted in the females with the mean dimensions at week zero being 11.5 mm × 6.5 mm (length × diameter) and increasing to 16.5 mm × 10 mm after four weeks of testosterone treatment. This, too, is indicative of elevated testosterone levels (Trimble and Herbert, 1968).

Estrogen and Progesterone Treatments. Two OVX female wolves (108, 116) and two castrated males (39, 103) were treated with 17β-estradiol [E_2 (120–140 mg/implant)] and then with E_2 and progesterone [P_4 (100 mg/implant)] through the use of subcutaneous silastic implants. Hormone treatment approximated the three phases of proestrus (only E_2 elevated), estrus (E_2 and P_4 elevated), and pregnancy (primarily elevated P_4 levels) (Seal et al., 1979). Urine samples were collected as described above. Although radioimmunoassay data indicated an increase in circulating plasma levels of these hormones, the estradiol levels were below those seen in normal proestrus females. However, the OVX females displayed typical signs of proestrus, including bloody vaginal discharge.

In addition, male dogs (although not male wolves) were able to discrimi-

nate between normal anestrous females and the hormone-treated OVX females. Male dogs tried to approach the E_2- and E_2 + P_4-treated OVX wolves but, because there was some doubt as to whether or not it was safe to let them be together, the behavior of the male dogs introduced instead to anesthetized female wolves was observed. The male dogs sniffed and licked at the treated OVX females' vaginal areas, then tried to mount them, exhibiting pelvic thrusting. The male dogs also sniffed longer at treated castrated males than at untreated males. The dogs appeared somewhat confused while investigating the castrated males treated with the female hormones. It is assumed that the lack of response by intact male wolves was due to their relatively low levels of testosterone, since this experiment was conducted during the summer, that is nonbreeding season.

Due to the more complex nature of the female hormone supplementation experiment, controls consisted of urine samples collected from the same animals (with one exception) one year prior. The control animals were OVX 108, OVX 116, castrate 39, and castrate 36. Castrated male 36 served as the control for male 103 because 103 had not been castrated in time to serve as a control in this experiment.

Analytical Procedures. The volatile constituents of the urine samples obtained under the described conditions were analyzed through a headspace sampling procedure (Novotny et al., 1974a,b). Following thermal desorption of volatiles from a porous polymer (Tenax GC, Applied Science Laboratories, State College, Pennsylvania) onto a 60-m × 0.25-mm ID, glass capillary gas-chromatographic (GC) column coated with a polypropylene glycol fluid, UCON-50-HB-2000, the separations were followed with the flame ionization detector (FID) at the column temperature from 30 to 160°C at a rate of 2°/min. Compound identifications were initially aided by the nitrogen-sensitive (thermionic) and the sulfur-sensitive (flame photometric) GC detectors. Peak areas were collected and tabulated through the use of a Perkin-Elmer Sigma 10 Chromatography Data Station. Data obtained from combined capillary gas chromatography–mass spectrometry (GC-MS) provided the major information leading to a positive identification of the urinary constituents. GC-MS data were obtained with a Hewlett-Packard 5982A dodecapole instrument in conjunction with an Incos 2300C GC-MS data system. The majority of the compound identifications discussed below were verified through both retention and mass-spectral data derived from synthetic compounds.

Representative chromatograms for normal (intact) male and female urine volatiles are shown in Figures 1 and 2. The urines sampled to produce these chromatograms were pooled from many animals from November through February, a time which spanned the breeding season. Mass spectral identifications for the numbered peaks of these figures are presented in Table 1.

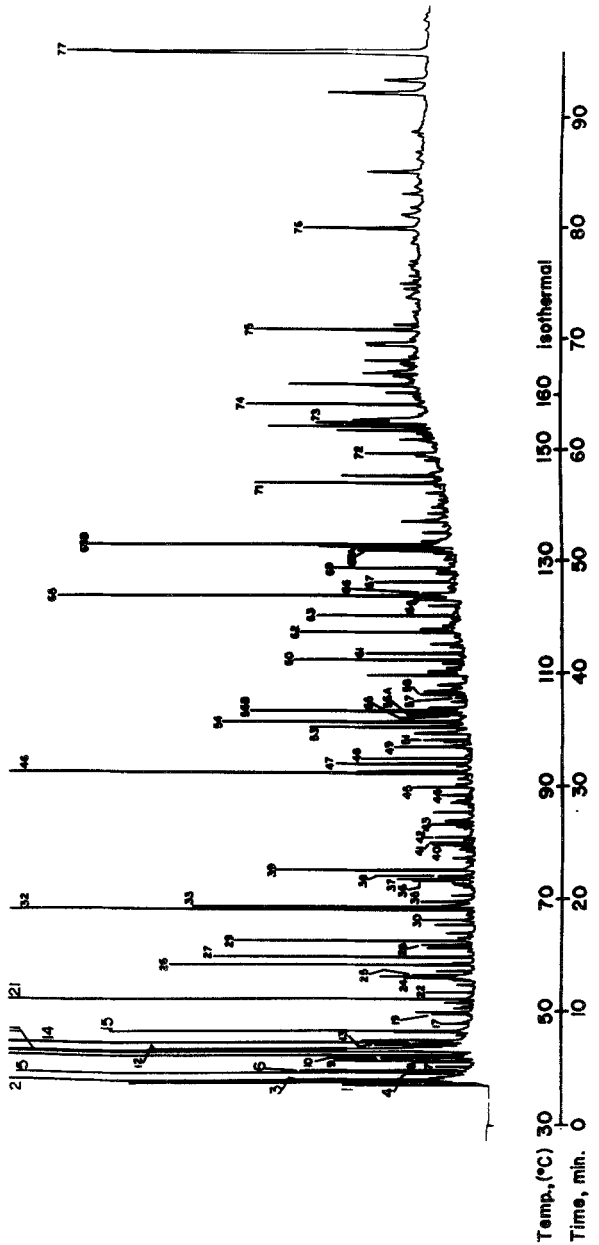


FIG. 1. Volatile profiles of normal male urine followed with the flame ionization detector. Conditions as in the text.

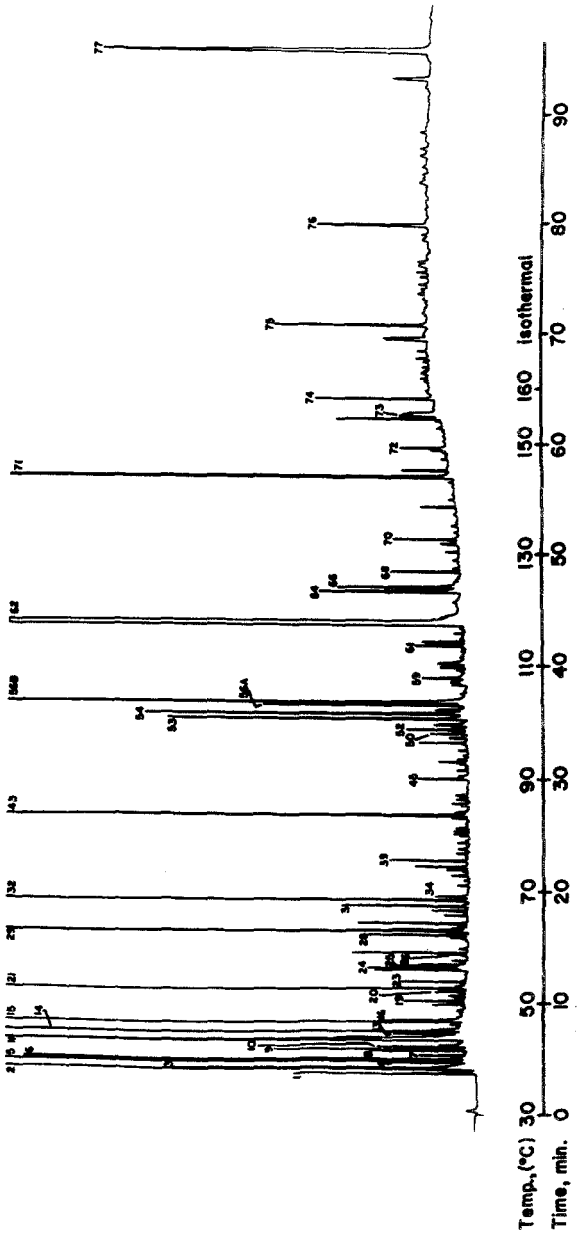


FIG. 2. Volatile profile of normal female urine followed with the flame ionization detector. Conditions as in text.

RESULTS

Testosterone Treatment

Chromatograms resulting from the separation of the volatile components of the urine were examined, and it was determined via this visual pattern recognition that of the many peaks present in each profile, 17 of them appeared to have been changing in intensity during the experiment. These peaks encompassed possible changes in both castrated males and OVX females. Subsequently, the area of each peak was plotted as a function of time in order to verify any trends apparently induced by the hormone treatment.

Male Response. Figure 3 shows the volatile profiles of the urine from an intact male wolf, a castrated male wolf, and finally a castrated male treated with testosterone. Both castrate profiles were derived from urine samples of the same animal. For the most part, this figure summarizes the results indicated by the plots mentioned in the previous paragraph. Figure 4 is a plot of peak area vs. week number following testosterone treatment for castrated male 103 for four of the ketones (numbers 33, 40, 60, and 63 in Figures 1 and 3 as well as Table 1) previously reported (Raymer et al., 1984). Similar trends were seen for castrated male 143. The delay before the increasing levels of these compounds were observed could be the result of "priming" the animal (e.g., induction of certain enzymes). A time delay may be necessary in order to induce the formation or reactivation of hormone receptor sites that have not been stimulated for some time. This testosterone dependence implies a possible importance of the four ketones in chemical communication. Indeed, behavioral testing with the wolves indicates that these compounds are of interest to the animals (C. Asa, unpublished observation).

Among the five sulfur compounds found in the urine of intact male (Raymer et al., 1984), only methyl propyl sulfide and methyl isopentyl sulfide achieved any abundance after testosterone treatment. The peak area corresponding to methyl propyl sulfide increased nearly ninefold for castrate 103 and nearly threefold for castrate 143. The increases observed for methyl isopentyl sulfide were less. However, the trend toward more elevated levels correlated very well with testosterone administration.

The levels of two compounds indicative of the normal state in females (3-ethylcyclopentanone and acetophenone), which have also been shown to become elevated in castrated animals as compared to normal males, were found to decrease somewhat after the animal had been treated with testosterone (Figure 3) (Raymer et al., 1984). Finally, 2-heptanone, a generally common mammalian urine component, which attains a slightly higher average level in normal male wolves than in normal females (unpublished observation), was found to exhibit an elevation in level in both of our castrated males after treatment with

TABLE 1[a]

Peak	Compound	Mol wt	Authenticated	Significant *m/e* (relative abundance)
1	Methyl chloride	50		40(100), 50(2)
2	Ethyl Chloride	64		64(100), 66(30), 49(25)
3	Ethylene oxide?	44		44(100), 43(44), 42(12)
4	Propionaldehyde	58		58(100), 57(32), 42(4)
	Furan	68		68(100)
5	Acetone	58		43(100), 58(29)
6	Acrolein	56		56(100), 55(84)
7*	Dimethoxydimethylsilane	120		105(100), 75(45), 59(18), 45(5), 89(5)
8	2-Methylfuran	82		82(100), 81(55), 53(53), 39(34)
9	Methylene chloride	84		49(100), 51(31), 84(55) 86(38);
	Butanone	72		43(100), 72(16), 57(5)
10*	Dimethoxymethylphenylsilane	182		167(100), 137(78), 69(8), 90(3)
11	Ethanol	46		45(100), 46(35)
12	3-Butene-2-one	70		55(100), 43(88), 70(33)
13	Acetonitrile	41		41(100), 40(66), 39(15)
14*	Hexamethylcyclotrisiloxane	222		207(100), 208(20), 209(12), 191(10)
15	Methyl propyl sulfide	90	A	61(100), 90(68), 48(32), 75(10)
16	2-Pentanone	86		43(100), 86(7), 58(6)
17	3-Pentanone	86		57(100), 86(15), 44(13)
18	4-Ethylcyclohexene	110		54(100), 41(65), 67(42), 39(36), 110(13), 81(10)
19	4-Methyl-2-pentanone	100		43(100), 58(20), 41(14), 57(9)
20	Toluene	92		91(100), 92(57), 55(5)
21	Methyl butyl sulfide	104	A	61(100), 56(70), 104(55), 41(53), 75(15)
22	3-Hexanone	100		57(100), 43(85), 71(30), 100(18)
23*	Octamethylcyclotetrasiloxane	296		281(100), 265(12), 249(8)
24	Hexanal	100		44(100), 56(58), 57(38), 72(12), 82(4)
25	3-Penten-2-one	84		69(100), 41(95), 43(72), 57(22), 84(15)

26	Methyl isopentyl sulfide	118	A	61(100), 70(70), 41(52), 118(50), 55(46)
27	3-Methyl-2-hexanone	114		43(100), 72(30)
28	Unknown	112?		41(100), 55(95), 67(68), 97(52), 43(50), 57(50)
29	4-Heptanone	114	A	43(100), 71(80), 41(23), 114(16), 58(7)
—	Methyl pentyl sulfide	118		54(100), 55(98), 57(98), 67(79), 70(64), 118(48)
30	3-Heptanone	114		57(100), 41(26), 72(13), 85(11)
—	Isopentenyl methyl sulfide	116		61(100), 116(81), 68(34), 67(30), 101(22)
31	3-Methylcyclopentanone	98		55(100), 42(86), 69(77), 98(61)
32	2-Heptanone	114	A	43(100), 58(48), 71(10)
33	4-Methyl-3-heptanone	128	A	57(100), 43(63), 71(42), 86(29)
34	Heptanal	114		43(100), 41(57), 57(35), 70(22)
35	Unknown	?		41(93), 56(89), 57(100), 70(22)
36	3-Methylheptanone	142		43(100), 72(20), 57(9)
—	Methyl propyldisulfide	122		122(100), 80(84)
37	Styrene	104		104(100), 78(30), 51(25)
38	2,4-Dithiapentane	108		61(100), 108(71), 45(38)
39	Cyclohexanone	98		55(100), 42(58), 98(22)
40	3,5-Dimethyl-2-heptanone	142		43(100), 72(40), 57(38), 41(32)
41	3-Octanone	128		43(100), 57(48), 72(24).
42	4-Methyl-2-octanone	142		43(100), 57(33), 71(12), 86(18)
43	3-Ethylcyclopentanone	112	A	55(100), 83(89), 41(57), 112(45), 70(18)
44	6-Methyl-5-hepten-2-one	126		43(100), 55(27), 69(13), 72(13), 108(7)
45	Furfural	96		95(100), 96(83), 39(48)
46	3,5-Dimethyl-2-octanone	156	A	43(100), 72(62), 57(22), 85(9)
—	Dipropyl disulfide	150		150(100), 43(82), 41(57), 108(55)
47	Like No. 46 but not a stereoisomer	156		43(100), 72(60), 57(23), 85(9)

TABLE 1[a] Continued

Peak	Compound	Mol wt	Authenticated	Significant *m/e* (relative abundance)
48	3-Nonanone	142	A	57(100), 43(74), 72(60), 85(43)
49	4-Methyl-3-nonanone?	156?		57(100), 43(28), 86(10)
50	2-Acetylfuran	110		43(100), 95(63), 58(60), 110(12)
51	2-Nonanone	142		43(100), 58(92), 71(15)
52	3-Propylcyclopentanone	126		55(100), 44(93), 83(65), 97(28), 126(20), 69(15)
53	Benzaldehyde	106	A	105(100), 106(87), 77(85), 51(45)
54	A Methylvinylpyrazine	120		120(100), 52(63), 94(10), 66(5)
55	6-Methyl-1-hepten-3-ol	128?		57(100), 72(23), 85(15), 109(6)
56A	*N*-Acetylpyrrole	109		67(100), 109(22), 40(21)
56B	1-Octen-3-ol	128		57(100), 55(30), 43(28), 70(15), 72(10), 85(5)
57	2-Methylpyrrole	81		44(100), 80(59), 81(23)
58	3-Methyl-2-decanone or 3,5-dimethyl-2-nonanone			43(100), 72(70), 57(15), 85(5)
59	2-Ethyl-1-hexanol	130		57(100), 35(65), 70(23), 83(13)
60	3,5,7-Trimethyl-2-nonanone	184	A	43(100), 72(80), 57(38), 85(16)
61	C_2-vinylpyrazine	134		52(100), 134(70), 93(10)
62	Acetophenone	120	A	105(100), 77(68), 51(26), 120(21)
—	Propyl pentyl disulfide	178		43(100), 178(91), 108(75), 71(63)
63	3,5-Dimethyl-2-decanone	184	A	43(100), 72(75), 57(30), 85(6)

64	2-Octen-1-ol	128		57(100), 41(79), 55(64), 67(20), 81(20)
65	3,5,7-Trimethyl-2-decanone	198	A	43(100), 72(73), 57(32), 85(20)
66	*o*-Chlorobenzaldehyde	140		139(100), 140(70), 141(40), 142(20), 111(25), 75(20)
67	3-Methyl-2-undecanone?	184?		43(100), 72(70), 57(20), 85(12)
68	Phenylacetone	134		43(100), 91(35), 65(10), 134(10)
69	4-Methyl-3-dodecanone	198		57(100), 86(62), 43(43), 71(6)
69A	(see text)	184		57(100), 72(92), 43(74), 95(60)
69B	(see text)	198		86(100), 57(70), 71(26), 85(31), 99(16)
70	1-Phenyl-2-propyl acetate	178		43(100), 121(38), 118(27), 117(20), 77(13)
71	*o*-Chlorobenzonitrile	139		137(100), 139(33), 102(22), 75(12), 50(10)
72	Benzothiazole	135		135(100), 108(23), 119(10)
73	Phenol	94		94(100), 66(15), 55(8)
74	Hexadecane?	226?		57(100), 43(82), 71(48), 85(20)
75	Heptadecane?	240?		57(100), 43(95), 71(46), 85(28)
76	Octadecane	254		57(100), 45(80), 71(45), 85(20)
77*	Diethyl phthalate	222		149(100), 177(19), 105(6), 104(5), 76(5)

[a] Identification of peaks numbered in Figures 1 and 2 based on 70 eV mass spectra. "A" indicates that the compound has been authenticated by retention times and mass spectra of synthetic substances. Compounds marked with an asterisk are artifacts of the methodology.

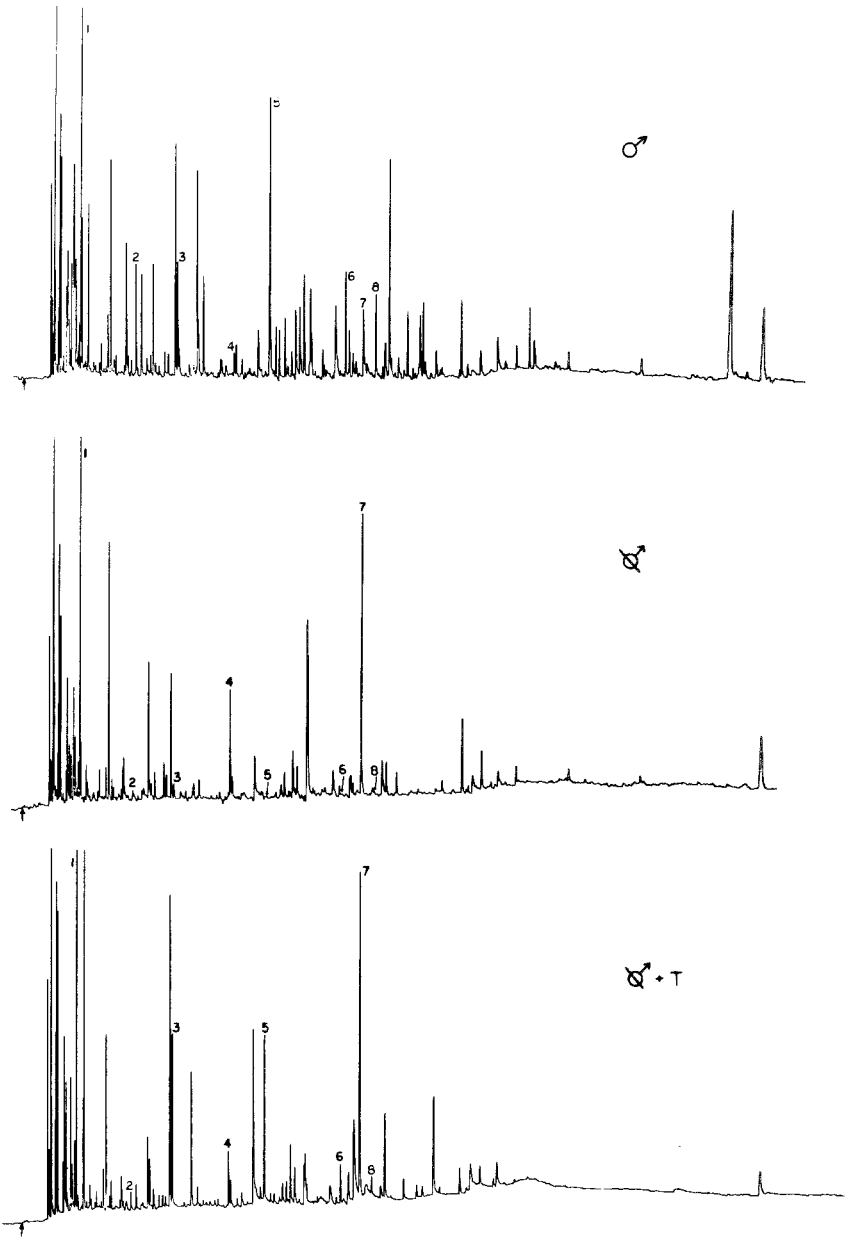


FIG. 3. Comparison of urinary volatile profiles of normal male (top), castrate male (center), and the same castrate male after treatment with testosterone. Compound identifications are: (1) methyl propyl sulfide, (2) methyl isopentyl sulfide, (3) 4-methyl-3-heptanone, (4) 3-ethylcyclopentanone, (5) 3,5-dimethyl-2-octanone, (6) 3,5,7-trimethyl-2-nonanone, (7) acetophenone, (8) 3,5-dimethyl-2-decanone.

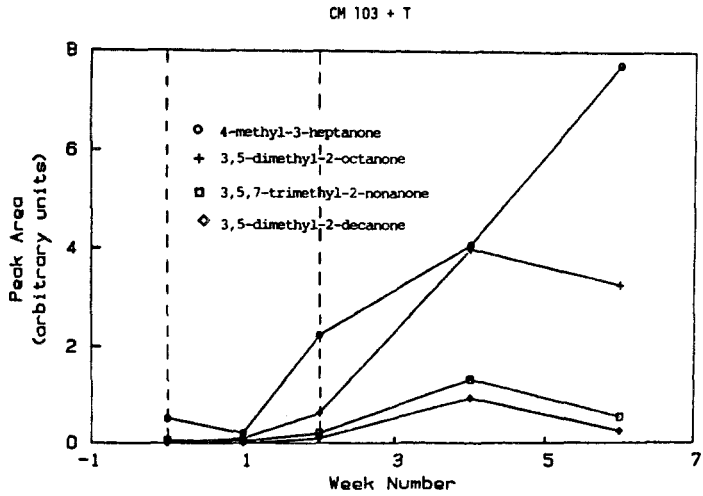


FIG. 4. Peak area vs. week number after testosterone treatment for the four carbonyl compounds induced by treatment of castrate male 103 with testosterone. The vertical dotted lines indicate when the animal was injected with the hormone.

testosterone. The same compound in the OVX females supplemented with testosterone showed no clear change.

The data presented above are entirely self-consistent since some compounds associated with intact males increased in the urine, while the compounds associated with intact females or castrate males were decreased, upon treatment with testosterone.

Female Response. The results of the experiment in which OVX females were treated with testosterone seemed, generally, to parallel the effects seen in the castrate males. The most striking change was the induction of increased urinary methyl propyl sulfide and methyl isopentyl sulfide (Figure 5). Some elevation of the ketones (for example, 3,5-dimethyl-2-decanone) was noted, although the relative amounts produced were much less than for the male. No changes were observed in the levels of 3-ethylcyclopentanone, but the acetophenone level was suppressed for at least three weeks beginning three weeks after the initial injection of testosterone. The average peak area (in arbitrary units) was 23.6 before the suppression and 0.7 afterward. These results clearly indicate that testosterone somehow inhibits the metabolic pathway leading to the formation of acetophenone.

Female Hormone Regimen

The volatile profiles for this part of the experiment were analyzed in the same manner as those above. Thirty-eight peaks were tabulated and compared to the same 38 peaks from the control data set.

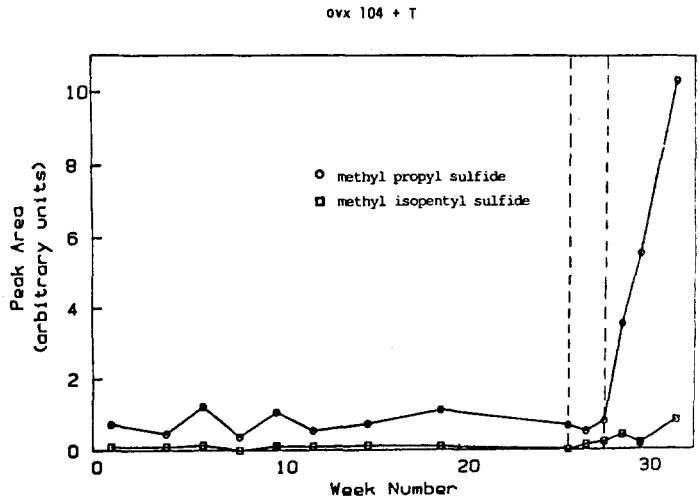


FIG. 5. Peak area vs. week number after testosterone treatment for the two sulfur-containing compounds induced by treatment of OVX 104 with testosterone. The vertical dotted lines indicate when the animal was injected with the hormone. The injections for both castrate and OVX animals took place at the same time. Therefore, week 0 of Figure 4 is not the same as week 0 here.

Female Response. Changes in the levels of various urinary compounds in treated females compared to the controls were relatively few. The compound exhibiting the most dramatic change was benzaldehyde (Figure 6). There is a clear elevation of this compound when estradiol is being released into the animals' bodies. During both E_2 and P_4 administration, regardless of which hormones were present, decreases were seen for acetophenone (although the greatest suppression was seen with progesterone alone) and peaks 11 and 29, which remain unidentified. (The mass spectrum for peak 11 suggests a $C_8H_{14}O$ ether or a $C_7H_{10}O_2$ diether. The mass spectrum for peak 29 suggests an alcohol with two positions of unsaturation). Therefore, there is evidence that the urine volatiles change as females approach estrus. This is further supported by behavioral observations that during this period of proestrus, the male wolves become very much interested in the urine of the females as evidenced by their sniffing and licking (C. Asa, unpublished observation).

Male Response. The most striking change in the castrated males treated with the female hormone E_2 was an increase in urinary benzaldehyde, as in the females (Figure 7). The fact that the response of castrated male 39 was not as clear as for animal 103 may be explained by one or both of the following. First, male 103 was castrated six months before the experiment, while male 39 had been castrated for about three years. This length of time may have altered his

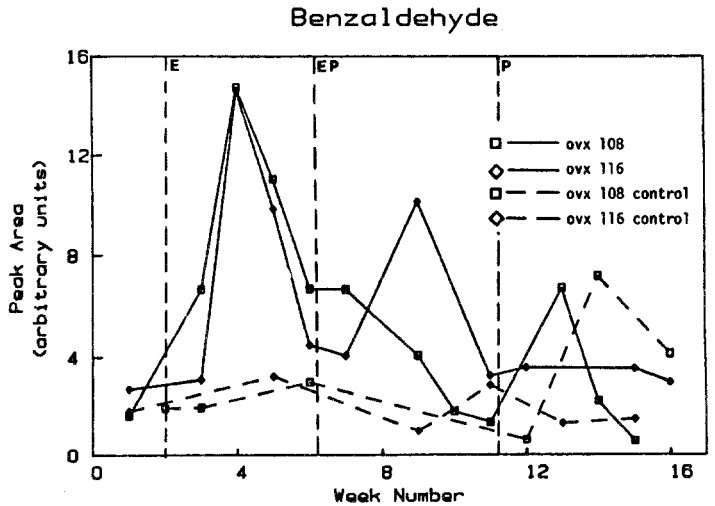


FIG. 6. Peak area of urinary benzaldehyde vs. week number for the experiment in which OVX females were treated with female hormones. The vertical dotted lines represent the time of implantation. E stands for 17β-estradiol, and P stands for progesterone. The letters indicate the hormones that were being introduced into the animal for the specified time period.

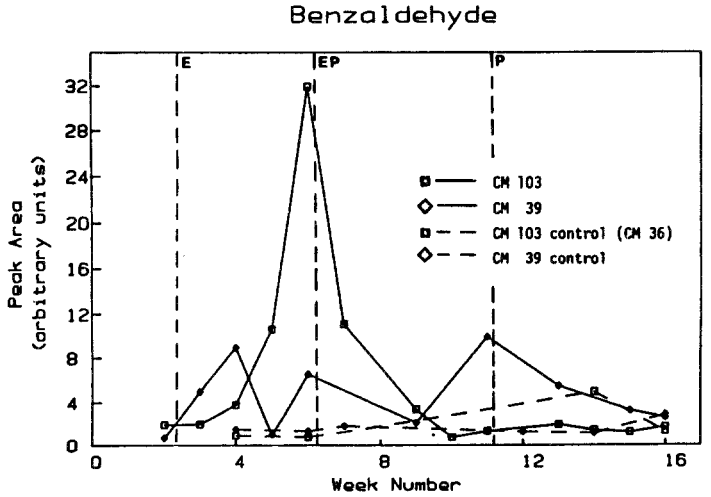
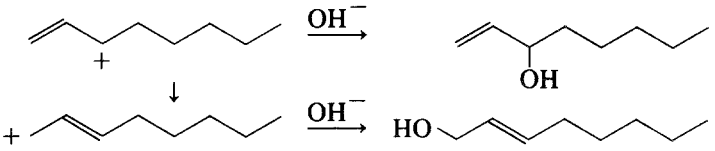


FIG. 7. Peak area of urinary benzaldehyde vs. week number for the experiment treating castrate males with estradiol and progesterone.

"ability" to respond. In addition, male 39 was considerably older than male 103.

Methyl propyl sulfide was generally decreased in both animals when both E_2 and P_4 were present. This observation becomes of interest when contrasted with the elevating effect of testosterone upon this compound. Finally, 1-octen-3-ol and 2-octen-1-ol were elevated in castrated male 103 (but not male 39, probably for one of the above-mentioned reasons) when both estradiol and progesterone were in the system. The fact that both alcohols follow the same trend can be explained by their common relationship to a single-resonance stabilized cation:



DISCUSSION

The testosterone dependence of a number of compounds found in the volatile fraction of normal male wolf urine, but absent or greatly diminished in castrated male urine has been demonstrated. This dependence opens the possibility that these compounds might serve to indicate to mature female wolves the degree of sexual maturity of prospective mates. The increased scent-marking that wolves perform as breeding season approaches (Peters and Mech, 1975), as well as greater frequency of marking in newly formed pairs (Rothman and Mech, 1979), is consistent with this hypothesis.

In addition, acetophenone and 3-ethylcyclopentanone, two compounds characteristically high in female urine and found to increase in male urine after castration, tended to decrease upon treatment of castrate males with testosterone. When OVX females were treated with testosterone, acetophenone decreased, and the animals began producing compounds characteristic of males. Treatment of OVX females with estradiol and progesterone caused changes in the urinary volatiles that could communicate to conspecifics their approaching estrus. The urinary volatiles of the castrate male treated with female hormones also changed but did not parallel exactly the changes observed in the female.

Treatment of animals with hormones of the opposite gender raised some questions concerning the biogenesis of the urine compounds. For example, why is benzaldehyde formation induced by estradiol treatment? The amounts seen in the urine are far too large to be explained simply as metabolites of the estrogen, indicating that a metabolic pathway common to both male and female wolves is important here. The trends elucidated in this work may help to clarify some questions about mammalian biochemistry.

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Book Review

Techniques in Pheromone Research. Hans E. Hummel and Thomas A. Miller (eds.). New York, Springer Verlag, 1984. $59.00, 464 pp.

This book is published within the Springer Series in Experimental Entomology edited by Thomas A. Miller. It contains 14 chapters written by leading experts on the topic they discuss. As it focuses on the techniques used in pheromone research and its advances during the last decade, it occupies a special place among the books on pheromones and chemical ecology. It is particularly useful for the specialist wanting an up-to-date account of available methods and research equipment which could serve his purpose. The book starts with a chapter written by two pioneers who inaugurated the field of pheromone research: E. Hecker and A. Butenandt. It is entitled "Bombykol Revisited" and makes not only interesting reading for the student of the present history of science, but also mentions the more recent identification of companion compounds of bombykol, suggested routes for the biogenesis of this pheromone, the metabolism of tritium-labeled bombykol in the antenna, and its importance with respect to the molecular mechanism of action of pheromones.

The next four chapters are concerned with assays. Chapter 2, by T.C. Baker and R.T. Cardé, on techniques for behavioral bioassays, includes general considerations for conducting bioassays and contains several examples, divided into assays with and without moving air, in both cases with and without monitoring of the displacement. Chapter 3, by T.C. Baker and C.E. Linn on wind tunnels in pheromone research, and Chapter 4, by R.T. Cardé and J.S. Elkinton on field trapping with attractants, are linked in subject matter to Chapter 2. Chapter 4 also contains data of practical importance in pest management, such as trap design and trap placement. Chapter 5 by W.L. Roelofs deals with electroantennogram assays. Their importance as rapid and convenient screening procedures is well-known. The technique, pioneered by Schneider in 1957 has been developed by Roelofs and others to the present almost indispensible tool for screening, isolation, and identification of pheromones. Its broad applicability is illustrated by numerous examples in this chapter.

The three chapters that follow describe the use of electrophysical or behavioral assays as detectors in gas chromatography. Gas chromatographic separation usually preceeds EAG in the investigation of natural pheromones. In Chapter 6, on combined gas chromatography and electroantennogram record-

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ing, D.L. Struble and H. Arn show how these two different techniques can be combined, using the antenna as a selective and sensitive detector (EAD) which, if desired, can be used simultaneously with an FID detector. Their paragraph on GC–single sensillum recording (GC-SSR) forshadows the discussion in Chapter 7 by L.J. Wadhams on the coupled gas chromatography–single cell recording technique (GC-SCR). These most recent aids in pheromone research, independently developed by Wadhams (1982), who worked with beetles, and Van der Pers and Löfsted (1983), working with a moth, are of particular interest in the study of some fundamental questions which arise when studying multicomponent pheromones. As GC-SSR and GC-SCR have the advantage of differentiating among responses of different receptor cells but provide information on only one type of sensillum or cell, these techniques offer a complement rather than a substitute for the GC-EAD technique.

H.E. Hummel, in Chapter 8 on the tandem gas chromatography-behavior assay, describes another coupling of two classical techniques in pheromone research. The author emphasizes the advantage of combining the virtues of the two methods, but although he does not ignore the inherent problems, he treats them as of little importance. The simultaneous presence of several compounds in a critical ratio is often necessary for activation of insects, and this is one of several factors that limits the applicability of coupling the two techniques. The others include memory effects resulting from previous runs and solvent effects.

The next five chapters deal with chemical techniques, analytical as well as synthetic. In Chapter 9, L.K. Gaston gives a brief but lucid account of techniques and equipment for collection of volatile chemicals. These techniques serve not only the identification of pheromones from natural sources but are of equal importance in developing a rational application of controlled release formulation where rates of evaporation have to be measured.

Collection of pheromones is also the subject of the much more elaborate Chapter 10 by M.A. Golub and I. Weatherston on techniques for extracting and collecting of sex pheromones. Their 52-page chapter is an extensive review containing many detailed drawings of a variety of collection apparatus and two long but conveniently arranged tables of the extraction and effluent collection methodologies as used by different authors.

Chapter 11, by R.R. Heath and J.H. Tumlinson on techniques for purifying, analyzing, and identifying pheromones, treats the processes usually following the collection of pheromones. Many of the techniques described were initially developed and are still used for much larger amounts of material and require modifications and refinements to make them useful for pheromone analyses. Without attempting to be complete, the authors give an adequate review of the present techniques and briefly give their view on future developments.

Chapter 12, by K. Mori, deals with the significance of chirality and methods for determining absolute configuration and optical purity. The wealth of

data and the many examples show how rapid the development of these methods has been and how complicated the relationship between chirality and pheromone activity can be. This chapter is not only important for pheromone scientists but for all those interested in chemistry of natural products and in structure–activity relationships.

Chapter 13, by P.E. Sonnet, tabulates selected methods of syntheses frequently employed for insect sex pheromones. It contains a rather complete survey of the relevant literature up to 1982.

The concluding Chapter 14, by D.G. Campion, surveys pheromone uses in pest control, the practical goal of most pheromone research. Although most of these uses are also found in other books or chapters on pest management by pheromones, the present book would be incomplete without this chapter.

This volume is recommended primarily to all scientists involved in pheromone research; it also contains data of general interest to ethologists, electrophysiologists, and analytical and organic chemists. Other fields of chemical ecology, such as those of microorganisms and mammals, may benefit from some of the techniques described, but it should be emphasized that the book only describes applications in the entomological field.

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BIOORGANIC CHEMISTRY OF COMMUNICATION SYSTEMS

This issue arose from a symposium which I organized for the Northeastern Regional Meeting (NERM-15) of the American Chemical Society held on June 24 and 25, 1985, at New Paltz, New York. It was my intention to bring together scientists in the northeastern United States and Canada who were engaged in studying biological chemistry of sensory systems of plants, vertebrates, and insects. A prejudice for the newer subdiscipline which I call pheromone biochemistry—the study of pheromone production, reception, and removal on a molecular level—will be evident in the topics selected.

This issue is organized into three main areas: (1) pheromone chemistry, biochemistry, and perception in insects; (2) chemical blends and their perception in vertebrates; and (3) biochemical events in plant–plant and plant–insect interactions.

Pheromone biosynthesis in female moths is examined in three different ways. Bjostad and Roelofs use fatty acyl precursors multiply-labeled with stable isotopes of carbon and hydrogen to examine the origins of (*E*)- and (*Z*)-11-tetradecenyl acetates in the red-banded leafroller moth (*Argyrotaenia velutinana*). Pheromones and glandular lipids were analyzed using GC-MS, with chemical ionization and selective ion monitoring to optimize sensitivity. The importance of functional groups in pheromone specificity is explored in the spruce budworm moth (*Choristoneura fumiferana*) enzymatic studies with radiolabeled precursors by Morse and Meighen. De novo fatty acid synthesis and interconversions of acid, ester, alcohol, and aldehyde functionalities can be observed in glandular tissues by specific enzymes. As Ding and Prestwich indicate, the biosynthetic and catabolic enzymes which catalyze these functional group interconversions are broadly distributed in tissues of the tobacco budworm moth (*Heliothis virescens*). Teal and Tumlinson describe the production of nonpheromone aldheydes by topical application of exogenous fatty alcohols to pheromone glands of female *Heliothis virescens* moths. They conclude that the supply of an alcohol precursor to an oxidase system is rate-limiting for pheromone production. Finally, Bell and Meinwald describe chiral pyrrolizidine aphrodisiac pheromones of two male arctiids (*Creatonotos* spp.) and their derivation from ingested plant alkaloids. They also document new alkatrienes and a chiral C_{21} diene epoxide in females of these arctiid moths.

Enzymes which degrade pheromones are essential for clearing sensory hairs

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and hydrophobically adsorbed pheromone on the integument. Prestwich, Vogt, and Riddiford describe experiments which clarify the specificity of the sensillar esterase of male wild silkworm moths (*Antheraea polyphemus*). With radiolabeled pheromone (6*E*, 11*Z*-16:Ac) and pheromone analogs of very high specific activity (58 Ci/mmol), physiologically meaningful concentrations (10 nM) were possible in these assays. In contrast to this soluble esterase found only in male antennal sensory hairs, Vogt reports that a second, cuticle-bound pheromone esterase is present in body scales of both sexes of this moth but not in unrelated species.

Pheromone processing in the aldehyde-producing moth *Heliothis virescens* is examined by Ding and Prestwich, using stoichiometrically tritiated Z9-14:Al to study tissue and subcellular location of the expected aldehyde dehydrogenase (ALDH). ALDH was highest in both male and female antennae and was also found as the most active enzyme together with alcohol oxidase (Z9-14:OH as substrate) and acetate esterase (Z9-14:Ac as substrate) in legs and glandular tissues. Similarly, Lonergan reported GC-MS evidence for the conversion of the pheromonal *E*11-14:Al to *E*11-14:Acid in all tissues of the male and female spruce budworm *Choristoneura fumiferana*. Many of the findings in these two papers were foreshadowed by related studies on *C. fumiferana* summarized by Morse and Meighen.

Pheromone reception is examined in two papers. O'Connell, Beauchamp, and Grant summarize their continuing work in characterizing electrical activity of receptor neurons in individual sensilla to single and multiple component stimuli. The neural mechanism for encoding of information from multicomponent pheromone blends is still poorly understood. Silk and Kuenen review the chemistry and behavioral responses for pheromones of the *Choristoneura species*, with specific attention to the unsaturated fatty acids in *C. fumiferana* glands. They describe the synchronized variations in Δ11-14:Acid, Δ11-14:Ac, and Δ11-14:Al, and search for additional pheromone components by screening biosynthetically related compounds for behavioral activity.

Next, two authors review work of their research groups on vertebrate chemical communication systems. Doty provides an overview of the relationships between psychophysiological measures of human olfactory perception as they are influenced by endocrine function in both sexes. Belcher presents a summary of the work in the Epple and Smith groups directed at decoding the complex information contained in scent marks of the tamarin *Saguinus fuscicollis*. Butyrate esters and squalene are found in skin glands in two subspecies and can be distinguished using chemical pattern recognition analysis of GC-MS data.

Finally, I felt that no symposium on the chemistry of communication systems would be complete without a look at selected studies on plant–plant and plant–insect interactions. Thus, Ahmad provides a review of the enzymic strat-

egies which insects and mites use to deal with phytochemicals. He offers specific data on the variation and multiplicity of MFOs in gypsy moth larvae during maturation. Finally, Lynn and Chang review the chemistry of host recognition in parasitic angiosperms, and the properties of a specific class of chemicals, the xenognosins, which induce development of the haustorium.

I hope that the readers of this *Journal of Chemical Ecology* symposium volume share vicariously in the stimulating research described in these papers. The ability to achieve a molecular-level understanding of production and regulation of communicative chemicals and the unraveling of the stepwise biochemical events involved in their reception, transduction, and catabolism are important goals for research in chemical communication in the next decade.

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BINDING AND HYDROLYSIS OF RADIOLABELED PHEROMONE AND SEVERAL ANALOGS BY MALE-SPECIFIC ANTENNAL PROTEINS OF THE MOTH *Antheraea polyphemus*

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Abstract—Sensory hair proteins from antennae of males of the wild silk moth, *Antheraea polyphemus* (Lepidoptera, Saturniidae) were incubated with radiolabeled 6*E*,11*Z*-hexadecadienyl acetate in the presence of unlabeled pheromone analogs as competitive inhibitors. The two extracellular proteins of importance, a highly active sensillar esterase and an abundant 15,000 mol wt binding protein, interact to degrade labeled pheromone less efficiently in the presence of certain unsaturated acetate analogs of the natural pheromone.

Enzymatic hydrolysis of the acetate (or diazoacetate) was also examined for three pheromone analogs: [11,12-$^3H_2$]-6*E*,11*Z*-hexadecadienyl diazoacetate, [11,12-$^3H_2$]-hexadecyl acetate, and [9,10-$^3H_2$]-9*Z*-tetradecenyl acetate. The former two are poor substrates at concentrations over four orders of magnitude. The 9Z–14 : Ac, however, is the best alternative substrate for this in vitro pheromone metabolism system. Unlabeled 9Z–14 : Ac is also the best competitive inhibitor of the hydrolysis of labeled 6*E*,11*Z*–16 : Ac. Whereas the tritiated natural pheromone shows a flat response (ca. 40% conversion) to increasing concentrations from 3×10^{-9} to 3×10^{-6} M, tritiated 9Z–14 : Ac is degraded more rapidly at higher concentrations.

Key Words—*Antherea polyphemus*, Lepidoptera, Saturniidae, wild silk moth, radiolabeled pheromone, pheromone binding, pheromone hydrolysis, antennal proteins.

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INTRODUCTION

The antennae of the male giant silk moth *Antheraea polyphemus* show remarkable sensitivity and specificity in detecting the sex pheromone of this species. The pheromone of this species consists of two chemical components, 6*E*,11*Z*-16:Ac and 6*E*,11*Z*-16:Ald, with the acetate component being the more active compound (Kochansky et al., 1975). Each of the approximately 55,000 pheromone-sensitive sensory hairs per antenna possesses a pair of neurons, with one neuron of each pair being excited by only one of these pheromone components (Boeckh and Boeckh, 1979).

Vogt and Riddiford (1981), in studying the biochemical components of these sensory hairs, identified two proteins uniquely situated in the extracellular receptor lymph of these pheromone-sensitive sensory hairs: a pheromone-binding protein and a sensillar esterase. The binding protein (mol wt = 15,000 daltons) is soluble and present in a concentration of 20 mM (Vogt and Riddiford, 1981). Although this binding protein shows a low affinity for the pheromone, most pheromone is protein bound in vivo because of the extremely high concentration of binding protein (Vogt and Riddiford, 1986). The sensillar esterase, mol wt 55,000 daltons (Kaissling et al., 1985), is present at a concentration at least 10^4 lower than the binding protein (Vogt, 1984). This esterase is extremely aggressive towards the acetate component of the pheromone. We estimated that the half-life of pheromone in the presence of a physiological concentration of this esterase would be approximately 15 msec (Vogt et al., 1985). Hydrolysis of *Z*7-12:Ac by antennal esterases in *Trichoplusia ni* has been previously studied by Ferkovich (1981) and coworkers.

We have now begun to study the biochemical components of these sensory hairs utilizing a photoaffinity analog of the natural pheromone (Vogt et al., 1985, 1986). The analog 6*E*,11*Z*-16:DZA, in which the diazoacetate (DZA) group is substituted for the normal acetate moiety, showed 10% of the electrophysiological activity of the acetate (Ganjian et al., 1978). Unlabeled unsaturated 16:DZA isomers associate with varying specificity to the same proteins with which the natural pheromone associates, i.e., the sensillar esterase, the binding protein, and the putative receptor protein. Furthermore, when a DZA-protein complex is irradiated with UV light, the analog may become covalently attached to the complexed protein. Indeed, Prestwich et al. (1984a) have prepared tritium-labeled 6*E*,11*Z*-16:DZA (58 Ci/mmol), and Vogt et al. (1986) have obtained evidence for pheromone-competable, light-activated irreversible attachment of the DZA to soluble and membrane-bound proteins. Photoaffinity-labeled JH analogs have also been shown to be useful in understanding insect protein–ligand interactions (Prestwich et al., 1984b).

In this paper, we report how several pheromone analogs interact with the sensillar esterase of *A. polyphemus*. Our purpose was twofold. First, we wished to know whether the photoaffinity analog would bind to or be degraded by the sensillar esterase. It is essential to know whether or not the probe would be

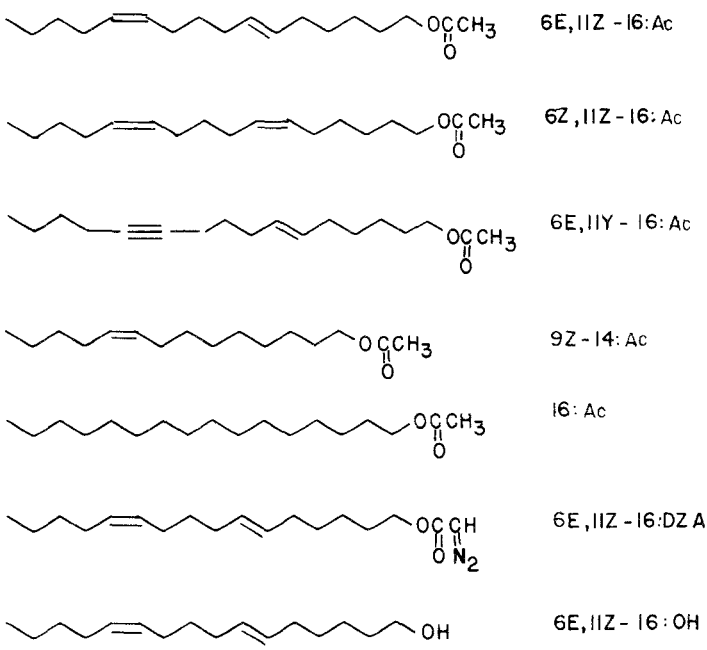

FIG. 1. *A. polyphemus* pheromone and pheromone analogs (unlabeled) used as competitors.

cleaved when complexed to a pheromone-relevant protein. Second, we wished to further examine the substrate selectivity of the soluble biochemical system responsible for pheromone transport and degradation within these sensory hairs. To these ends, a variety of unlabeled pheromone analogs were examined in enzymatic competition studies (Figure 1), and three radiolabeled pheromone analogs were synthesized and tested as alternative substrates (Figure 2).

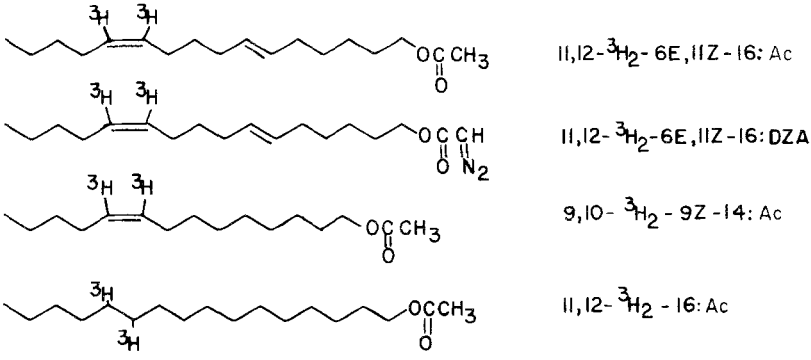

FIG. 2. High-specific-activity tritium-labeled *A. polyphemus* pheromone and pheromone analogs used as alternative substrates.

METHODS AND MATERIALS

Animal and Enzyme Sources. Antheraea polyphemus were purchased as pupae, stored in diapause at 4°C, and reared to adults at 25°C on a 18:6 light–dark photoperiod.

The enzyme preparation used in these studies was the unpurified homogenate of pheromone-specific sensory hairs isolated from the antennae of freshly emerged male moths. The technique of isolating sensory hairs was modified from Klein (Klein and Keil, 1984; Vogt, 1984, Vogt et al., 1983a,b). Branches of antennae were mixed with equal volumes 0.1-mm and 0.5-mm diameter glass beads (B. Braun, West Germany) in a lyophilization flask. The flask was lowered into liquid nitrogen and shaken violently by hand for 5–10 sec. The flask was then rechilled and the process was repeated several times. Freezing made the antennal tissue very brittle, and shaking the flask caused the glass beads to break the sensory hairs from the branches at the hair bases.

Sensory hairs were separated from glass beads and branches by repeatedly pouring the lyophilized material down a trough made of Mylar film. The sensory hairs adhered to the film, and the remaining glass beads and antennal branches were shaken away. Hairs were scraped from the Mylar film using the edge of a coverslip, and were stored in sealed microfuge tubes at −70°C. This preparation routinely required additional removal of branches using forceps under a dissecting microscope. One hundred fifty antennae would yield on the order of 20 mg lyophilized sensory hairs.

Sensory hair homogenates were prepared using an ice-cooled ground-glass microhomogenizer (Bolab), first dry and then with repeated washings with 200-μl aliquots of pH 7.2 mM Tris HCl buffer. A final concentration of 1 mg hair/ml buffer was routinely used (220 μg protein/ml). Residual hair cuticle was removed by centrifugation (12,000 *g*, 5 min, 4°C). Fresh protein solutions were prepared for each day's experiments. In addition, dilutions of the stock hair protein solutions were made just prior to a given substrate of competition assay.

Synthesis of Pheromone Analogs. [11,12-$^3H_2$]-Hexadecyl acetate was prepared by complete reduction of a partially overreduced sample of [11,12-$^3H_2$]-6*E*,11*Z*-hexadecadienyl acetate, prepared as described previously (Prestwich et al., 1984a). Thus, 5 mg of PtO_2 and several milligrams of tritium-labeled acetate (ca. 500 mCi) were stirred 16 hr in 2 ml methanol under an atmosphere of H_2. The product was purified by flash chromatography (5% ethyl acetate–hexane) (Still et al., 1978) in a disposable pipet to give TLC-homogeneous material with a nominal specific activity of 58 Ci/mmol.

[9,10-$^3H_2$]-(*Z*)-9-Tetradecenyl acetate was prepared by reductive tritiation of the 9-tetradecyn-1-yl acetate. The latter was prepared by Y.-S. Ding (Stony Brook) by alkylation of 1-tetrahydropyranyloxy-8-bromooctane with the acetylide of 1-hexyne in THF–HMPA (Henrick, 1977). Acid hydrolysis of the THP ether (CH_3CO_2H–THF–H_2O = 4:2:1), acetylation (Ac_2O, Py), and flash chromatography provided the necessary starting material.

The tritiation was carried out at the NIH Tritiation Facility at Lawrence Berkeley Laboratory. To a sample of 10 mg of 9-tetradecyn-1-yl acetate in 5 ml of hexane was added 3 mg of 5% $Pd/BaSO_4$ and 10 μl of a 100 $\mu g/\mu l$ hexane solution of quinoline. The reaction vessel was freeze-degassed and the reaction was stirred 20–30 min under 1 atm of carrier-free T_2 gas. The reaction was stopped at ca. 60% conversion. Prior experience indicated that taking the reaction to completion generated hard-to-remove overreduction products. The tritium and volatiles were removed in vacuo to reduce the volume by half, and the crude product mixture was centrifuged to remove the catalyst. The supernatant was concentrated under N_2 and chromatographed on flash silica gel with 3% ethyl acetate–hexane and then on 20% $AgNO_3$-coated flash silica gel with 6% ethyl acetate–hexane to give complete separation of the desired [9,10-$^3H_2$]-9*Z*-14:Ac (58 Ci/mmol) from the alkyne precursor.

[11,12-$^3H_2$-Hexadecadienyl diazoacetate (58 Ci/mmol) was prepared from the corresponding radiolabeled acetate by hydrolysis and diazoacetylation as described previously (Prestwich et al., 1984a).

Unlabeled analogs were available as intermediates from prior synthetic work. Thus, 6*E*,11*Z*-hexadecadien-1-ol, 6*E*-hexadecen-11-ynyl acetate, and 6*E*,11*Z*-hexadecadienyl diazoacetate were prepared as described elsewhere (Prestwich et al., 1984a). 6*Z*,11*Z*-Hexadecadienyl acetate was obtained by $AgNO_3$–SiO_2 chromatography of an 85:15 6*Z*:6*E* mixture obtained from a Wittig coupling performed by Dr. F.A. Golec (unpublished results). Unlabeled 6*E*,11*Z*-hexadecadienyl acetate and 9*Z*-tetradecenyl acetate were prepared during model studies for the tritiation experiments using H_2 in place of T_2.

All unlabeled pheromone analogs were prepared as 2.1×10^{-2} M stock solutions in heptane. For the experiments, ethanol stock solutions at 2.1×10^{-4} M were prepared by transferring 20 μl of heptane stock to a clean glass vial, blowing off solvent with N_2, and vortexing with 2.0 ml of absolute ethanol. Aqueous pheromone working solutions were prepared less than 2 hr prior to use in experiments to minimize concentration changes, since pheromones and analogs slowly adsorbed onto the glass. Preliminary experiments with labeled pheromones had indicated that this method of preparing working solutions gave stable and reproducible pheromone concentrations in the 10^{-5} to 10^{-9} M range. Thus, 50 μl of the 2.1×10^{-4} M ethanol stock was added to 1.0 ml of 10 mM Tris HCl buffer (pH 7.2) with vigorous vortexing to give a 1×10^{-5} M working solution from which serial dilutions of 1:10 (10^{-6} M), 1:100 (10^{-7} M), and 1:1000 (10^{-8} M) were rapidly prepared.

Solutions of labeled pheromone and pheromone analogs were prepared from hexane stock solutions of materials synthesized three months prior to these experiments and stored at −70°C. When necessary (i.e., when purity as assessed by TLC plate scraping was <98%), compounds were repurified by flash chromatography. Elution with 5% ethyl acetate–hexane gave radiochemically homogeneous materials which were used within three to four weeks. To prepare working solutions, an intermediate ethanol stock was prepared of each material

by evaporation of 100 μl of the hexane or heptane stock (1–3 mCi in each case) under N_2 and redissolving the radiolabeled analog in 200 μl of absolute ethanol. The ethanol stocks ($\sim 10^{-4}$ M) were stored at 4°C between daily preparations of aqueous solutions. A fresh working solution was made by addition of 10 μl into 1.0 ml Tris HCl buffer at room temperature and then prompt preparation of 1:10, 1:100, and 1:1000 dilutions. Unused solutions were discarded daily, but the vials were reused for the same analog and same dilutions to minimize adsorption problems for these 10^{-6} to 10^{-9} M solutions.

Analog Metabolism Assays. Enzyme dilution, labeled analog degradation, and competition studies were all performed following the same general protocol. First, protein solutions from sensory hair homogenates were freshly prepared and fresh aqueous pheromone analog and labeled pheromone solutions were prepared. Next, protein solutions (60 μl) were added to eight 10 × 75-mm borosilicate tubes. At 1-min intervals, 60 μl of radiolabeled pheromone solution was added. Each incubation (duplicates of four concentrations) was for 10 min at 20°C. A 3-μl aliquot (to determine [^{3}H]pheromone concentration) was removed from the aqueous solution using a calibrated, disposable glass micropipet, and 3 ml of Aquasol was added for LSC. At t = 10 min, 180 μl of ethyl acetate was added and vortexed vigorously for 15 sec. A 3-μl aliquot of the upper layer was removed to determine recovery (LSC), and a second 3-μl aliquot was spotted on a 4 × 8-cm Machery-Nagel Polygram Sil G/UV 254 plate. Plates were divided into four vertical strips and were prespotted with unlabeled 6*EZ*,11Z–16:Ac (substrate marker) and 6*EZ*,11Z–16:OH (product marker). The plates were developed in 20% (v/v) ethyl acetate–hexane; the substrate (R_f 0.61) and product (R_f 0.33) spots were visualized with I_2, and the spots were scraped into scintillation vials and counted in 3 ml Aquasol using a Beckman LS230 scintillation counter (39% efficient for tritium with minimal quenching).

The enzyme dilution experiment was performed using a 1:10 ($\sim 10^{-7}$ M) solution of [^{3}H]-6*E*,11Z–16:Ac, a fresh 1 mg/ml sensory hair preparation (220 μl protein/ml), and 1:10, 1:100, and 1:1000 dilutions of this sample. Incubation and TLC assay were performed as above.

Degradation of labeled analogs was performed with undiluted sensory hair homogenates and serial dilution (1:10 to 1:1000) of labeled analog working solutions. For these experiments no competitor was added, and 120 μl of labeled analog solution was used instead of 60 μl.

RESULTS AND DISCUSSION

Enzyme Dilution. We found that the hydrolytic activity of the sensory hair homogenates declined during the course of 8 hr of experimentation; this was most severe in diluted samples. The maximum conversion observed for a 10-

min incubation at 20°C was 76% with an undiluted fresh protein solution; the minimum was 38%. Thus, dilutions of the stock (1.0 mg sensory hairs/ml) were prepared immediately prior to a given assay.

In initial attempts to observe displacement of [^{3}H]-6*E*,11*Z*-16:Ac by the unlabeled pheromone, we observed essentially no competition at the highest concentration (3×10^{-6} M), indicating that we had not saturated the enzyme at this concentration. Since it was impossible to raise the pheromone concentration without exceeding the limits of solubility (in the absence of detergents or cosolvents), it was necessary to reduce the quantity of enzyme in the assay. A dilution series of undiluted, 1:10, 1:100, and 1:1000 gave conversions to the 6*E*,11*Z*-16:OH of 76%, 68%, 20%, and 4%, respectively. Subsequent competition experiments were run with the enzyme concentration which gave ~50% conversion, that is, a 1:30 dilution of stock. Vogt (1984) estimated that the sensillar esterase comprised less than 0.01% of total soluble protein of a sensory hair. Therefore, based on a maximum estimate of 0.1 μg enzyme/ml in stock, the enzyme concentration in the 1:30 dilution is at most 3 ng/ml or ca. 5×10^{-11} M.

Competition Studies. Using a 1:30 dilution of the stock sensory hair protein solution, the relative competitive abilities of different pheromone analogs and homologs (Figure 1) was examined. In these assays, the concentration of [^{3}H]-6*E*,11*Z*-16:Ac was held constant at 1.8×10^{-7} M and the competitor concentration varied from 3×10^{-9} M to 3×10^{-6} M. Conversions were calculated as the ratio of cpm of [^{3}H]-6*E*,11*Z*-16:OH (product) to total recovered cpm of this alcohol plus the acetate substrate. The concentration of [^{3}H]-6*E*,11*Z*-16:Ac used in this assay was below the estimated K_m of 2.2×10^{-6} M for the hydrolysis of the natural pheromone by the sensillar esterase (Vogt et al., 1985). In addition, approximately 30,000 cpm were present in the 3-μl aliquot assayed by TLC, so that our detection limit for low hydrolysis rates was on the order of a few percent. In all cases, the recovery from the TLC plates was in the range 85–95% of the cpm applied as determined by counting an equal aliquot of the applied ethyl acetate layer. All assays were performed in duplicate and are expressed as mean values normalized to maximal conversion at the lowest concentration of competitor (Figure 3).

There appear to be two main subsets among the competitors assayed. The first group consists of those analogs that possess competitive abilities equivalent to the natural pheromone. In this subset one finds the 6*Z* isomer of the natural pheromone, the diazoacetate analog of the natural pheromone, and 9*Z*-14:Ac. Each of these has a "kinked" hydrocarbon chain as well as an acetoxy or diazoacetoxy group as a terminal substituent.

The "poor" competitors include the 11-triple bond analog, the saturated hexadecyl acetate, and the product 6*E*,11*Z*-16:OH. These lack either the acetoxy group or the kinked hydrocarbon chain. It appears that the length of the chain is less important than the presence of a cisoid *Z*-linkage in the chain. This

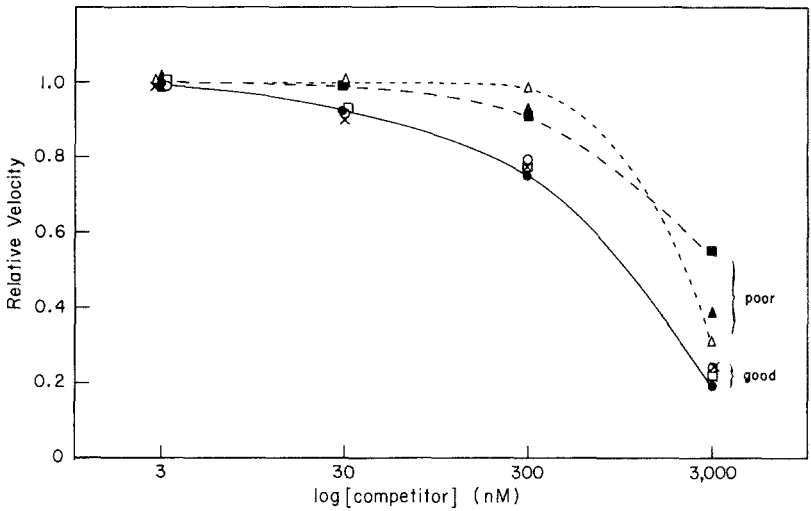

FIG. 3. Competitive displacement of [11,12-$^3H_2$]-6*E*,11Z–16 : Ac by unlabeled analogs. Percent hydrolysis (mean of two determinations) was normalized in each set to relative velocity = 1.0 for the lowest concentration of competitor to correct for fluctuations in sensillar esterase activity during a series of experiments. Good competitors: (●) 6*E*,11Z–16 : Ac; (○) 6*E*,11Z–16 : DZA; (×) 6Z,11Z–16 : Ac; (□) 9Z–14 : Ac. Poor competitors: (△) 6*E*,11-yne-16 : Ac; (▲) 16 : Ac; (■) 6*EZ*,11Z–16 : OH.

is consistent with the data of Priesner et al. (1975), which demonstrated that the pheromone analogs of highest behavioral activity are those in which the Z-alkene is correctly located relative to the methyl end of the chain instead of the acetate end.

Hydrolysis of Alternative Substrates. Using an undiluted stock sensory hair preparation, the hydrolysis rates of three pheromone acetate and diazoacetate analogs (Figure 2) were compared with that of the natural pheromone. This experiment is complementary to the competition study in that it defines the catalytic site selectivity of the sensillar esterase, while the competition study focuses on the enzyme's general preference for substrate analogs. In other words, this experiment provides direct evidence on the selectivity of the sensillar exterase in hydrolyzing substrate analogs.

Each radiolabeled substrate was examined in duplicate at three or four concentrations, and the percent hydrolysis was calculated from the ratio of product alcohol to total alcohol plus analog cpm recovered from the TLC plate. The substrates were all synthesized by reduction of an acetylenic bond with carrier-free tritium (Prestwich et al., 1984a) and have nominal specific activities of 58 Ci/mmol. Substrates were rechromatographed within three weeks of use and were examined for homogeneity by TLC just prior to incubations.

The results of the enzymatic hydrolysis of the tritium-labeled natural pheromone, the 9Z–14:Ac, the saturated hexadecyl acetate, and the 6*E*,11Z–16:DZA are shown in Table 1. In agreement with their relative competitive abilities, [^{3}H]-9Z–14:Ac is a good substrate and [^{3}H]-16:Ac and [^{3}H]-6*E*,11Z–16:DZA are poor substrates.

It is evident from Table 1 that the rate of hydrolysis of [^{3}H]-9Z–14:Ac is not constant at low concentrations, increasing from 19% to 62% (in 10 min) as the concentration increases three orders of magnitude. In evaluating these data, it is important to recall that the sensory hair preparation contains a pheromone binding protein (mol wt 15,000) at a concentration at least 10,000-fold greater than that of the enzyme (Vogt, 1984), and with an in vivo concentration of about 20 mM (Vogt and Riddiford, 1981). Unlike the very aggressive sensillar esterase (Vogt et al., 1985a), this relatively smaller binding protein apparently

TABLE 1. ALTERNATIVE SUBSTRATES FOR SENSILLAR ESTERASE

Substrate	Concentration (M)[a]	Hydrolysis (%)[b]
[11,12-$^3H_2$]-6*E*,11Z–16:Ac	1.8×10^{-9}	36
	1.8×10^{-8}	39.8
	2.8×10^{-7}	42.1
	3.5×10^{-6}	38.3
With boiled enzyme	9.7×10^{-8}	2.6
With no protein	9.7×10^{-8}	4.7
(Substrate only on TLC[c])	—	0.9
[9,10-$^3H_2$]-9Z–14:Ac	5.0×10^{-10}	19
	6.8×10^{-9}	33.5
	1.3×10^{-7}	53.3
	2.5×10^{-6}	61.9
Substrate only[c]	—	1.0
[11,12-$^3H_2$]-16:Ac	6.0×10^{-7}	5.6
	1.1×10^{-6}	3.7
	1.6×10^{-5}	2.2
Substrate only[c]	—	0.7
2 hr with aq. NaOH–MeOH	—	50
[11,12-$^3H_2$]-6*E*,11Z–16:DZA	1.2×10^{-7}	2.6
	4.5×10^{-7}	2.4
	3.3×10^{-6}	1.8
Substrate only[c]	—	0.8

[a] Calculated from the dpm in a 3-μl aliquot of the aqueous incubation mixture, using a specific activity of 58 Ci/mmol for each substrate.

[b] Percent hydrolysis is calculated from the duplicate assays cpm (alcohol product) ÷ [cpm (substrate) + cpm (product)].

[c] This value reflects the radiochemical purity of the starting acetate.

has a very low affinity for the pheromone. Vogt and Riddiford (1986) have proposed that this sensillar binding protein buffers the pheromone against enzymatic degradation through low specific protein–pheromone interactions enhanced by the extremely high endogenous binding protein concentration. This combination of a high concentration of a low-affinity binding protein and a low concentration of an aggressive esterase could allow modulation of the amount of pheromone reaching the receptor over some six or more decades of phero mone concentration (Vogt et al., 1985; Vogt, 1984; Vogt and Riddiford, 1986). We suggest that the low rate of degradation of [^{3}H]-9Z–14:Ac at its lowest concentrations may reflect this buffering capacity of the binding protein.

CONCLUSION

Studies with analogs of 6*E*,11*Z*–16:Ac, the major component of the *A. polyphemus* pheromone, show that the sensillar esterase in male antennae has a distinct preference for substrates with conformations most similar to the natural pheromone. This is consistent with previous findings (Vogt et al., 1986; Vogt and Riddiford, 1986), and supports the hypothesis that the principal function of this esterase is to inactivate the sex pheromone.

Although the diazoacetate analog most similar to the natural pheromone proved to be a reasonably good competitive inhibitor of pheromone degradation, it proved to be a rather poor substrate for enzymatic hydrolysis. This means that, while the active site on the sensillar esterase clearly recognizes this analog, it is not capable of binding it in a manner that allows for the analogs' hydrolysis. This is a positive finding in terms of the usefulness of utilizing this probe to label pheromone-relevant proteins. However, UV irradiation of this 6*E*,11*Z*–16:DZA–sensillar esterase complex does not provide detectable amounts of labeled enzyme, although photoattachment of this diazoacetate analog to the soluble and membrane-bound protein constituents of these pheromone-sensitive sensory hairs does occur (Vogt et al., 1986).

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PHEROMONE BIOSYNTHESIS AND ROLE OF FUNCTIONAL GROUPS IN PHEROMONE SPECIFICITY

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Abstract—The sex attractants for many Lepidoptera are long-chain, monounsaturated acetate esters, alcohols, and aldehydes. In vivo metabolic studies and enzyme analysis in vitro have provided evidence that the aldehyde pheromone of the eastern spruce budworm is synthesized de novo via an acetate ester precursor. Interconversion of the functional groups (ester, alcohol, aldehyde) can explain differences in the pheromone blends used by closely related species.

Key Words—Acetate esters, alcohols, aldehydes, fatty acid biosynthesis, insect pheromones, Lepidoptera, pheromone biosynthesis, spruce budworm.

INTRODUCTION

Although the pheromone complement of a given insect is typically a complex mixture of two or more components, the building blocks, or components, of lepidopteran pheromones are relatively simple molecules with many structural similarities (Inscoe, 1982). Most pheromones of Lepidoptera are composed of a limited number of long-chain monounsaturated acetate esters, alcohols, or aldehydes, which vary with respect to chain length as well as position and configuration of the double bond.

Consideration of the lepidopteran sex attractants, listed in a recent review (Inscoe, 1982) for almost 400 species, shows that the majority (77%) of species have pheromones with monounsaturated carbon chains between 10 and 16 carbons in length. These compounds are displayed in a three-parameter diagram in Figure 1. The number of species attracted to a given compound is shown in the vertical dimension for each combination of chain length, double-bond po-

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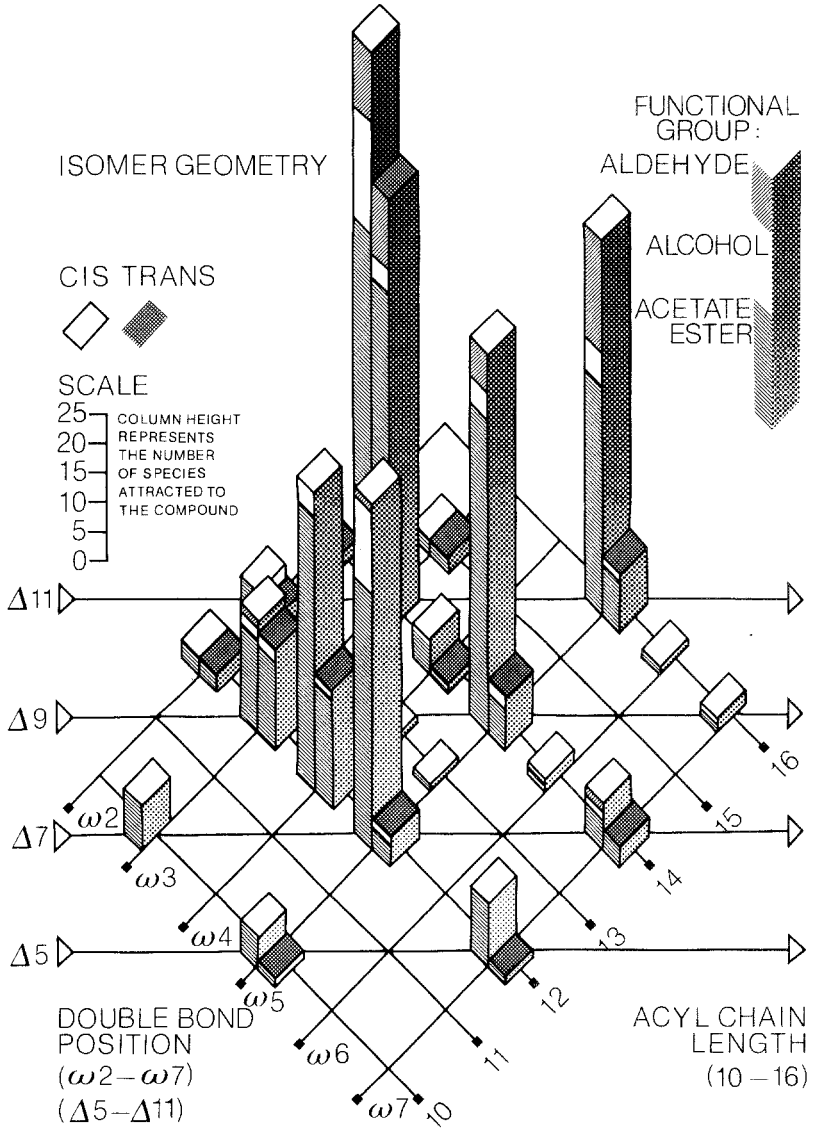

FIG. 1. Common lepidopteran sex attractants. Dependence of the number of lepidopteran species that use monounsaturated fatty acid derivatives as sex attractants on the chain length, double-bond position (ω and Δ) and configuration (*trans* or *cis*) and functional group (acetate ester, alcohol, or aldehyde). The data was compiled from a recent listing of insect sex attractants (Inscoe, 1982), and includes attractants for 293 of the 379 lepidopteran species. Saturated or polyunsaturated compounds which are the attractants for most of the remaining species were not included.

sition and configuration, and functional group. The horizontal and oblique axes define the chain length and the double-bond position with respect to either the terminal carbon atom of the chain (ω1) or the carbon containing the functional group (Δ1). This presentation emphasizes the close metabolic relationships between the different pheromones by grouping the six chemical structures resulting from two configurations at the double bond (*cis* or *trans*) and the three different functional groups (ester, aldehyde, or alcohol). These groupings are based on the assumptions that the two configurational isomers are synthesized by similar means and that the functional groups can be readily interconverted in the insect.

A strong metabolic relationship also exists between the components along each ω axis since, once the double bond is formed, its position relative to the terminal carbon (ω value) is independent of the processes of chain shortening or elongation as these occur at the functional Δ1 carbon. Consider, for example, all compounds on the ω3 axis. A Δ11-specific desaturase (Bjostad and Roelofs, 1981), active on 14-carbon acyl chains, could produce all compounds on this axis if coupled with mechanisms for chain shortening and elongation. Similarly, a Δ3-desaturase active on six-carbon acyl chains could produce the same range of compounds if coupled with a mechanism for chain elongation.

Evidence for de novo synthesis of pheromones in Lepidoptera is supported by both morphological and biochemical data (Blomquist and Dillwith, 1983). In vivo labeling studies of the eastern spruce budworm (*Choristoneura fumiferana*) have shown that an acetate ester is synthesized de novo and serves as a precursor to the aldehyde pheromone (Morse and Meighen, 1984b). The release of pheromone coincides with extrusion of the pheromone-producing gland (Sanders, 1969) which is morphologically identical to pheromone glands of other insects (Roelofs and Feng, 1968).

Recent enzyme and metabolic studies on the eastern spruce budworm have shown that the three major classes of functional groups (esters, alcohols, aldehydes) are interconverted during biosynthesis of its aldehyde pheromone, *trans/cis*-11-tetradecenal (96:4) (Morse and Meighen, 1984a, b; unpublished data). From these results, a metabolic pathway for de novo synthesis of the spruce budworm pheromone can be formulated (Figure 2) which includes those enzymes that have so far been implicated in pheromone biosynthesis. The proposed location of these enzymes is based on subcellular fractionation experiments (Morse and Meighen, 1984a; unpublished data) and ultrastructural studies of the gland (Percy, 1974) as well as information concerning the location of similar enzymes in other tissues. This biosynthetic pathway is of particular value since it can explain how distinct pheromone signals utilizing different functional moieties (ester, alcohol, and aldehyde) could have arisen for members of the budworm genus and also may explain pheromone differences in species of other genera of the Lepidoptera. The goal of this work is to summarize the evidence

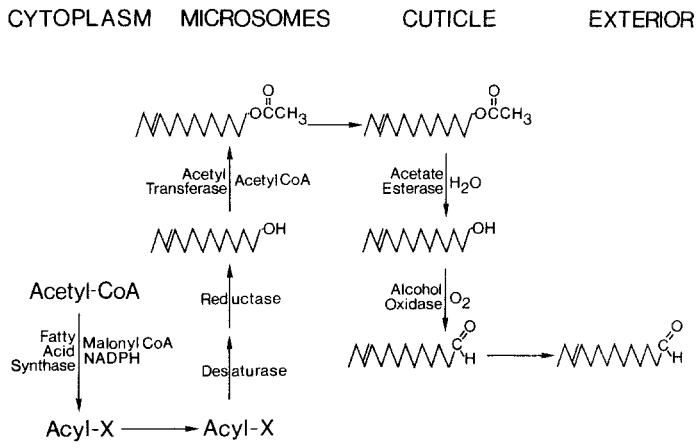

FIG. 2. Proposed pathway for pheromone biosynthesis in the eastern spruce budworm. This model incorporates the results from metabolic and enzyme studies on the spruce budworm into a framework consistent with morphological and biochemical observations in other systems. The pathway begins with fatty acid synthesis and ends with release of the aldehyde pheromone (96:4, *trans*:*cis*-11-tetradecenal) from the insect.

for specific steps in this pathway and to define more closely the role and metabolic relationship of the pheromone functional groups of Lepidoptera.

METHODS AND MATERIALS

Materials. [9,10-^{3}H]Myristic acid (23 Ci/mmol) was prepared by New England Nuclear by catalytic reduction of *cis*-9-tetradecenoic acid. [1-^{14}C]Myristate was purchased from Amersham; [^{3}H]acetyl-CoA, [2-^{14}C]malonyl-CoA, and [1-^{14}C]acetate were from New England Nuclear. Labeled tetradecanol was prepared by $LiAH_4$ reduction of labeled myristate in dry ether and purified by thin-layer chromatography. All other reagents were purchased from Sigma. Phosphate buffers were prepared by diluting appropriate amounts of 1.0 M NaH_2PO_4 and 1.0 M K_2HPO_4.

Pupae of female eastern budworms (*Choristoneura fumiferana*) were kindly supplied by Dr. Gary Grant of the Forest Pest Management Institute, Sault Ste Marie. The pupae were allowed to emerge under natural lighting. Glands were excised from the female moths and homogenized in 0.05 M phosphate, pH 7.0, with a motor-driven pestle at 500 rpm for 2 min (10 glands/ml).

In Vivo Labeling of Insects. Radioactive compounds ($\sim$1 nmol) were applied in dimethyl sulfoxide ($\leq$0.1 μl) to the glands of individual female moths (Bjostad and Roelofs, 1981; Morse and Meighen, 1984b). After 1 hr, the glands were excised and extracted with hexane.

Enzyme Assays. Acetate ester, alcohol oxidase, and aldehyde dehydrogenase assays were conducted as described previously (Morse and Meighen, 1984a). No difference in enzyme levels in extracts was observed between glands excised in the photophase or scotophase.

Thin-Layer Chromatography (TLC) and Autoradiography. Silica gel chromatography was performed on Machery Nagel Sil N-UV_{254} TLC plates, heat activated for 60 min at 110°C and developed for 90 min in hexane–ether–acetic acid (90:10:2). Argentation chromatography was performed in the same solvent on silica gel TLC plates sprayed with a 7.5% solution of $AgNO_3$ in 90% ethanol and then heated for 60 min. Reversed-phase chromatography was performed on Whatman KC18-F TLC plates, which were used without heating and developed for 20 min in CH_3CN–tetrahydrofuran–methanol (65:1:10). TLC plates containing ^3H-labeled ligands were generally treated with ENhance (NEN) prior to exposure. However, this procedure cannot be used for plates containing $AgNO_3$. Autoradiography was performed by exposing the TLC plate to Kodak X-AR OMAT film at −70°C.

RESULTS AND DISCUSSION

Fatty Acid Biosynthesis. Biosynthesis of the carbon chain backbone is the first step in the de novo formation of the pheromones in Lepidoptera. Fatty acid biosynthesis involves the stepwise addition of two carbon units from malonyl-CoA to the carboxyl functional group of an acyl derivative starting with acetyl-CoA (Volpe and Vagelos, 1973, 1976; Bloch and Vance, 1977). In animal cells, fatty acid synthase (FAS) is found in the cytoplasm (Volpe and Vagelos, 1973; 1976). Eukaryotic systems also contain two other systems believed to function in chain elongation of existing fatty acids (Bloch and Vance, 1977); a mitochondrial system which operates by reversal of β-oxidation using acetyl-CoA, and a microsomal system which appears to have a mechanism similar to the cytosolic FAS system (Khan and Kolattukudy, 1973; Sanchez and Harwood, 1981).

Fatty acid synthase activity has been measured in extracts of the budworm gland by the incorporation of [^3H]acetyl-CoA and [^{14}C]malonyl-CoA into hexane-soluble material with an isotope ratio ($^{14}C/^3H$) of approximately 8:1. The fatty acids, synthesized in vitro, appear to be saturated since chromatography on silver nitrate thin-layer plates, which resolves saturated and unsaturated fatty acids, gave only one product that comigrated with saturated fatty acid (Figure 3).

Free fatty acids have also been observed during in vivo labeling of the budworm gland with ^{14}C-labeled acetate, myristate, and tetradecanol. Analysis of the labeled fatty acids in a reversed-phase TLC system indicates that the fatty acids have chain lengths of between 12 and 18 carbons (Figure 4). This TLC

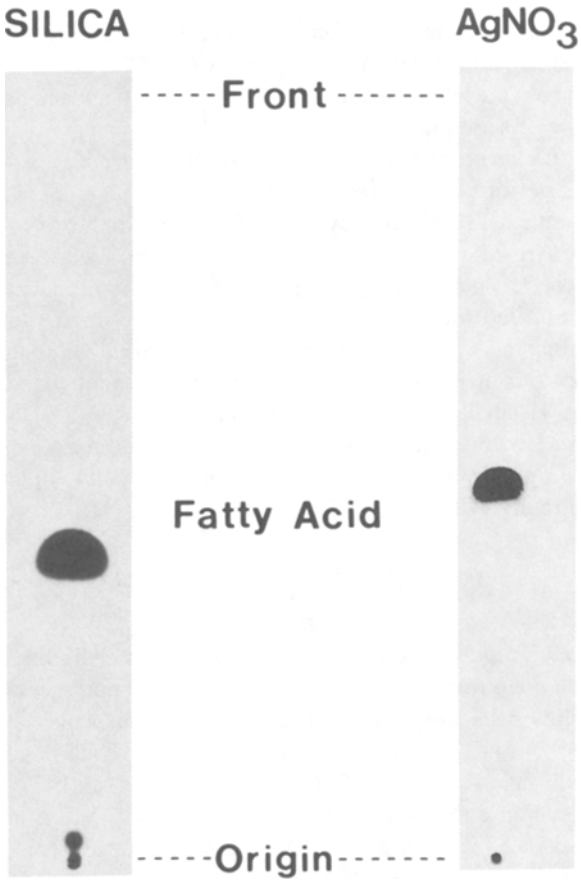

FIG. 3. In vitro fatty acid synthase activity in gland extracts. The reaction mixture (at 37°C) contained 15 μM [^{3}H]acetyl-CoA, 10 μM [^{14}C]malonyl-CoA, 500 μM NADPH and 2 μg gland homogenate in 1.0 ml 0.05 M phosphate buffer (pH 7.0), 1 mM dithiothreitol. Fatty acids, extracted into hexane after 20–60 min, were chromatographed on silica gel and silica gel plates sprayed with a 7.5% solution of $AgNO_3$. The radiolabeled product comigrates with saturated fatty acids in both systems. Minor amounts of radiolabel at or near the origin are acetyl-CoA and acetate.

system cannot distinguish between a monounsaturated acid and a saturated acid two carbons shorter. For the samples labeled with [1-^{14}C]myristate or [1-^{14}C]tetradecanol, it appears that 14:1 and not 12:0 acids are formed, as the radiolabel would be lost on direct chain shortening. Furthermore, degradation followed by de novo synthesis would not account for the apparently higher level of incorporation of label into free fatty acid (14:1/12:0) from myristate and

tetradecanol than from [1-^{14}C]acetate. Interestingly, the labeled fatty acids, released by saponification of the triglycerides, have the same distribution of chain lengths as the free fatty acids (Figure 4), suggesting that these fatty acids can be used directly in triglyceride biosynthesis in vivo.

The formation of free fatty acids has been observed in insect tissue homogenates (Municio et al., 1972; Ryan et al., 1982) as well as in mammalian systems (Bloch and Vance, 1977). In the mammalian systems, palmitic acid is the major product due to a thioesterase associated with FAS that cleaves acyl chains containing 16 carbon atoms. In several other systems, a thioesterase distinct from the FAS has been shown to produce short-chain fatty acids by cleavage of the growing acyl chains (Rogers et al., 1982; Ryan et al., 1982). Short-chain fatty acids can also be formed by limited β-oxidation of the palmitate normally formed by the FAS (Chang and Holman, 1972). Chain shortening has been implicated in the biosynthesis of the cabbage looper pheromone, as radiolabel from a ω5 16-carbon acid was found to be incorporated in vivo into the ω5 12-carbon backbone of the pheromone (Bjostad and Roelofs, 1983).

Although free fatty acids can be formed by the spruce budworm, the incorporation of label from fatty acid ([^{14}C]myristate) into the acetate ester precursor of the budworm pheromone (Morse and Meighen, 1984b) indicated that this fatty acid must first be degraded before label could be incorporated by de novo synthesis. Not only was the incorporation of label from other fatty acids into the acetate ester the same as from myristate but radiolabel incorporation from [9,10-^{3}H]myristate was fivefold less than from [1-^{14}C]myristate. The differential incorporation of the two labels provides a sensitive indicator for β-oxidation and degradation followed by resynthesis since the tritium label is preferentially lost under these conditions. It appears that the free exogenous fatty acids cannot be efficiently used in the budworm to produce the pheromone, suggesting that the fatty acyl chains may be channeled directly from the FAS system, possibly by transacylation rather than by hydrolysis (Knudsen and Grunnet, 1982). Interestingly, other work on insect systems has suggested that exogenous (dietary) fatty acids are different than newly formed acids (Keith, 1967). Furthermore, fatty acids released in an activated form (as acyl derivatives) can be used directly in desaturation or reduction steps whereas free fatty acids must first be activated (Groot et al., 1976).

Fatty Acid Desaturation and Reduction. Very little is known about the mechanisms of desaturation and reduction of fatty acids to form the unsaturated pheromones of Lepidoptera. In mammalian systems, a desaturase that acts specifically on acyl-CoAs in the presence of NADPH and molecular oxygen to introduce a double bond at the Δ9-position has been widely studied (James, 1977; Jeffcoat, 1979). All mammalian Δ9-desaturases have been localized in the microsomal fractions on subcellular fractionation.

An alternative method of desaturation is used in bacteria and involves the introduction of a double bond into a growing acyl chain (Scheuerbrandt and

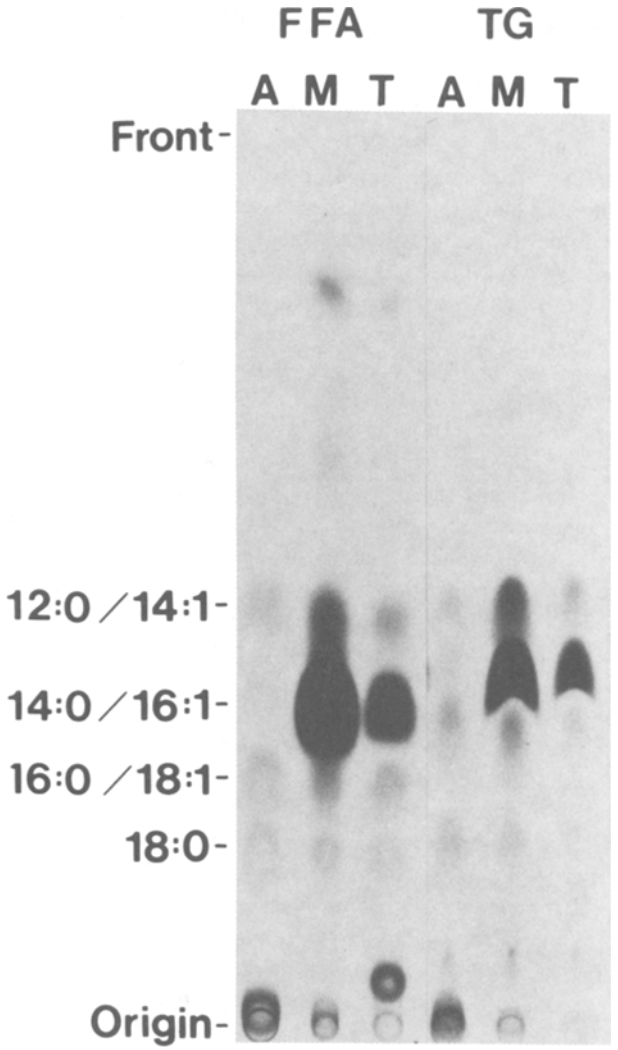

FIG. 4. Analysis by TLC of fatty acids synthesized in vivo. The glands of 20 female budworm moths were labeled with 0.8 nmol/gland [1-^{14}C]acetate (A) (56 mCi/mmol), 1.7 nmol/gland [1-^{14}C]myristate (M) (31 mCi/mmol), or 1.2 nmol/gland [1-^{14}C]tetradecanol (T) (31 mCi/mmol). After a 60-min incubation, the glands were excised, extracted with hexane, and the radiolabeled lipids separated by thin-layer chromatography (TLC) on silica gel. The free fatty acids (FFA) and the triglycerides (TG) were then extracted from the silica and rerun on a reversed-phase TLC system as shown. The positions of fatty acid standards run in parallel are indicated on the ordinate. The TG sample was saponified before reversed-phase TLC.

Bloch, 1962). A specific β-hydroxydecanoyl thioester dehydrase produces a Δ3-decenoyl derivative that can be further elongated to form palmitoleic acid (Kass et al., 1967; Brock et al., 1967).

A Δ11-desaturase has been proposed to be involved in the biosynthesis of the pheromone of the redbanded leafroller based on a higher incorporation of myristate vs. acetate label into the unsaturated pheromone, whereas other lipids were preferentially labeled by acetate (Bjostad and Roelofs, 1981). In contrast, a similar level of incorporation of label from either myristate or acetate was observed for the unsaturated ester of the spruce budworm (Morse and Meighen, 1984b). These results could reflect differences in the relative rates of desaturation and degradation in the two insects. Alternatively, the differences could be due to the shorter labeling period (2 hr) used for the budworm moth compared to that used for the redbanded leafroller moth (24 hr) which encompasses its pheromone release period. Thus, the possibility that the most stable metabolic pool (e.g., myristate) preferentially labels those lipids which are turned over at the highest rate during the labeling period cannot be excluded.

Although the reduction of fatty acids and fatty acyl-CoAs has been studied in other systems (Griffith et al., 1981; Moore and Snyder, 1982; Riendeau et al., 1982; Rodriguez et al., 1983), this activity has not been detected in extracts of insects. The reduction of fatty acids by extracts from eukaryotic cells is generally catalyzed by microsomal fractions. Fatty alcohols, and not fatty aldehydes, are the major product as aldehyde reductases appear to be associated with the fatty acid reductases (Riendeau and Meighen, 1985), presumably to prevent the accumulation of the relatively reactive, and thus toxic, aldehyde.

Aldehyde reductase, but not fatty acid reductase, activity has been detected in extracts of the gland of the spruce budworm (Table 1). In this experiment, the labeled aldehyde substrate is produced in situ by a bacterial fatty acid reductase (Riendeau and Meighen, 1981). In the presence of the gland homogenate, the aldehyde could be reduced to the alcohol with NADPH. The amounts of the different substrates and products were determined by scintillation counting after separation by thin-layer chromatography on silica gel. Unfortunately, this assay cannot be used to characterize the aldehyde reductase since the observed properties reflect those of the coupled system. An alternate coupled assay involving the conversion of the alcohol product into a labeled acetate ester with [^{3}H]acetyl-CoA has also shown that aldehyde can be reduced by gland extracts.

The specificity of the reductase system in the gland for unsaturated fatty acids will be important since it has been suggested that the ratio of unsaturated isomers (*cis* and *trans*) in the pheromone may also be controlled by this enzyme (Blomquist and Dillwith, 1983). In some species, the ratio of isomers of unsaturated fatty acids differs from the isomer ratio of the pheromone (Wolfe et al., 1981). It appears likely that the desaturase and reductase function in concert, with the reductase modifying the isomeric ratio produced by the desaturase.

TABLE 1. ALDEHYDE REDUCTASE ACTIVITY IN GLAND EXTRACTS

Added components[a]	Product (%)		
	Acid	Aldehyde	Alcohol
Reaction mixture	82	4	8
+ Fatty acid reductase (FAR)	28	56	8
+ Gland homogenate	85	4	5
+ FAR + gland homogenate	12	51	30

[a] The reaction mixture contained 0.4 μM [9,10-^{3}H]myristate (22.8 Ci/mmol), 1 mM ATP, 1 mM NADH, 1 mM NADPH, 0.1 mM CoA, and 0.1 mM $MgCl_2$ in 1 ml of 0.05 M phosphate buffer (pH 7) containing 1 mM β-mercaptoethanol. After a 60-min incubation, the aqueous medium was extracted with ether, and the products separated on silica TLC. The fatty acid reductase from *Photobacterium phosphoreum* produces significant levels of aldehyde from the acid, whereas the gland homogenate could not reduce the fatty acid directly but could reduce the aldehyde to alcohol.

Biosynthesis of Acetate Esters. The activity directly responsible for synthesis of the acetate ester has been measured both in gland extracts of the budworm moth (manuscript in preparation) and in the living insect (Morse and Meighen, 1984b). In vivo, the activity was detected by the incorporation of [1-^{14}C]tetradecanol directly into a saturated ester product. In vitro, the enzyme was found to be specific for long-chain alcohol acceptors with acetyl-CoA as the acyl donor (designated fatty alcohol: acetyl-CoA O-acetyl transferase). Activity was found only in gland extracts from the female moth, indicating that this enzyme was exclusively involved in pheromone biosynthesis. On subcellular fractionation, the acetyltransferase sedimented in the microsomal fraction with the smooth endoplasmic reticulum (SER), which is involved in lipid metabolism and is present in large amounts in the gland cells (Percy, 1974). As the SER is generally associated with the storage and secretion of material, this result is consistent with the high levels of 11-tetradecenyl acetate (96:4, *trans-cis*) in the gland and their dependence on diurnal rhythm (Silk et al., 1980) and support the proposal that the acetyltransferase synthesizes a stored acetate ester precursor that is secreted to form the aldehyde pheromone (Figure 2).

Formation of Aldehyde Pheromone. An acetate esterase, which hydrolyzes the ester to form the long-chain alcohol, and an alcohol oxidase, which catalyzes fatty aldehyde formation, have been found in extracts of the pheromone gland of the spruce budworm (Morse and Meighen, 1984a). The release of the volatile labeled aldehyde pheromone from the insect, synthesized de novo from [^{14}C]acetate, correlated with a decrease in the level of labeled acetate ester in the gland itself (Morse and Meighen, 1984b).

The acetate esterase and alcohol oxidase, in contrast to those described above, are soluble. Only water and O_2 are required for conversion of the acetate ester to aldehyde in reactions that are essentially irreversible and easily distin-

guished from the reverse pathway used for ester formation. The soluble nature of the acetate esterase and alcohol oxidase are consistent with the enzymes being physically separated in the cell from the enzymes involved in the formation of the acetate ester which appear to be associated with the endoplasmic reticulum. It is probable that the acetate esterase and alcohol oxidase are exterior to the gland cells in the cuticle since metabolic energy and cellular cofactors are not necessary for conversion of the acetate ester to the aldehyde pheromone, only water and oxygen. Ultrastructural studies of the gland cuticle have revealed the presence of pore canals (Percy, 1974; Percy and Weatherston, 1974), which in other insects have been shown to contain oxidase activity (Locke and Krishnan, 1971) and esterase activity (Locke, 1961). The location of these enzymes in the cuticle woulde be advantageous as it would clearly separate the pathways of ester synthesis and degradation, which pass through a common 11-tetradecenol intermediate.

Pheromone Degradation. Since removal of the signal molecule from the environment of the receptor is necessary for a response to new pheromone molecules, degradative mechanisms have generally been associated with the process of pheromone reception (Blomquist and Dillwith, 1983). A similar mechanism could also be associated with the biosynthetic machinery in the budworm gland in order to remove the reactive, and potentially toxic, aldehydes that are not released from the cell surface. Such a mechanism would be presumably located in a different subcellular compartment from the biosynthetic enzymes to avoid degradation of pheromone about to be released.

A NAD-specific aldehyde dehydrogenase has been found in relatively high levels in the budworm gland and is associated with the membrane in gland extracts (Morse and Meighen, 1984a). This activity was also found in homogenates of the male antennae, raising the possibility that the same enzyme is responsible for aldehyde removal in the gland and the antennae. As shown in Table 2, the levels of aldehyde dehydrogenase activity measured in gland homogenates far surpass the levels observed for the pheromone biosynthetic ac-

TABLE 2. GLANDULAR LEVELS OF ENZYME ACTIVITIES[a]

Enzyme	Product	Activity
Fatty acid synthase	Fatty acid	300
Acetyltransferase	Ester	6
Acetate esterase	Alcohol	80
Alcohol oxidase	Aldehyde	20
Aldehyde dehydrogenase	Fatty acid	400

[a] The catalytic rates of selected enzymes, given in picomoles of product per minute per insect gland, were based on standard assays and represent velocities at saturating substrate concentrations.

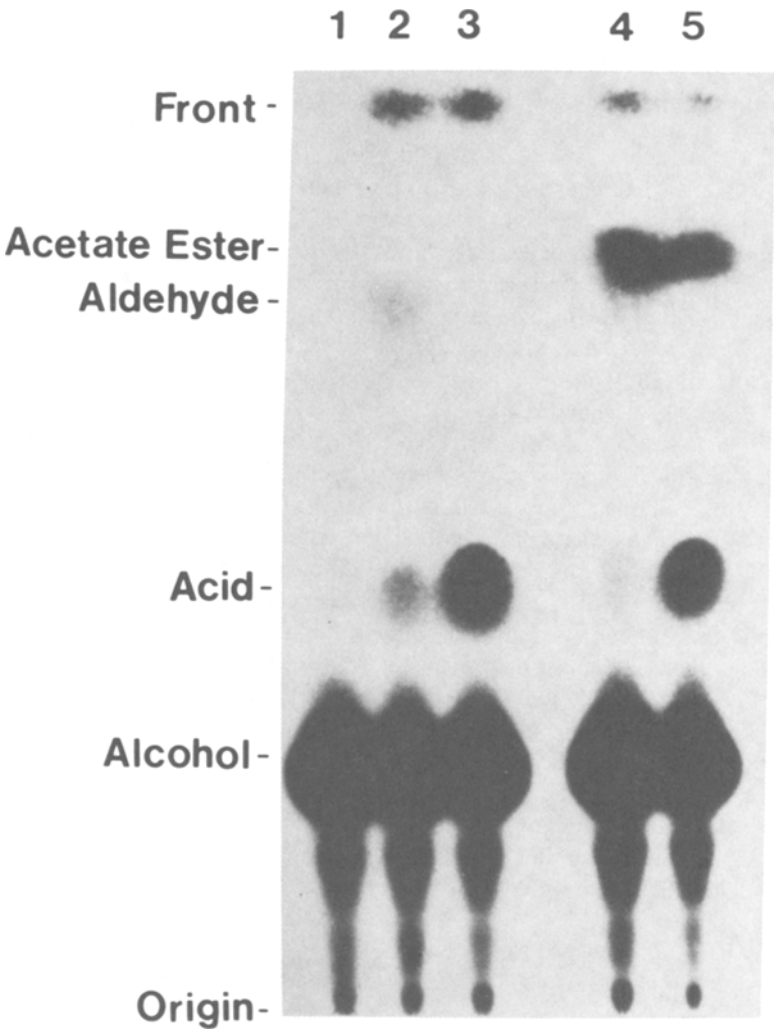

FIG. 5. Metabolism of tetradecanol by gland homogenates. The standard reaction mixture (lane 2) contained 1.2 μM [^{3}H]tetradecanol and 20 μg of gland homogenate in 1 ml of phosphate buffer, pH 7.0. Labeled compounds were extracted with hexane after a 60-min incubation and analyzed by TLC on silica gel and autoradiography. (1) Control minus gland homogenate; (3) plus 2 mM NAD; (4) plus 31 μM acetyl-CoA; (5) plus NAD (1 mM) and acetyl-CoA (31 μM). The positions of standards of tetradecanol, tetradecanoic acid, tetradecanol, and tetradecanyl acetate are indicated on the ordinate.

tivities. However, as substrate concentrations in the insect have not been measured, these activities may not reflect the rates in vivo.

A more direct comparison of the relative rates is given in Figure 5 where the radiolabeled products obtained on addition of [^{3}H]tetradecanol to a gland extract are analyzed by TLC. In the absence of other exogenous substrates, the alcohol is converted into both fatty aldehyde and fatty acid due to the presence of alcohol oxidase and aldehyde dehydrogenase activities, respectively. Addition of NAD^+ to the extract greatly stimulates the aldehyde dehydrogenase activity, as there is apparently only a low level of endogenous cofactor (NAD^+) in the extract. Consequently, the intermediate aldehyde product can no longer be detected. The large increase in the amount of fatty acid partially reflects the difficulty in extracting aldehyde from the aqueous reaction mixture compared to the other long-chain products. If acetyl-CoA is added to the extract containing tetradecanol, the alcohol is preferentially channeled into the acetate ester rather than being oxidized. Addition of NAD to the mixture containing acetyl-CoA now stimulates the oxidation pathway, and under these particular conditions, approximately equal amounts of the two products (acetate ester and fatty acid) are formed. These results illustrate that, even though a common intermediate (fatty alcohol) is being utilized in the biosynthesis and degradation of acetate esters, the relative flux in these two pathways can be regulated. Furthermore, as proposed in Figure 2, the biosynthetic and degradative pathways would be separated within the cell.

Role of Functional Groups in Pheromone Specificity. The preceding sections have summarized the evidence for a series of enzyme activities in the pheromone-producing gland of the spruce budworm based on a combination of in vitro and in vivo techniques. From these results, a biosynthetic pathway has been proposed for the spruce budworm pheromone (Figure 2) in which the final

TABLE 3. THEORETICAL DEPENDENCE OF PHEROMONE COMPOSITION ON ACETATE ESTERASE AND ALCOHOL OXIDASE ACTIVITY LEVELS

Enzyme levels		Pheromone composition[a]		
Esterase	Oxidase	Ester	Alcohol	Aldehyde
High	High	−	−	+
High	Low	−	+	+
High	Absent	−	+	−
Low	High	+	−	+
Low	Absent	+	+	−
Low	Low	+	+	+
Absent		+	−	−

[a](+), present or (−) absent in the pheromone.

TABLE 4. FUNCTIONAL GROUP DIFFERENCES PROVIDING SPECIES-SPECIFIC ATTRACTANTS

Genus	Species	Functional group[a]			Carbon chain backbone
		Ester	Alcohol	Aldehyde	
Acleris	*emargana*			+	*trans*-11 14:1
	paridiseana	+			
Argyrotaenia	*citrana*			+	*trans/cis*-11
	dorsalana	+	+		14:1
	velutinana[b]	+	−		
Autographa	*biloba*	+			cis-7 12:1
	californica	+	+		
Choristoneura	*biennis*			+	*trans/cis*-11
	fumiferana	−	−	+	14:1
	fractivittana		+	+	
	occidentalis	+		+	
	rosaceana	+	+	+	
Croesia	*conchyloides*			+	*cis*-11 14:1
	askoldana	+		+	
Epiblema	*desertiana*	+			*cis*-8 12:1
	scudderiana	+	+		
Eurois[c]	*occulta*	+	−		*cis*-11 16:1
	astricta	+		+	
Grapholitica	*prunivora*	+	−		*trans/cis*-8
	molesta	+		+	12:1
Heliothis[d]	*armiger*			+	*cis*-11 16:1
	virescens[e]		+	+	
	punctiger	+		+	
Oidaematophorus	*guttalus*			+	*trans*-11 14:1
	mathewianus	+			
Pandemis	*limitata*	+			*cis*-9/*cis*-11
	heparana	+	+		14:1
Platynota	*flavedana*	−	+		*trans/cis*-11
	sultana	+	−		14:1
Synanthedon	*alleria*	+			*trans,cis*-3,13
	biblionipennis	+	+		18:2
Thyris	*maculata*	+			*trans*-11 14:1
	usitata		+		

[a] (+), sex attractant; (−) inhibitory to the sex attractant. Data compiled from the review of Inscoe (1982).
[b] Pheromone of this species also contains dodecanol.
[c] Pheromone of this genus also contains *cis*-9-tetradecenyl acetate.
[d] Pheromone of this genus also contains *cis*-9-tetradecenal.
[e] Pheromone of this species also contains tetradecanol.

step involves conversion of an acetate ester precursor to the aldehyde via a fatty alcohol intermediate. If the same mechanism of pheromone biosynthesis is applicable to other genera of the Lepidoptera, then it might be predicted that pheromone specificity in closely related species could be modulated by differences in their functional groups (ester, alcohol, aldehyde) rather than by alterations in the backbone chain.

Differences in pheromone specificity can easily be accomplished by alterations in the relative levels of the acetate esterase and the alcohol oxidase enzymes. Seven distinct pheromone blends of ester alcohol and aldehyde can be predicted depending on the relative activities of these enzymes (Table 3). Of course, a wide range of pheromone blends can be produced in which the relative amounts of acetate ester, alcohol, and aldehyde are different.

Support for this proposal was obtained by an examination of the insect sex attractants for different species within a genus (Inscoe, 1982). Table 4 shows that a large number of genera contain species that use attractants which differ only in their functional groups and are composed of a blend of ester, alcohol, and/or aldehyde. All seven possible combinations listed in Table 3 have at least one illustration. In some cases (i.e., in the *Platynota* species), not only does one species exclusively use a sex attractant with a different functional group than the other species, but the compound is inhibitory to the attraction of the other species.

The development in insects of a biosynthetic pathway to form acetate ester pheromones would have been greatly favored due to the high chemical stability of esters. Hydrolytic and oxidative steps to form alcohol and aldehyde pheromones could have then evolved during species differentiation. Thus specific communication systems composed of pheromones containing functional group blends would have arisen without additional energy or metabolic factors being required from the cell.

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TERMINAL STEPS IN PHEROMONE BIOSYNTHESIS BY *Heliothis virescens* AND *H. zea*[1]

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Abstract—In vivo application to the sex pheromone gland of *Heliothis virescens* and *H. zea* of large quantities of alcohols normally present in small amounts resulted in the preferential conversion of the alcohols to the corresponding pheromonal aldehydes. Amounts of the minor component aldehydes were increased up to 15-fold by selectively applying large quantities of the alcohol precursors. Using this technique, we have induced *H. virescens* to convert "bombykol," the sex pheromone of the silkworm, to the corresponding aldehyde, "bombykal," and have induced female *H. zea* to produce the same sex pheromone components used by *H. virescens* by applying tetradecanol and (*Z*)-9-tetradecenol to the surface of the gland. Further, treated *H. zea* females were attractive to *H. virescens* males and caused males to attempt interspecific copulation repeatedly. We have also found that the enyzme involved in this conversion is dependent on the presence of molecular oxygen, indicating that a nonspecific alcohol oxidase is responsible for the terminal biosynthetic step in pheromone production by both *H. virescens* and *H. zea*.

Key Words—Sex pheromone biosynthesis, *Heliothis virescens*, *H. zea*, Lepidoptera, Noctuidae.

INTRODUCTION

Recent developments in the collection and analysis of airborne organic molecules have enabled researchers to accurately identify the volatile pheromone blends released by a number of insect species (Golub and Weatherston, 1984).

[1] Mention of a commercial product or proprietary does not constitute endorsement by either the University of Guelph or U.S.D.A.

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The volatile pheromone blends released by several species of Lepidoptera differ from those found in gland extracts not only with respect to the ratios of components but also in that extracts contain other compounds that are often closely related to the volatile pheromone components. These related compounds often lack a behavioral function, but in some instances they have been shown to have an inhibitory effect on male behavior. This is exemplified by the phermone blends released by females of the genus *Heliothis* (Lepidoptera: Noctuidae), major pests of cotton in North and South America.

Early studies by Roelofs et al. (1974) and Tumlinson et al. (1975) indicated that pheromone gland extracts of *H. virescens* contained a 16:1 ratio of (Z)-11-hexadecenal (Z11–16:AL) and (Z)-9-tetradecenal (Z9–14:AL) and that the pheromone gland of *H. zea* contained Z11–16:AL. Although these compounds were active, Tumlinson et al. (1975) suggested that the sex pheromone of *H. virescens* was more complex than that identified because the two-component blend was not as attractive as were calling females in bioassays. Subsequently, Klun et al. (1980a) identified five additional components from pheromone gland rinses of female *H. virescens*. These compounds included: tetradecanal (14:AL), hexadecanal (16:AL), (Z)-7-hexadecenal (Z7–16:AL), (Z)-9-hexadecenal (Z9–16:AL), and (Z)-11-hexadecenol (Z11–16:OH). Further, the gland of *H. zea* was found to contain the same 16 carbon aldehydes that were present in the pheromone gland of *H. virescens* (Klun et al., 1980b).

Although the above seven components were identified from *H. virescens* gland extracts, field trapping studies indicated that the 16:1 ratio of Z11–16:AL to Z9–14:AL was as efficient as the seven-component blend (Hartstack et al., 1980). Therefore, Tumlinson et al. (1982), Pope et al. (1982), and Teal et al. (1985) collected and analyzed volatiles released by sexually attractive *H. virescens* females and were able to identify only the aldehyde components. Vetter and Baker (1984) assessed the behavioral function of each of the seven components identified by Klun et al. (1980a) in laboratory flight tunnel studies and were unable to ascribe a behavioral function to Z11–16:OH. Further, analysis of gland extracts of *H. zea* calling females revealed the presence of Z11–16:OH, but field studies indicated that as little as 0.1% of this compound in pheromone lures reduced trap captures (Teal et al., 1984). Thus, although both species produce this alcohol, there was no evidence that it was released as a volatile pheromone component. A third species of this genus, *H. subflexa* (Gn.), also maintains Z11–16:OH and other alcohols in the pheromone gland (Teal et al., 1981; Klun et al., 1982) but, as occurs with *H. zea*, the incorporation of these alcohols into pheromone lures caused significant reductions in the capture of males (Teal et al., 1981). These results suggested that the alcohols produced by all three species were not released as volatile pheromone components but were involved in pheromone biosynthesis. The following reports the results of in vivo studies on the terminal steps of pheromone biosynthesis by *H. virescens*

and *H. zea* females which indicate that the identified alcohols are immediate precursors of their analogous aldehyde pheromone components.

METHODS AND MATERIALS

General. Heliothis virescens and *H. zea* females used in these studies were obtained from laboratory colonies maintained at the University of Guelph and and Insect Attractants, Behavior and Basic Biology Research Laboratory. Pupae were allowed to emerge in 30 × 30 × 30-cm clear plastic boxes under both reversed and standard 16:8 light–dark cycles at 26°C (day) 24°C (night) and 55% relative humidity. Newly emerged adults were transferred to new cages daily and were provided with a 10% sugar solution soaked onto cotton. Insects used in all studies were virgin females between 2 and 5 days old.

In all experiments, the terminal abdominal segments which contain the sex pheromone gland (Teal et al., 1983) were extended by applying pressure to the thorax and anterior abdominal segments. Ethyl ether extracts were prepared by removing the terminal abdominal segments of five females and extracting the tissue for 120 sec in 25 μl of diethyl ether (Fisher, anhydrous reagent grade). Ten microliters of isooctane (Fisher, 99 mole %) containing the appropriate internal standards (Teal et al., 1985) were then added and the volume was reduced to 5 μl under N_2 prior to gas chromatographic (GC) and GC–mass spectral analysis.

In whole insect preparations, the terminal segments were clamped in an extended position using the technique described by Bjostad and Roelofs (1983). A 0.5-μl drop of the test chemicals dissolved at various concentrations in dimethyl sulfoxide (DMSO) was then applied to the surface of the pheromone gland using a 1.0-μl syringe and allowed to penetrate for various lengths of time. In experiments conducted to assess the need for molecular oxygen by the enzyme system, the extended terminal abdominal segments were removed from the abdomens of noncalling females during either the light or dark period and placed in a 0.5-ml conical microvial under either N_2 or air. The glands were then treated with the test alcohols as described above. In these experiments the treated glands were maintained for 30 min under either air or N_2 or under N_2 for 15 min then under air for 15 min. After incubation, the terminal abdominal segments were extracted for 2 min by addition of 5 μl of isooctane containing 10 ng each of tridecan-1-ol acetate and pentadecan-1-0l acetate as internal standards. The total volume of the extract was then injected onto the capillary gas chromatographic columns.

In experiments to determine if the enzyme was present on the surface of the cuticle overlying the glands, emulsions of 200 μg of the test alcohols per milliliter of distilled H_2O were prepared by sonication on a daily basis. One

microliter (200 ng) of the emulsion was applied to the gland surface of non-calling *H. virescens* females and the preparations were allowed to incubate for 15 min prior to extraction with 10 μl of isooctane. Approximately 10 min prior to GC analysis, ca. 2 mg of granular anhydrous $MgSO_4$ was added to the extract to absorb any H_2O. Half the extract was then chromatographed on each capillary column. Experimental controls included extracts of untreated glands and extracts of 1-μl drops of the alcohol–H_2O emulsion.

Chemical Analysis. Gas chromatographic (GC) analyses were conducted using Hewlett-Packard 5792 and 5890 GCs equipped with splitless capillary injectors and flame ionization detectors. Data were recorded and processed using Hewlett-Packard 3390 reporting integrators. Fused silica capillary columns used for analyses included 30-m × 0.25-mm (ID) SPB1 and SPB10 (Supelco) and a 25-m × 0.22-mm (ID) BP20 (SGE). Conditions of chromatography were as follows: initial temperature = 80°C, splitless injector purge at 0.5 min, oven temperature increased at 25°/min after 1 min to final temperatures of 160°C or 165°C (SPB1 and SPB10) and 150° (BP20). Hydrogen was used as the carrier gas at a linear flow velocity of 38 cm/sec. Retention times of compounds eluting during the analyses of extracts were compared with those of synthetic standards and amounts of individual components were calculated.

Mass spectral analyses (MS) were conducted using VG 1212F and Finnigan 3200 chemical ionization mass spectrometers interfaced to capillary GCs having splitless injectors. Both methane and isobutane were used as reagent gases and helium was used as the carrier gas. The SPB1 column was used in the Finnigan system while a 50-m × 0.25-mm (ID) DB5 column (J&W) was used in the VG 1212F. Spectra of natural compounds were compared with those of authentic standards.

Synthetic Compounds. Saturated aldehydes including pentadecanal (15:AL), hexadecanal (16:AL), and octadecanal (18:AL) were prepared by oxidation of the corresponding alcohols obtained from the Sigma Chemical Company (St. Louis, Missouri) with pyridinium chlorochromate (Corey and Suggs, 1975). Bombykol [(*E*)-10-(*Z*)-12-hexadecadienol] (BKOH), bombykal [(*E*)-10-(*Z*)-12-hexadecadienal] (BKAL), 2-tetradecanol (2–14:OH), 3-tetradecanol (3–14:OH), and 5-tetradecanol (5–14:OH) were synthesized by R.E. Doolittle (Insect Attractants Laboratory, USDA, Gainesville, Florida). All other compounds were obtained from Albany International Ltd. (Needham Heights, Massachusetts). All saturated compounds were recrystallized from pentane while mono- and diunsaturated compounds were purified by high-performance liquid chromatography using a 25 × 1.2-cm (OD) $AgNO_3$ impregnated silica column eluted with toluene (Heath and Sonnet, 1980). GC analyses of all compounds using the SPB1 and BP20 capillary columns indicated that the compounds were at least 99% pure and were free of the corresponding alcohols or aldehydes.

Flight-Tunnel Studies. Flight-tunnel studies were conducted in the 2.0-m (l) × 1.0-m (w) × 0.5-m (h) wind tunnel used by Teal et al. (1985). Airflow

through the tunnel was 0.5 m/sec and was exhausted out from the building using a laboratory fume hood. In these experiments the terminal abdominal segments of actively calling *H. zea* females were clamped in an extended position as described earlier and the alligator claps positioned in the holes of an aluminum concentrator block so that the glands were pointing up. A 1-μl drop of a solution containing 10 ng each of 14:OH and Z9–14:OH in DMSO was then applied to the gland surface and the concentrator block was immediately positioned 1 m upwind from the male release point on a platform which raised the preparations to the middle of the tunnel. Three females were used in each test. Individual male *H. virescens* were then released in the downwind end as described by Teal et al. (1985), and the behaviors of the males were recorded over a 3-min period. Females were replaced after three males had been flown. The behaviors elicited by the volatiles released from the surface of the treated glands were compared to the male behaviors which occurred when presented with *H. zea* females which had been treated with DMSO alone.

RESULTS

Identification of Pheromone Related Alcohols. A full discussion of the identification of 14:OH, Z–14:OH, 16:OH, and Z11–16:OH is given in Teal et al. (1985). In brief, GC and GC-MS analyses using a variety of different columns, including those reported in this paper, proved that the sex pheromone gland of calling *H. virescens* females contains the above alcohols (Teal et al., 1985). The ratio of the alcohols was 83% Z11–16:OH, 7% 16:OH, 3% Z9–14:OH, 3% 14:OH.

We also conducted a study on the production of Z11–16:OH during the calling period. In this study we extracted the pheromone glands of groups of five *H. virescens* females in ethyl ether at 30-min intervals throughout the dark period. Capillary GC analysis of the extracts indicated that the amounts of Z11–16:AL and Z11–16:OH increased and peaked during the calling period and that the ratio of these compounds remained relatively constant. However, the ratio favored the alcohol in precalling females.

Application of Alcohol Pheromone Analogs. In initial studies, large amounts (0.5–1.0 μg) of 16:OH were applied to the surface of the pheromone glands of both *H. virescens* and *H. zea* ca. 1 hr prior to the onset of pheromone release (Tingle et al., 1978) and the glands were extracted 2 hr later. As indicated in Figure 1, GC analyses of the extracts on the three columns revealed that there was an average 12-fold increase in the concentration of 16:AL in *H. virescens* extracts ($N = 20$) and a tenfold increase in the *H. zea* extracts ($N = 15$) with respect to glands treated with DMSO alone. These increases were coupled with four- to fivefold reductions in the concentrations of the other aldehydes in the extracts. Application of similar amounts of Z9–14:OH, 14:OH,

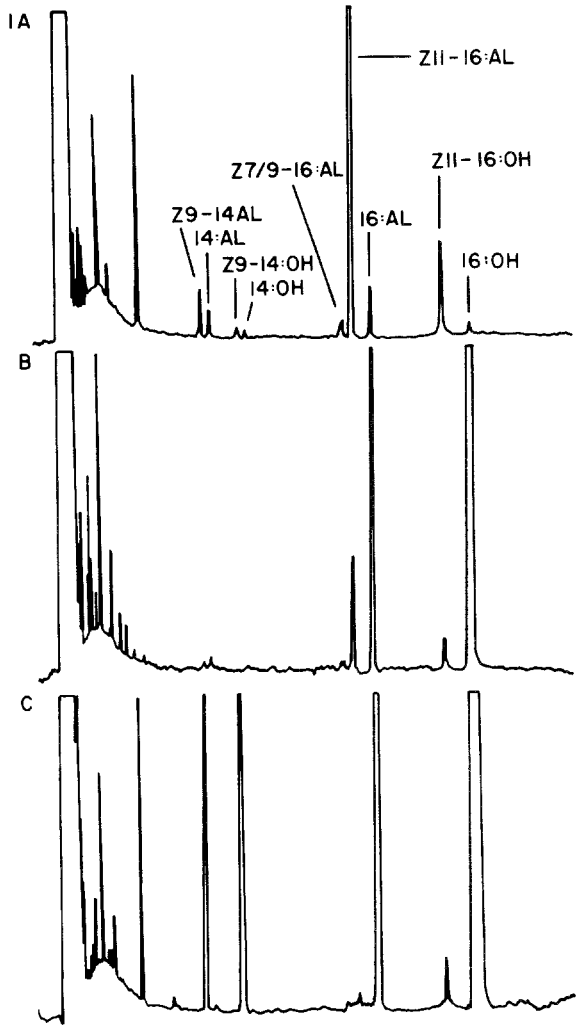

FIG. 1. Chromatograms of gland extracts of *H. virescens* on the SPB1 column. (A) Extract of untreated gland. (B) Extract of gland treated with 1 μg of 16:OH in DMSO. (C) Extract treated with a 3:1 ratio of 16:OH and Z9–14:OH.

and (Z)-7-hexadecenol resulted in 10- to 14-fold increases in the amounts of the corresponding aldehydes. Similarly, the application of a mixture of 0.5 μg Z9–14:OH and 1.5 μg of 16:OH resulted in large increases and a 1:3.5 ratio of (Z)-9-tetradecenal (Z9–14:AL) and 16:AL (Figure 1).

The initial studies had indicated that large nonphysiological amounts of the alcohols were preferentially converted to the aldehydes. However, the large

amounts applied may have disrupted the biosynthetic system within the pheromone gland. Therefore, we applied 50 ng of 16:OH to the surface of the pheromone glands of *H. zea* females and extracted them after 15 min. Extracts of the treated glands contained 8–12 ng ($N = 5$) of 16:AL while extracts of untreated glands contained 0.5–1.2 ng of 16:AL ($N = 10$).

Enzyme specificity and Functioning. Enzyme specificity was studied by applying several alcohols unrelated to any of the aldehyde pheromone components present in the pheromone glands of either *H. zea* or *H. virescens*. In initial studies, 1.0 μg of 18:OH was applied to the surface of precalling *H. virescens* glands ($N = 15$). The glands were extracted and analyzed after a 2-hr incubation period. All of the treated extracts contained substantial amounts of 18:AL as indicated by GC and GC-MS analyses. Similarly, GC ($N = 10$) and GC-MS analyses of glands treated with 15:OH indicated that this alcohol was converted to the corresponding aldehyde (Figure 2).

To assess the specificity of the enzyme system for "*Z*" configurational double bonds and for monounsaturated alcohols, glands of *H. virescens* were treated with 1 μg of either (*E*)-11-tetradecenol ($N = 10$) or BKOH ($N = 20$). As indicated in Figure 2, between 15 and 27 ng of BKAL were produced by the BKOH preparations after a 1-hr incubation period. Similarly, (*E*)-11-tetradecenal was produced when the corresponding alcohol was applied. We also studied the specificity of the enzyme for primary alcohols by applying 1-μg amounts of 2–14:OH ($N = 10$), 3–14:OH ($N = 10$), and 5–14:OH ($N = 15$)

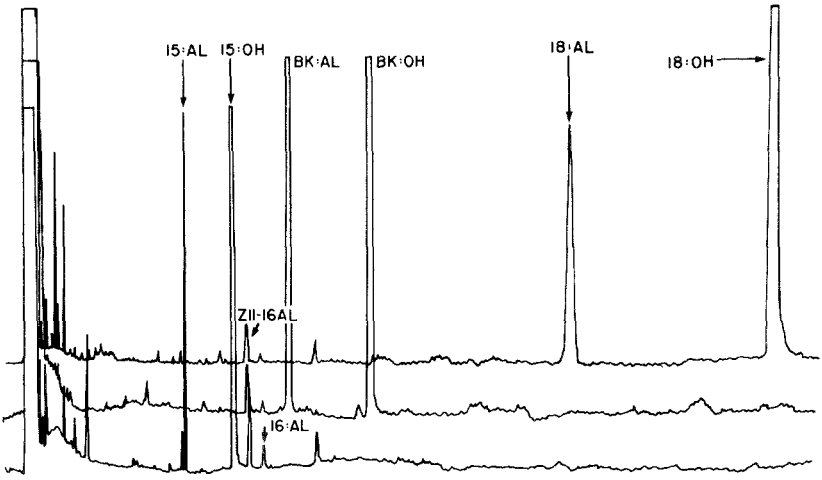

FIG. 2. Chromatograms of extracts of glands treated with octadecanol, pentadecanol and bombykol on the SPB1 column. Octodecanal = 18:AL, octadecanol = 18:OH, pentadecanal = 15:AL, pentadecanol = 15:OH, BKAL = bombykal, BKOH = bombykol.

to the glands as described above. GC analyses indicated that none of the secondary alcohols were converted to their corresponding ketones.

Studies on the effect of the circadian rhythm governing pheromone release on enzyme functioning were conducted by applying 50 ng of 16:OH in DMSO to the surface of the glands of noncalling *H. virescens* females during the photophase. Glands were removed, extracted, and analyzed after a 15-min incubation period. Results of this study indicated substantial amounts of 16:AL($\bar{x}$ = 13.3 ng, N = 5) were produced by treated glands, while control glands did not contain detectable amounts of this aldehyde.

Application of 100 ng of 16:OH to glands removed from females during the photoperiod and maintained under atmospheric conditions in microvials for 30 min prior to extraction resulted in the production of 15–30 ng of 16:AL (N = 15). However, application of 100 ng of 16:OH to glands removed during the photoperiod and maintained under N_2 for 30 min did not result in the production of 16:AL (N = 15) (Figure 3). When air was exchanged for N_2 after 15 min of incubation and the glands extracted 15 min later, substantial amounts of 16:AL (12–25 ng, N = 15) were produced.

Rate of Aldehyde Production and Release. The above studies had indicated that the alcohols were converted to the corresponding aldehydes very rapidly but gave little information about the actual rate of biosynthesis. To study this we applied 500 ng of (*E*)-11-tetradecenol to the surface of glands of *H. zea* females which had been removed and placed in microvials. Glands were extracted 1, 2.5, and 5 min after application of the alcohol. Analysis of the extracts indicated that 7.9 ng (±2.2 SE), 12.5 ng (±1.7 SE), and 18.6 ng (±2.4 SE) of (*E*)-11-tetradecenal were produced in 1, 2.5, and 5 min, respectively.

Application of 200 ng of *Z*9–14:OH in 1 μl of distilled H_2O to the gland surface of noncalling females (N = 10) resulted in the production of ca. 20 ng of *Z*9–14:AL after a 30-min incubation period. As in other experiments, little if any of the other pheromone components were detected in these extracts. This study was repeated using 16:OH (N = 15), 14:OH (N = 10), and *E*11–14:OH (N = 5) with similar results.

Flight-Tunnel Studies. In flight-tunnel studies, used to discover if the artificially produced aldehydes were released as volatiles from the surface of the pheromone gland, 65% of the male *H. virescens* entered taxis and landed on the platform which held the treated *H. zea* females and 40% attempted to copulate with these females (N = 20) (Figure 4). No males entered taxis in response to *H. zea* females treated only with DMSO.

DISCUSSION

Several studies on the actual volatile pheromone blend released by *H. virescens* have indicated that only aldehydes are released (Tumlinson et al., 1982;

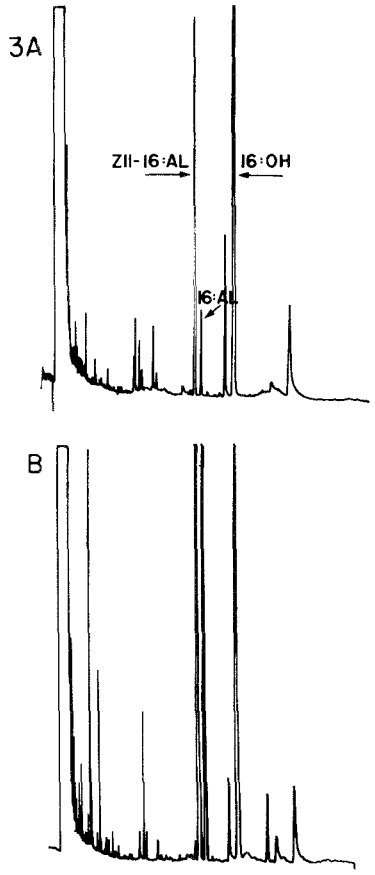

FIG. 3. Chromatograms of glands of calling *H. virescens* on the SPB1 column treated with 100 ng of 16:OH in DMSO and maintained (A) under N_2 for 30 min, and (B) under N_2 for 15 min and then air for 15 min.

Pope et al., 1982; Teal et al., 1985). However, both *H. virescens* and *H. zea* maintain significant amounts of Z11-16:OH in the pheromone glands and, in the case of *H. virescens*, 14:OH, Z9-14:OH, and 16:OH have been identified in pheromone gland extracts (Teal et al., 1983, 1985). Until now no function could be ascribed to these alcohols. Results of our study have demonstrated that the alcohols present in the pheromone gland of these species are rapidly converted to the corresponding aldehydes and are the immediate precursors of the pheromone components. The alcohol oxidase responsible for the conversion was found to be specific for primary alcohols but appears to show little specificity for either the level of unsaturation or geometric configuration of double bonds. Further, as indicated by the reduced amount of Z11-16:AL present in

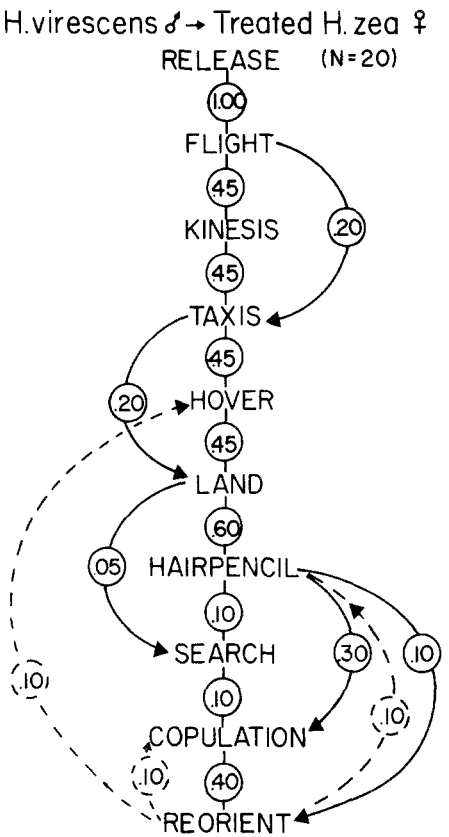

FIG. 4. Ethogram of the responses of *H. virescens* males ($N = 20$) to *H. zea* females treated with 20 ng of Z9–14 : OH and 14 : OH and placed in the upwind end of the 2-m-long flight tunnel. Numbers indicate the probabilities that each transition will occur.

extracts treated with alcohols other than Z11–16 : OH and production of a 1 : 3.5 ratio of Z9–14 : AL to 16 : AL when a 1 : 3 ratio of the corresponding alcohols was applied, substrate level competition for the enzyme regulates the blend of aldehydes produced. This is consistent with the ratio of alcohols to aldehydes in gland extracts found in earlier studies (Teal et al., 1985).

Wolf et al. (1981) documented a correlation between the ratios of unsaturated 14- and 16-carbon fatty acids present in the pheromone gland of *H. virescens* with the corresponding aldehyde pheromone components. They also found the saturated acids and suggested that the fatty acids were intermediates in pheromone biosynthesis. Subsequent work on other species indicated that the acids were intermediate precursors for the sex pheromones of those species and that double-bond position was the result of a specific Δ-11-desaturase and sub-

sequent chain-shortening step (Bjostad et al., 1981; Bjostad and Roelofs, 1981, 1983). Our hypothesis is that the free acids are converted to their corresponding alcohols which are subsequently converted to the aldehyde pheromone components by a cuticular bound oxidase. The limiting step occurs prior to the production of the aldehydes, as is indicated by the relatively constant ratios and amounts of alcohols present in the glands of calling *H. virescens* females (Teal et al., 1985). This is supported by the fact that the oxidase converts primary alcohols to aldehydes without regard to the diel periodicity of pheromone release and that the levels of the alcohols rise just prior to the onset of calling and then fall to essentially zero at the end of the scotophase.

The dependence of the enzyme on molecular oxygen demonstrated that it is a primary alcohol oxidase. This finding was supported by results of unpublished in vitro experiments conducted as described by Morse and Meighen (1984) in which various cofactors including NAD, NADP, and FAD failed to stimulate enzyme activity. A similar biosynthetic system employing an alcohol oxidase has been identified for the spruce budworm moth (Morse and Meighen, 1984), and therefore oxidases may be common among Lepidoptera species using aldehyde pheromone components.

The production of aldehydes within the first minute after application of the analogous alcohols was surprising because we anticipated that penetration into the gland would take a longer period. This suggested that perhaps the oxidase was present either within or on the surface of the cuticle. Insect cuticle contains a layer of apolar hydrocarbons which make it impermeable to water (Locke, 1974). Therefore, the alcohols would remain on the cuticular surface if applied in H_2O, and any conversion could be attributed to a cuticular bound alcohol oxidase. The fact that approximately the same amounts of the aldehydes in question were produced in these studies as when the alcohols were applied in DMSO strongly supported our hypothesis that the terminal step in aldehyde pheromone biosynthesis occurs within and on the surface of the cuticle overlying the pheromone gland. Cuticular bound enzymes have been identified from a number of insect species (Locke, 1974; Kapin and Ahmad, 1980; Ferkovich et al., 1982). Ferkovich et al. (1982) also demonstrated that cuticular esterases in the male cabbage looper [*Trichoplusia ni* (Hübner)] were involved in degradation of the female sex pheromone. However, ours is the first study indicating that cuticular enzymes are involved in pheromone production.

Although evidence pointed to the fact that a cuticular bound alcohol oxidase was responsible for the conversion of the alcohols to the actual aldehyde pheromone components in both *H. virescens* and *H. zea*, we did not know if the artificially produced aldehydes were released as volatile compounds. Therefore, we conducted flight-tunnel studies using *H. zea* females and *H. virescens* males. *H. zea* females do not produce Z9–14 : AL or 14 : AL, while *H. virescens* does, and these aldehydes have been demonstrated to be necessary for effective

sexual communication by *H. virescens* (Vetter and Baker, 1984; Teal et al., 1985). Further, these 14-carbon aldehydes appear to be responsible for semiochemically induced reproductive isolation between *H. virescens* and *H. zea* (Klun et al., 1980a,b). Therefore, by treating the *H. zea* pheromone gland with the 14-carbon alcohols, we had not only induced the production and release of 14:AL and Z9–14:AL into the air but had also eliminated semiochemically induced reproductive isolation between the two species.

The development of a cuticle-bound alcohol oxidase suggests that conversion of the alcohols to aldehydes within the pheromone gland cells is an inefficient method of aldehyde production. This is reasonable when considering the biochemical instability of aldehydes within cellular systems and ease of interconversion between aldehydes and alcohols (Snyder, 1972). It is probable that if aldehydes were produced directly from the acids within the pheromone gland cells, they would be rapidly converted to the corresponding alcohols (Reichwald-Hacker, 1983). Therefore, the aldehydes would not be available for use as pheromone components. The use of the cuticle-bound alcohol oxidase ensures that the aldehydes are available for pheromone release and that all of the alcohols are converted as they pass through the cuticle. At present, the precursor steps in alcohol biosynthesis by *H. virescens* and *H. zea* are undetermined. However, it is unlikely that alcohol biosynthesis proceeds via the production of the corresponding acetates, as occurs in the spruce budworm moth (Morse and Meighen, 1984), because none of the acetates have ever been identified in pheromone gland extracts of either *H. virescens* or *H. zea*. We are investigating the biosynthetic route responsible for the production of the alcohols at the present time.

In conclusion, our study has demonstrated that alcohols present in the pheromone glands of both *H. zea* and *H. virescens*, which have no behavioral function, are the immediate biosynthetic precursors of the aldehyde pheromone components. Further, we have demonstrated that the aldehydes produced are released as volatiles from the surface of the pheromone gland. These studies have added considerable knowledge to our understanding of the semiochemical-mediated biology of *Heliothis* species.

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SPRUCE BUDWORM (*Choristoneura fumiferana*)[1] PHEROMONE CHEMISTRY AND BEHAVIORAL RESPONSES TO PHEROMONE COMPONENTS AND ANALOGS

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Abstract—This paper reviews the sex pheromone chemistry and pheromone-mediated behavior of the spruce budworm and related coniferophagous (*Choristoneura*) budworms. In *C. fumiferana*, temporal changes in pheromone-gland monounsaturated fatty acids (pheromone precursors) enable the prediction of the primary sex pheromone components. This technique may also be applicable for predicting additional pheromone components. Tetradecanal (14:Ald), previously shown to enhance close-range precopulatory behavior, lowers the threshold of response by males for upwind flight to a pheromone-component source. Spruce budworm males maintain upwind flight to 95:5 (*E*/*Z*)-1,12-pentadecadiene (diolefin analog) after initiating upwind flight to a primary-component pheromone source (95:5 *E*/*Z*11-14:Ald). This is the first demonstration of apparently normal male flight responses to a pheromone analog.

Key Words—*Choristoneura fumiferana*, spruce budworm, Lepidoptera, Tortricidae, sex pheromone, behavior, flight tunnel, pheromone analog, pheromone fatty acid precursors.

INTRODUCTION

Lepidopteran female sex pheromones are, with few exceptions, multicomponent, consisting of a blend of two or more chemicals emitted at fairly consistent

[1] Lepidoptera: Tortricidae

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ratios and release rates. These chemical blends elicit in conspecific males sequences of behaviors that include upwind flight which brings males to within close proximity of females. These behaviors are often readily observed in laboratory wind tunnels, and responses by males to synthetic pheromone sources can be compared to their responses to natural pheromone or females.

The existence of a pheromone communication system in the spruce budworm (*Choristoneura fumiferana* Clemens) was demonstrated by Greenbank (1963), but elucidation of the blend of chemicals comprising the female sex pheromone is still incomplete. However, recent progress has been made in defining the sex-pheromone chemistry and pheromone-mediated behavior of this insect. This paper reviews the history and some current research related to the spruce budworm pheromone communication system.

BACKGROUND

In the genus *Choristoneura* (Lepidoptera: Tortricidae), known primary sex pheromone components are comprised of Δ11-unsaturated C_{14} carbon-chain compounds. Different species' primary sex pheromone components have different oxygenated functional groups and varying blends of these components (see Inscoe, 1982). Specifically, the coniferophagous spruce budworms comprise a group of closely related (*Choristoneura*) species, native to North America (Freeman, 1967; Freeman and Stehr, 1967; Powell, 1980). Six species have been studied in terms of their pheromone specificity (Sanders, 1971; Sanders et al., 1974, 1977). The components of these female-produced sex pheromones form a group of congeneric Δ11-C_{14} aldehydes (Δ11–14 : Ald), acetates (Δ11–14 : Ac), and alcohols (Δ11–14 : OH), with blends, geometrical isomer ratios, and release rates specific to each species (Table 1).

In attempting to characterize the sex pheromone of budworm species through cross-attraction studies, Sanders et al. (1977) concluded that *C. fumiferana*, *C. occidentalis*, and *C. biennis* apparently had similar pheromones while *C. pinus pinus*, *C. orae*, and *C. retiniana* (= *C. viridis*; Powell, 1980) were mutually cross-stimulating but did not appear to have sex pheromone components in common with the former group. This was an accurate assessment; subsequent research has shown that the former group utilizes Δ11–14 : Ald's as primary sex pheromone components, whereas the latter, utilize Δ11–14 : Ac's or Δ11–14 : Ac/Δ11–14 : OH blends (Table 1). It has yet to be verified that the *C. biennis* sex pheromone is Δ11–14 : Ald, although present evidence is strongly supportive (Sanders, 1971; Sanders et al., 1974, 1977). Sex pheromone components of the remaining five species have been documented: *C. fumiferana* (Sanders and Weatherston, 1976; Silk et al., 1980) and *C. occidentalis* (Cory et al., 1982; Silk et al., 1982) have been shown to release *E*/*Z*11–14 : Ald as

TABLE 1. SEX PHEROMONE COMPONENTS[a] OF CONIFEROPHAGOUS *Choristoneura* spp.

Species	Primary	Additional (Secondary)	References
C. fumiferana	*E*11-14:Ald		Weatherston et al., 1971
	96:4 *E*/Z11-14:Ald		Sanders and Weatherston, 1976
	95:5 *E*/Z11-14:Ald	14:Ald	Silk et al., 1980 Alford et al., 1983
C. occidentalis	92:8 *E*/Z11-14:Ald		Cory et al., 1982
	92:8 *E*/Z11-14:Ald	89:11 *E*/Z 11-14:Ac 85:15 *E*/Z 11-14:OH	Silk et al., 1982 Alford and Silk, 1983
C. biennis	*E*/Z11-14:Ald[b]		Sanders, 1971
	E/Z ratio unknown		Sanders et al., 1974
C. orae	82:9:9, *E*11-14:Ac, Z11-14:Ac, *E*11-14:OH		Gray et al., 1984
C. pinus pinus	90:10, 85:15 *E*/Z11-14:Ac, 85:15 *E*/Z11-14:OH		Silk et al., 1985b
C. retiniana	92:8, *E*/Z11-14:Ac	*E* or Z11-14:OH (enhances trap capture)	Daterman et al., 1984

[a] *E*/Z11-14:Ald = (*E*/*Z*)-11-tetradecenal; *E*/Z11-14:Ac = (*E*/*Z*)-11-tetradecenyl acetate. *E*/Z11-14:OH = (*E*/*Z*)-11-tetradecen-1-ol; 14:Ald = tetradecanal.
[b] Inferred from cross-attraction studies (Sanders, 1971).

primary sex pheromone components. In contrast, *C. pinus pinus* (Silk et al., 1985b), *C. retiniana* (Daterman et al., 1984), and *C. orae* (Gray et al., 1984) release *E*/Z 11-14 : Ac or *E*/Z11-14 : Ac/*E*/Z11-14 : OH blends as primary components (Table 1).

However, all species have Δ11-14 : Ac in common as the major component in the pheromone gland (in references above). In *C. fumiferana* Δ11-tetradecenyl acetate is synthesized *de novo* only in the pheromone gland, the Δ11-14 : Ac is the direct biosynthetic precursor to the aldehyde pheromone (Morse and Meighen, 1984). Morse and Meighen (1984) also found that the female diel aldehyde-emission period was synchronous with acetate production, and this supports their hypothesis that in this (coniferophagous) *Choristoneura* group a metabolic relationship exists between the aldehyde, acetate, and alcohol. It seems likely, therefore, that species specificity in pheromone production is controlled by species-specific metabolic processes, giving rise to different functional groups, ratios, and release rates from the common acetate precursor.

REVIEW OF SEX PHEROMONE CHEMISTRY OF SPRUCE BUDWORM

Among coniferophagous budworms, the spruce budworm, *C. fumiferana*, has been the most intensively studied. Early work indicated that spruce budworm females release a pheromone that "attracts" males (Greenbank, 1963). Subsequently, *E*11–14 : Ald was identified (Weatherston et al., 1971) as a pheromone component. The importance of adding the *Z* to the *E* isomer was determined upon reanalysis of female volatiles and a (96:4) *E/Z*11–14:Ald blend was shown to maximize trap captures (Sanders and Weatherston, 1976). Solvent extracts of excised pheromone glands were inactive in eliciting male response (Sanders, 1971). Reanalysis of gland extracts identified *E*11–14:OH (Weatherston and MacLean, 1974) and *E*11–14 : Ac (Wiesner et al., 1979), both of which inhibited trap capture (Sanders and Lucuik, 1972; Sanders et al., 1972; Sanders 1976).

More detailed chemical analyses (Silk et al., 1980) of pheromone gland extracts showed that Δ11–14 : Ac (20–40 ng/insect), Δ11–14 : Ald (1–3 ng/insect), and Δ11–14 : OH (1–3 ng/insect) were all present in 95 : 5 *E/Z* ratios; the saturated analogs of each functionality were also present at ca. 1% of the corresponding *E* isomer. In contrast, effluvia from "calling" females were found to contain *E/Z*11–14 : Ald (95 : 5; 10–40 ng/insect/night) and the saturated analog, tetradecanal (14 : Ald) at ca. 2% of the *E*11–14 : Ald (Silk et al., 1980). In addition, traces of (*E*)11–14 : Ac were found; no alcohols were detected.

In the same study, field testing showed that there were no significant differences in trap captures between traps baited with all four components (formulated in PVC at female effluvial ratios) compared to captures using the primary components (95 : 5 *E/Z*11–14 : Ald) alone. The reduction of trap capture by admixture of Δ11–14 : Ac with 95 : 5 *E/Z*11–14 : Ald (Sanders et al., 1972) was confirmed; however, this effect appeared to be negated by the presence of 14 : Ald, but only when all components were present in synthetic sources in female effluvial ratios (Silk et al., 1980). Pheromone release rate by "calling" females, measured by a specific and sensitive bioluminescent assay technique (Morse et al., 1982, Meighen et al., 1981, 1982), occurs mainly during scotophase in a series of "bursts" at rates as high as 50 ng/hr with considerable individual variability.

REVIEW OF PHEROMONE-MEDIATED BEHAVIOR IN SPRUCE BUDWORM

Behavioral patterns involved in mate location in feral insects generally involve upwind flight, apparent close-range orientation, and copulation (Roelofs and Carde, 1977). As in most moths, spruce budworm males locate conspecific females by flying upwind along a pheromone plume. Often, the effects of putative pheromone components on these male behaviors has been inferred from

a chemical's effect on trap capture (until recently, this was also true for spruce budworm); however, observations and quantitation of some of these behaviors can be conducted in a sustained-flight wind tunnel (e.g., Miller and Roelofs, 1978). We review here recent field and wind-tunnel work, and present some recent progress.

Sanders (1981a) showed that synthetic 95 : 5 *E*/*Z*11–14 : Alds were equivalent in "attraction" to virgin females in field trapping experiments when using a 0.03% PVC source, which releases these primary components at a rate close to that of a "calling" virgin female (Silk et al., 1980). Furthermore, in preliminary wind-tunnel work, some males demonstrated an apparent full range of precopulatory behaviors, e.g., upwind flight, courtship, and copulatory attempts in response to only these two primary components (Sanders, 1979). However, more detailed observations in the wind tunnel (Sanders *et al.*, 1981) showed that males exposed to pheromone produced by "calling" females exhibited a higher incidence of upwind flight and made more rapid upwind progress than males exposed to a similar concentration of synthetic pheromone (95 : 5 *E*/*Z*11–14 : Ald).

Sanders and Seabrook (1982) concluded that it was unlikely that other chemicals were involved in the "attraction" phase of the mating process. However, recent observations in our laboratory's wind tunnel and in the field (Alford et al., 1983) indicated an effect of tetradecanal (14 : Ald) on the behavior of male spruce budworm. A greater number of males initiated upwind flight and continued on to contact a source with ca. 5% 14 : Ald added to the Δ11–14 : Ald's than when only the Δ11–14 : Alds were present.

The addition of *E*11–14 : Ac to the Δ11–14 : Alds decreased the males' responsiveness to the aldehydes, but when present at low levels, its effect appeared to be attenuated when 14 : Ald was also present (Alford et al., 1983). This latter effect was also seen in earlier field-trapping experiments (Silk et al., 1980) and was subsequently confirmed by further laboratory wind-tunnel studies (Sanders, 1984). In addition, Sanders (1984) found that duration of sustained flights was significantly longer in response to "calling" females than to any synthetic sources (with or without 14 : Ald), implying that the synthetic blends are incomplete with respect to the female-emitted blend.

RECENT PROGRESS

It is apparent that not all the chemicals involved in the sex pheromone communication system of spruce budworm are known. In monitoring and mating disruption programs, however, it may not be essential to know every minor component although, as Roelofs has pointed out (1978), trap specificity and potency may be greatly increased as the synthetic lure more closely duplicates the natural pheromone, and it is presumed that the efficacy of mating disruption

would likewise be enhanced by a "more complete pheromone." Sanders (1984) concluded that, although single components can cause considerable mating disruption in noctuids (Campion et al., 1981), incomplete blends are considerably less effective against tortricids (Charlton and Cardé, 1981; Roelofs and Novak, 1981; Sanders, 1981b). This has led many to the conclusion that elucidation of the "complete" pheromone blend for budworm is of importance prior to further major mating-disruption tests. Recent work in our laboratory has, therefore, focused on the identification of the "complete" spruce budworm pheromone.

Previous analyses of female spruce budworm effluvia (Silk et al., 1980), using a Porapak® Q collection technique, did not indicate, at least from a chemical perspective, the presence of other components. This method, however, does introduce relatively large amounts of contamination and minor components may have been obscured by this background contamination.

The fact that, chemically, minor components are present in very low quantities in spruce budworm prompted the use of indirect techniques to ascertain component identity. Recently, Bjostad and Roelofs (1981, 1983) demonstrated that fatty acid precursors of female sex pheromone components can be identified and used to predict the presence of minor pheromone components (Bjostad and Roelofs, 1983; Bjostad et al., 1984). A technique for rapidly identifying these monounsaturated fatty acids in pheromone glands excised from female moths, using GC-MS analysis after dimethyldisulfide derivatization (DMDS) of gland extracts has been developed. A detailed analysis of monounsaturated fatty acids present in the pheromone glands of *C. fumiferana*, *C. occidentalis*, and *C. pinus*

TABLE 2. MONOUNSATURATED FATTY ACID ESTERS OBTAINED BY DMDS DERIVATIZATION OF PHEROMONE GLAND EXTRACTS FROM THREE *Choristoneura* SPECIES[a]

DMDS[b] Δ-Fatty ester	*C. fumiferana*	*C. occidentalis*	*C. pinus pinus*
9-12:Me	T	T	T
5-14:Me	VS	VS	T
7-14:Me	T	T	T
9-14:Me	VS	VS	VS
11-14:Me	M	L	S
7-16:Me	M	M	S
9-16:Me	L	L	L
11-16:Me	M	M	S
12-16:Me	T	VS	ND[c]

[a] Quantitative estimate relative to the most abundant fatty ester DMDS adduct (C_{12}–C_{16}). L 100–50; M 50–30; S 30–10; VS 10–1; T < 1 (L, M, S, VS, T: large, medium, small, very small, and trace, respectively; in %). Data from Dunkelblum et al., 1985.

[b] Methanolyzed samples of chloroform–methanol extracts.

[c] ND, not detected.

pinus was carried out; the techniques and results are discussed in detail elsewhere (Dunkelblum et al., 1985) and are summarized in Table 2. As can be seen, the major pheromone-gland components (which have been previously identified) are readily correlated with their corresponding fatty acids. For example, in these three budworm species, the *E*/*Z*11–14:Me (*E*/*Z*11-tetradecenoic acids, methyl esters) are correlated with the Δ11-C_{14} moieties which comprise the primary pheromone components (Tables 1 and 2). The fatty acid profile is very similar for all three species. The other unsaturated fatty acids may be precursors to as yet unidentified secondary sex pheromone components in these species.

In some female moths, pheromone titer appears to be quite low on eclosion and increases to a maximum in a few days [e.g., *Trichoplusia ni* Hübner (Shorey and Gaston, 1965)]. Based on this fact, analysis of monounsaturated fatty acids in spruce budworm females by the DMDS method was repeated with the view of ascertaining whether changes in fatty acid profile occurred with moth age. Pheromone glands were obtained from groups of ca. 20 females that were in the pupal stage (just prior to emergence), 1 hr post-emergence, and 48 hr postemergence (all pupae and adults were maintained on a 16:8 light–dark cycle and glands were excised during the 2nd and 3rd hr of scotophase, coincident with mature females' peak "calling" period). Pheromone glands (three replicates) excised from these three groups were extracted with chloroform–metha-

TABLE 3. MONOUNSATURATED FATTY ACID ESTERS OBTAINED BY DMDS DERIVATIZATION OF EXTRACTS FROM PUPAL AND 48-HOUR POSTECLOSION SPRUCE BUDWORM PHEROMONE GLANDS[a]

DMDS[b] Δ-Fatty ester	Pupal glands[c]	48 h post-eclosion
5–14:Me	T	T
7–14:Me	T	T
9–14:Me	S, but largest 14:Me	S
11–14:Me	T	M, both *E*/*Z* isomers present; largest 14:Me
7–16:Me	S	M
9–16:Me	L, both *E*/*Z*; largest 16:Me	L, both *E*/*Z* isomers; largest 16:Me
11–16:Me	VS	M
12–16:Me	T	ND[d]

[a] Kuenen, Dunkelblum, and Silk, unpublished data.
[b] L, M, S, VS, and T same meaning as footnote in Table 3.
[c] Results for 1-h postemergence extracts were not significantly different from preemergence extracts and are not included in the table.
[d] ND, not detected.

nol (2:1) and subjected to acid-methanolysis followed by the DMDS reaction and capillary GC-MS (as in Dunkelblum et al., 1985).

The results are presented in Table 3. A large increase is seen for Δ11-14:Me (from <1% to 30–50%), and this is to be expected since this fatty acyl moiety is most likely the direct biosynthetic precursor to Δ11-14:Ac and this component to the pheromone, Δ11-14:Ald (Morse and Meighen, 1984). Perhaps more significantly, a large increase in titer occurred with two other monounsaturated fatty acids: Δ7-16:Me and Δ11-16:Me with the latter showing the largest increase. This prompted the supposition, following the corollary of Δ11-14:Me → Δ11-14:Ac → Δ11-14:Ald, that a similar sequence of events may be occurring in the pheromone gland of the spruce budworm for these Δ16 components. Although this does not preclude the other fatty acids (and certainly only monounsaturated moieties would be detected by this method), these two components were considered a suitable starting point for further chemical and behavioral analyses.

Reanalysis of female gland extracts (Dunkelblum and Silk, unpublished data) indicated that, indeed, Δ11-16:Ac is present in the spruce budworm sex pheromone gland (<0.1% of *E*11-14:Ac) but that Δ7-16:Ac could not be detected. However, neither Δ11-16:Ald nor Δ11-16:Ac was detected in effluvial material obtained by the Poropak Q collection method (Silk et al., 1980), although background contamination probably precluded detection. Because Δ11-14:Ac is the biosynthetic precursor to Δ11-14:Ald (primary pheromone components), it was assumed, as a first step, that since Δ11-16:Ac was determined as a gland component, it might be emitted as the aldehyde. However, initial wind-tunnel tests to determine the possible function of Δ11-16:Ald's were inconclusive; further tests will be warranted if Δ11-16:Ald's are found in female pheromone-gland volatiles.

The effects on behavior of putative pheromone components are usually assessed by adding them to compounds with known activity, (e.g., Linn and Gaston, 1981; Baker and Cardé, 1979). Alternatively, changes in male behavior have been measured in response to removing one or more components from the believed complete volatile blend, or by giving males a choice between two merging odor plumes (e.g., Teal et al., 1986). We have recently tested another approach relating to male response to low dosages of pheromone blends. More specifically, we hypothesized that males would have a lower response threshold to "more complete" pheromone blends, i.e., the active space (Bossert and Wilson, 1963) would become greater as the pheromone blend being tested more closely approached the natural female-emitted blend.

To test this hypothesis in our sustained-flight wind tunnel, we employed two "pheromone" sources (Figure 1). Both sources (rubber septa) were suspended, one behind the other, by thread (as in Baker and Kuenen, 1982; Kuenen and Baker, 1983) 15 cm above the center of the tunnel floor. The downwind

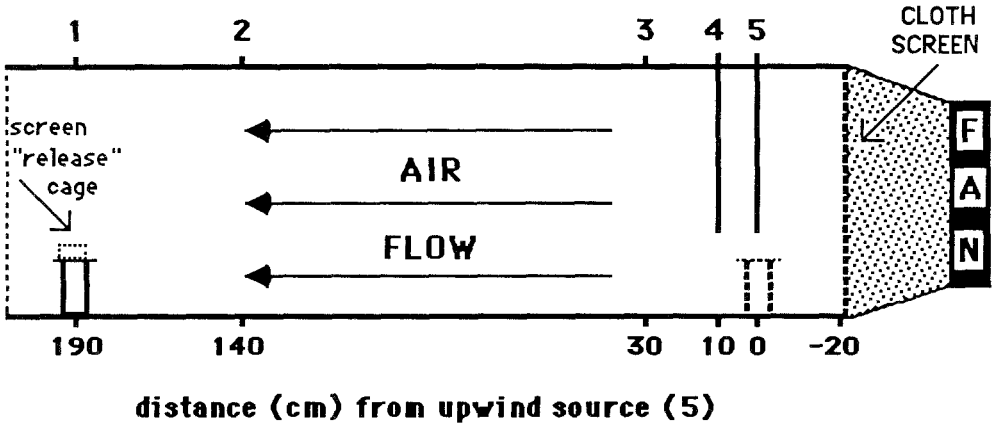

FIG. 1. Schematic side view of sustained-flight wind tunnel. In "two-source" experiments males were released from cages (at 1); as they flew upwind along the pheromone plume past (2) the downwind source (4) was pulled quickly upward, and the males' subsequent flight behaviors to the upwind source (5) were recorded.

source (DS) was constant at 3 or 10 μg 95:5 *E*/*Z*11–14:Ald, while the upwind source (US; the test source) ranged from 3 μg (95:5 *E*/*Z*11–14:Ald) down to 30 ng in half-$\log_{(10)}$ steps or the US was a blank. In a second experiment, the US contained 100 ng, 30 ng, or 10 ng Δ11–14:Ald or the same three with 5% 14:Ald added.

During the experiments, individual males were released from screen cages at the downwind end of the tunnel where some initiated upwind flight along the pheromone plume. When males, flying in or near the plume, were 140 cm from the US, the DS was quickly raised to the top of the tunnel by pulling on its supporting thread. After raising of the DS, males were observed for their up-tunnel progress and scored for whether or not they flew to within 30 cm of the US or if they continued upwind flight and contacted the US. A test flight was considered to be over as soon as males flew out of the back of the tunnel or if they touched any of the tunnel's surfaces.

Initially, we wanted to determine the threshold value for the US to which males continued to fly upwind after removal of the DS (10 μg Δ11–14:Ald). This threshold value was approximately 100 ng source dosage (Table 4). This is clearly seen by the reduction in the percentage of moths flying to within 30 cm of the US when the dosage was <100 ng (Table 4; $P < 0.05$). Additionally, the time required for males to fly to within 30 cm of the US tended to increase when the US dosage was 30 ng or 100 ng Δ11–14:Ald [Table 4; flight times were not analyzed statistically since numbers of moths flying close to the US was very low (1 or 2) when the US contained a low pheromone dose].

With the threshold value determined using Δ11–14 : Ald alone, we next tested whether this threshold would be lowered by the addition of 5% 14 : Ald to the US. Using identical procedures, we saw an apparent increased male responsiveness to low-dosage sources when tetradecanal was present. When dosage of the US was 100 ng Δ11–14 : Ald alone or plus 5% 14 : Ald, approximately the same number of moths flew to within 30 cm of the US. Most of these moths reached this source and flight times were similar. At 30-ng source loadings, however, more males continued flight to the US when 5% 14 : Ald was present ($P < 0.05$), and their flight times to both 30 cm-from-the-US and to the source were much less than flight times for males flying toward a source containing only the Δ11–14 : Ald's. (Table 5).

It appears from this data that secondary sex pheromone components, which act to increase the number of males approaching a pheromone source, also lower the response threshold for flight continuation to a similar pheromone component blend after initiation of upwind flight.

PHEROMONE ANALOGS

In some moth species, structural analogs of pheromone components have shown biological activity in terms of mating disruption (e.g., Shorey et al. 1974; Carlson and McLaughlin, 1982a,b). Activity of analogs has been predicated on the hypothesis that they are similar (isoelectronic or isosteric) to the natural compound(s) although these effects are difficult to predict. For the spruce budworm, for example, Δ9-dodecenyl formate, structurally very similar to Δ11–14 : Ald, has proven inactive in the field (Sanders et al., 1980), although formate analogs of the pheromones of the corn earworm *Heliothis zea* (Boddie) (Mitchell et al., 1975) and the tobacco budworm *Heliothis virescens* (F.) (Beevor et al., 1977) are active mating disruptants.

The response of insects to these pheromone analogs is generally difficult to assess except when examining for behaviors similar to those elicited by pheromones. Therefore, a simple and indirect assessment employing sticky traps has often been used to determine if the analogs (1) produce trap capture by themselves or (2) reduce or enhance trap capture when combined with known sex attractants, sex pheromones, or virgin females. Analogs have also been tested for their effect on mating (disruption) in field plots where analog sources have been distributed (e.g., Carlson and McLaughlin, 1982a,b).

We recently reported biological activity of a diolefin analog [95 : 5 (*E/Z*)-1,12-pentadecadiene] of Δ11–14 : Ald's (Silk et al., 1985a). Briefly, the results were as follows. Diolefin formulated in PVC vial-caps produced trap capture by itself and enhanced trap capture when combined with the Δ11–14 : Ald's. When formulated in PVC rods in a later test the same season, the diolefin produced little or no trap capture. However, when six PVCs (3.0% diolefin) were

TABLE 4. RESPONSES OF MALE SPRUCE BUDWORM TO DIFFERENT DOSAGES OF Δ-11-14:Ald[a] AFTER FLIGHT INITIATION TO 10 μg Δ11-14:Ald PLUS INDICATED DOSAGES OF Δ11-14:Ald

	Dosage Δ11-14:Ald on the upwind source (US)					
	Blank	30 ng	100 ng	300 ng	1 μg	3 μg
Mean % moths activated[b]	86.6 (29.96)	100 (−)	96.0 (8.94)	88.0 (10.95)	100 (−)	96.0 (8.94)
Mean % moths initiating flight	82.6 (29.05)	96.0 (8.94)	92.0 (10.95)	88.0 (10.95)	100 (−)	92.0 (17.89)
Mean % moths flying upwind	58.6 (21.90)	44.0 (16.73)	60.0 (20.00)	60.0 (24.49)	48.0 (22.8)	56.0 (21.91)
Mean % moths flying to within 30 cm of US[c]	8.0b (17.89)	12.0b (10.95)	40.0a (14.14)	56.0a (26.08)	44.0a (26.08)	52.0a (17.89)
Mean % moths contacting US	4.0b (8.94)	8.0b (10.95)	40.0a (14.14)	56.0a (26.08)	40.0a (24.49)	52.0a (17.89)
Flight duration from 140 cm to 30 cm downwind of US (sec)[d]	54.3 (12.84)	43.4 (20.60)	16.0 (3.06)	9.0 (1.88)	8.0 (2.59)	9.5 (3.71)
Flight duration from 140 cm downwind, to the US (sec)[d]	58.0 (4.47)	57.6 (4.24)	22.2 (4.99)	16.6 (4.86)	14.4 (5.31)	17.4 (5.75)
Mean closest approach to US (cm)	83.5a (20.35)	49.5b (18.11)	22.9c (16.01)	9.3c (20.88)	4.0c (7.15)	1.8c (4.02)

[a] Δ11-14:Ald = 95:5 *E*/Z11-14:Ald. $N = 25$ (five groups of five); values are means (± SD) calculated from means of each group of five moths tested.

[b] First three behavioral categories are indicators of male moth responsiveness used to assess the appropriateness of evaluating the subsequent behaviors; therefore no analyses were conducted on these data.

[c] Means in each horizontal row having no letters in common are significantly different; $P < 0.05$, Duncan's new multiple-range test.

[d] Means were not analyzed since only one or two moths flew to within 30 cm or closer to the "pheromone" source at the lower dosages.

TABLE 5. RESPONSES OF MALE SPRUCE BUDWORM TO THREE DOSAGES OF Δ11-14:Ald[a] AND THREE DOSAGES OF Δ11-14:Ald + 5% 14:Ald AFTER FLIGHT INITIATION TO 3 μg Δ11-14:Ald PLUS INDICATED DOSAGES

	Dosage Δ11-14:Ald on the upwind source (US)					
	10 ng	30 ng	100 ng	10 ng + 5% 14:Ald	30 ng + 5% 14:Ald	100 ng + 5% 14:Ald
Mean % moths activated[b]	100 (−)	100 (−)	96.0 (8.94)	92.0 (10.95)	100 (−)	100 (−)
Mean % moths initiating flight	100 (−)	100 (−)	92.0 (10.95)	84.0 (26.08)	96.0 (8.94)	96.0 (8.94)
Mean % moths flying upwind	56.0 (29.66)	52.0 (17.89)	52.0 (30.33)	44.0 (8.94)	64.0 (26.08)	68.0 (30.33)
Mean % moths flying to within 30 cm of US[c]	8.0c (10.95)	20.0abc (14.14)	44.0ab (21.91)	20.0bc (20.00)	32.0abc (10.95)	52.0a (36.33)
Mean % moths contacting US	4.0b (8.94)	4.0b (8.94)	44.0a (21.91)	4.0b (8.94)	28.0a (10.95)	40.0a (37.42)
Flight duration from 140 cm to 30 cm, downwind of US (sec)[d]	26.6 (26.30)	49.9 (26.89)	19.0 (13.44)	25.5 (22.45)	16.5 (8.86)	15.7 (6.60)
Flight duration from 140 cm downwind, to the US (sec)[d]	52.7 (−)	48.9 (−)	29.3 (18.98)	31.8 (−)	26.8 (11.02)	30.8 (22.31)
Mean closest approach to US (cm)	49.0a (31.98)	43.1ab (18.70)	6.7b (9.31)	43.1ab (25.69)	27.0ab (19.58)	19.2ab (24.00)

[a] All details as in Table 4.
[b] See Table 4, footnote *b*.
[c] See Table 4, footnote *c*.
[d] See Table 4, footnote *d*.

TABLE 6. RESPONSES OF MALE SPRUCE BUDWORM TO (1) Δ11-14:Ald (2) DIOLEFIN ANALOG, AND (3) 1:1 MIXTURE OF BOTH[a]

	(1) 10 ngΔ11-14:Ald	(2) 10 ng DO	(3) (1) + (2)
Mean % moths activated[b]	84.0a (16.80)	8.0b (11.00)	92.0a (17.80)
Mean % moths initiating flight	92.0a (17.80)	36.0b (7.20)	92.0a (11.00)
Mean % moths flying upwind	48.0a (11.00)	4.0b (9.00)	48.0a (11.00)
Mean distance flown per moth flying upwind (cm)[c]	150.8 (69.90)	185 (−)	150.8 (51.16)

[a] Δ11-14:Ald = 95:5 *E*/*Z*11-14:Ald; DO = diolefin analog. $N = 25$ (five groups of five); values are means (± SD)
calculated from means of each group of five moths tested.
[b] Means in each horizontal row having no letters in common are significantly different; $P < 0.05$, Duncan's new multiple-range test.
[c] Means were not analyzed since only one moth flew up-tunnel (center column) when diolefin alone was present.

placed in a circle around traps baited with Δ11-14:Ald's, trap capture was reduced to a level not significantly different ($P > 0.05$) from traps surrounded by equally spaced PVCs containing 0.03% Δ11-14:Ald's.

From this we concluded that the diolefin had biological activity, but the trap capture data were ambiguous. We then tested the diolefin in our wind tunnel. Male spruce budworm responses to (1) Δ11-14:Ald's, (2) diolefin, and (3) 1 + 2 were examined. In measures of activation, flight initiation, flight distance, and percentage source contact, males responded similarly (Table 6; $P > 0.05$) to Δ11-14:Ald's alone and when in admixture (1:1) with the diolefin, but by itself the diolefin did not elicit upwind flight in males. [Although one male reached the upwind portion of the tunnel (Table 6), its flight path appeared random and was rarely in or near the analog plume.) In another study, male responses to the diolefin alone were not distinguishable ($P > 0.05$) from responses to a blank (control) source (Kuenen and Silk, unpublished data). Thus the diolefin did not enhance or diminish the responsiveness of males flying upwind toward a "pheromone" source.

However, we reasoned that trap capture in diolefin-baited traps (mid-season study) may have occurred after males responded to female-emitted pheromone, while no pheromone sources (virgin females) were present in our late season study where diolefin did not elicit male activity (trap capture). As a first test of this hypothesis, we allowed individual males to initiate flight to two chemical sources (see Figure 1). The upwind source contained diolefin alone or was blank, while the downwind (15 cm) source contained Δ11-14:Ald's. After males had flown to within 140 cm of the upwind source, the downwind source was quickly pulled upward by its supporting thread. Males' progress was then recorded. Most notably, males continued their normal upwind ap-

proach to the diolefin source [upwind source; mean distance flown 163.3 cm ± 45.73 (SD); $N = 24/50$ tested], while, as expected, they soon initiated cross-wind casting (Kennedy and Marsh, 1974; Marsh et al., 1978) when the upwind source was blank [mean distance flown 119.0 cm ± 29.75 (SD); $P < 0.05$; t test; $N = 30/50$ tested]. The males' flights to the diolefin source were not visually distinguishable from their flight responses to a Δ11–14 : Ald source, and 16 of the 24 moths flying upwind continued their upwind flight to reach and land on the diolefin source.

CONCLUSIONS

Progress in understanding the sex pheromone chemistry of the spruce budworm in relation to male behavior has been made in recent years. However, the sex pheromone blend of the budworm is not completely defined. Very low emission rates of additional pheromone components have made it difficult to identify these components. Identification of pheromone gland fatty acids, and particularly their temporal variation in relation to pheromone production, may become a very useful technique in identifying these additional components.

The data we have presented on male flight thresholds is not definitive at this point, but represents an approach to the study of the spruce budworm communication system. Our analyses of male responses to a pheromone analog are also preliminary; however, the demonstration of normal male upwind flight in response to the diolefin analog is, to the best of our knowledge, the first report of the observation of this behavioral response to a pheromone analog.

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PHEROMONES OF TWO ARCTIID MOTHS (*Creatonotos transiens* AND *C. gangis*): Chiral Components from Both Sexes and Achiral Female Components

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Abstract—The two major components of the female sex pheromones of two *Creatonotos* species have been identified as an achiral C_{21} triene, (*Z,Z,Z*)-3,6,9-heneicosatriene, and a chiral epoxide, (*Z,Z*)-2(2,5-octadienyl)-3-undecyloxirane. The ratios of these components in the two species fall into nonoverlapping ranges. Two additional achiral minor components, (*Z,Z*)-6,9-heneicosadiene and (*Z,Z,Z*)-3,6,9-tricosatriene, were also identified in the female sex gland extracts. The male pheromone of both species consists of hydroxydanaidal, a chiral dihydropyrrolizine derived from pyrrolizidine alkaloids in the larval diet. *Creatonotos transiens* was found to convert dietary heliotrine into (*R*)-(−)-hydroxydanaidal, with inversion at the single asymmetric carbon atom. The possible biological and biosynthetic significance of the chiral pheromone components are discussed, and they are compared with known examples of chiral lepidopteran pheromones.

Key Words—Lepidoptera, pheromone, chirality, epoxide, hydroxydanaidal, biosynthesis, polyene, alkaloid, *Creatonotos transiens*, *Creatonotos gangis*, *Arctiidae*.

INTRODUCTION

It is now generally recognized that the sex pheromones of most lepidopteran insects consist of complex mixtures of chemical components (Jacobsen et al., 1970; Roelofs and Cardé, 1974; Tumlinson et al., 1982; Schneider, 1984a,b; Boppré, 1984). Long-range attraction of male moths is usually elicited by a

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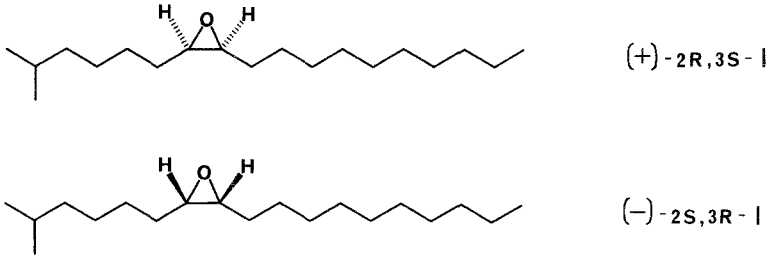
blend of chemicals released by the calling female. The responding male often deploys another set of compounds in short-range interactions with the female; these "aphrodisiac" pheromones increase the likelihood of successful courtship. Female lepidopteran sex pheromones usually have simple molecular structures; they are often unsaturated straight-chain alcohols, esters, or aldehydes consisting of 10–18 carbon atoms (Baker and Evans, 1979; Baker and Bradshaw, 1981, 1983; Baker and Herbert, 1984). Contrary to earlier belief, the sex attractants produced by a particular species are not necessarily unique to that species; some components may be shared even by sympatric species with overlapping activity patterns. For the apparent purpose of species identification, hence reproductive isolation, pheromone components often occur in characteristic ratios (e.g., Roelofs and Cardé, 1974). These components frequently have closely related structures that differ only in functionality. Alternately, pheromone components may differ only in their stereochemistry. This often involves a specific mixture of *E* and *Z* stereoisomers of unsaturated components (Klun et al., 1973; Roelofs et al., 1975; Roelofs, 1979).

Most lepidopteran pheromone components have achiral structures; accordingly, they cannot exist in optically active enantiomeric forms. While many examples of chiral coleopteran pheromones are known, only a small group of lepidopteran pheromones have chiral structures (Silverstein, 1979, 1982). Chirality, then, presents an alternative basis for variation of odor components, since enantiomer discrimination (enantioselectivity) in chemoreception is well known. With respect to olfactory discrimination of enantiomeric pheromones, the first single-cell electrophysiological recordings were performed by Kafka et al. (1973). An important example of chirality in species identification is provided by enantiomer ratios in aggregation pheromones of bark beetles (genus *Ips;* Silverstein, 1982). In the following sections we describe known examples of chiral lepidopteran sex pheromones and present our study of two Asian arctiid species in which both sexes produce chiral pheromone components.

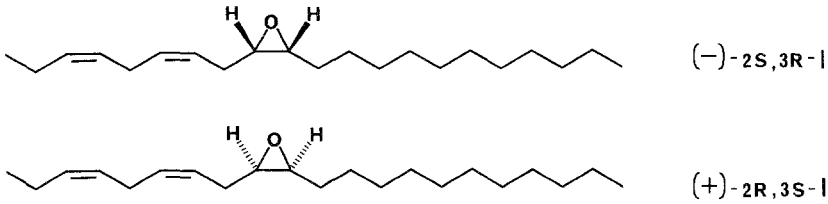
Chiral Female Pheromones in Lepidoptera. Disparlure (I) provides an interesting example of a chiral lepidopteran sex pheromone. This unsymmetrical epoxide was found to be the sex pheromone of the female gypsy moth, *Lymantria dispar* L. (Bierl et al., 1970, 1972; Beroza et al., 1971; cf. Jacobson et al., 1960, 1961). Subsequent to its isolation from the gypsy moth, disparlure was also identified as the sex attractant of the nun moth, *Lymantria monacha* L., which lives sympatrically with the gypsy moth in parts of Europe (Bierl et al., 1975). The suspicion that enantiomeric composition could be the key to species recognition led to the discovery of distinctly different enantiomer response patterns in the two species (Vité et al., 1976). More recently, single-cell and electroantennogram (EAG) recordings (Hansen, 1984; Schneider, 1984a) have suggested that the gypsy moth produces nearly enantiomerically pure (+)-2*R*,3*S*-I, whereas the nun moth pheromone contains only 10% of this (+) isomer, along with 90% of the (−)-2*S*,3*R* enantiomer. This is consistent



SCHEME 1.

with the surprising observation that both species are attracted to (+)-disparlure, whereas the gypsy moth alone, which has specialized receptor cells for both enantiomers, is inhibited by (−)-disparlure. The male nun moth has receptors for (+)-I but none for (−)-I (Scheme 1). Its attraction to the gypsy moth pheromone may be avoided in nature by the partial difference in the activity rhythms of the two species (Hansen, 1984; Schneider, 1984a). Since it is clear that insights of this sort require extremely good analytical techniques, it is unfortunate that no general methods exist for the determination of optical purity on the minute scale necessitated by most lepidopteran pheromone studies (Mori, 1984).

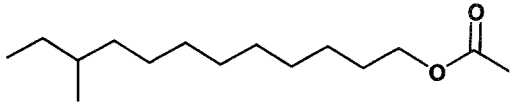
A second chiral epoxide, [2(2*Z*,5*Z*),3]-2(2,5-octadienyl)-3-undecyloxirane (II), is known as the major pheromone component of both the saltmarsh caterpillar moth, *Estigmene acrea* (Hill and Roelofs, 1981) and the fall webworm moth, *Hyphantria cunea* (Hill et al., 1982; Einhorn et al., 1982). Although the ranges of these two species overlap in North America, the female sex attractants used by both are blends of the same three compounds: epoxide II, (*Z*,*Z*)-9,12-octadecadienal and (*Z*,*Z*,*Z*)-9,12,15-octadecatrienal. Hill and Roelofs (1981) and Hill et al. (1982) used the EAG responses of males to the related epoxides, (+)- and (−)-disparlure, in order to probe receptor enantioselectivities in both species. In both cases, the EAG responses to (−)-disparlure exceeded those of the (+) enantiomer. By correlating the molecular structures of I and II, these investigators predicted that the active pheromones of both species would have the 2*S*,3*R* configuration. This prediction has been confirmed by EAG experiments with synthetic samples of 2*S*,3*R*-II and 2*R*,3*S*-II (Scheme 2) (Hill et al., 1982). The only assumption involved in the configurational as-



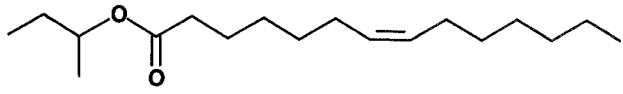
SCHEME 2.

signment is the enantioselectivity of the Sharpless epoxidation step used in synthesis of epoxide II (Mori and Ebata, 1981). In this case, enough of the natural pheromone was obtained from *Estigmene acrea* to produce a negative optical rotation, corresponding to that of synthetic 2*S*,3*R*-II. More recent work on female arctiids has uncovered this same 21-carbon epoxide in several species and a related 20-carbon epoxide occurs in one of these (*Antichloris viridis;* Meyer, 1984).

There are two known female lepidopteran pheromone components possessing a single asymmetric carbon atom. These are 10-methyldodecyl acetate (III), a minor component of the lesser tea tortrix moth, *Adoxophyes* sp. (Tamaki, 1979; Tamaki et al., 1979), and 2-butyl (*Z*)-7-tetradecenoate (IV), the major sex pheromone component of the western grapeleaf skeletonizer, *Harrisina brillians* (Myerson et al., 1982). In the case of III, neither EAG nor behavioral experiments indicated significant discrimination between synthetic enantiomers (Tamaki et al., 1983, 1984). The stereochemical compositions of natural III and IV remain to be determined (Scheme 3).



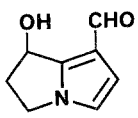
III



IV

SCHEME 3.

Chiral Male Pheromones in Lepidoptera. While female Lepidoptera produce chiefly fatty acid-derived pheromones (Roelofs and Bjostad, 1984), male moths and butterflies release scent chemicals having a greater variety of structures (Birch, 1974; Schneider, 1984a; Boppré, 1984). These male odor components are, in many cases, derived from natural products found in larval food sources or from compounds sequestered from plants by the adult. Perhaps the most widely distributed chiral male substance is hydroxydanaidal (V, Scheme 4), which can be derived from various pyrrolizidine alkaloids. Hydroxydanaidal has been isolated from the sex glands (abdominal hair pencils and alar wing patches) of many species of danaine butterflies (Edgar, 1975, 1982; Komae et al., 1982, 1983) and from coremata of arctiid moths belonging to the genus



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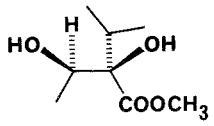

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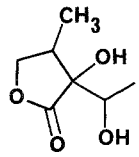


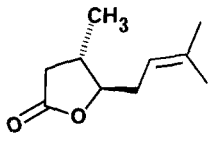
VII

SCHEME 4.

Utetheisa (Culvenor and Edgar, 1972; Connor et al., 1981). The discovery of hydroxydanaidal in the giant coremata of two other arctiids, *Creatonotos gangis* and *C. transiens* (Schneider et al., 1982) led to the first examination of optical activity in natural hydroxydanaidal. The results of this work have appeared in preliminary form (Bell et al., 1984) and are discussed later in this report. The conversion of pyrrolizidine alkaloids to dihydropyrrolizine scent components must be accompanied by removal of various esterifying acid moieties (e.g., viridifloric and trachelanthic acids). Esters derived from these acids occasionally appear as male sex organ constituents, for example methyl viridiflorate (VI) from *Euploea boisduvalii fraudulenta* (Edgar, 1982) and lactone (VII) from various ithomiine butterflies (Edgar et al., 1976) (Scheme 4).

Several chiral monoterpenes have been found as components of male pheromone mixtures and these have been summarized elsewhere (Birch, 1974; Boppré, 1984). Particularly noteworthy is eldanolide (VIII, Scheme 5), the wing gland pheromone of the male African sugar cane borer, *Eldana saccharina* (Wlk.) (Kunesch et al., 1981). This terpenoid lactone was proven to possess the 3*S*,4*R* configuration by comparison of its chiroptical properties with those of the synthetic enantiomers (Vigneron et al., 1982). Also of interest are the three chiral short-range sex attractants isolated from the hairpencils of the Oriental fruit moth, *Grapholitha molesta* (Baker et al., 1981). These were identified as (*R*)-(−)-mellein (IX), methyl jasmonate (X), and methyl 2-epijasmonate (XI) by comparison with authentic samples (Scheme 5).

We conclude this summary of chiral lepidopteran pheromones with a few unique male scent components. Particularly striking is the chiral saturated hydrocarbon, 13-methylheptacosane (XII, Scheme 6), a pheromone found on the wings of male sulfur butterflies, *Colias eurytheme* (Grula et al., 1980). The absolute configuration of this substance is apparently unknown and would be extremely difficult to determine because of the very small difference between the two enantiomers. Recently, hepialone (XIII), the major pheromonal component of the male ghost moth, *Hepialus californicus*, has been discovered as the long-range sex attractant toward the female (Kubo et al., 1985). The absolute configuration has been found to be *R*, as shown, by independent synthesis (Uchino et al., 1985). Finally, the main components of the pheromone blend



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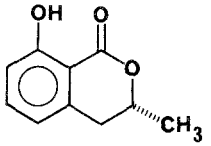

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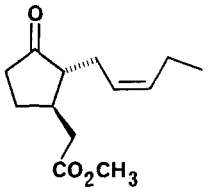
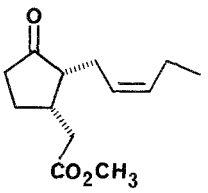
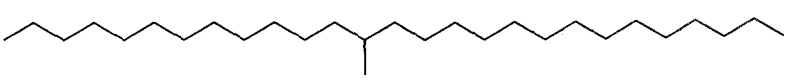


X

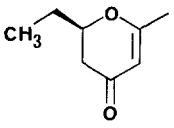


XI

SCHEME 5.



XII



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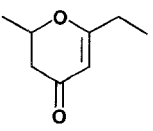

XIV

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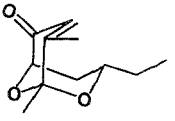


XVI

SCHEME 6.

of the male swift moth, *Hepialus hecta* L., have been identified very recently as dihydropyranone (XIV) and bicyclic ketals (XV and XVI) (Francke et al., 1985; Sinnwell et al., 1985). It is interesting to note that whereas in hepialone the chiral center bears the methyl substituent and the ethyl group is attached at the unsaturated carbon, the positions of these groups are exactly transposed in the substance produced by *Hepialus hecta* (XIV). The absolute configurations of XIV–XVI have not yet been reported.

METHODS AND MATERIALS

General. Creatonotos gangis L. and *C. transiens* (Walker) were captured mostly in North Sumatra and were reared in the laboratory (Seewiesen) as described by Wunderer et al. (1986). Details concerning the preparation of scent organs and electrophysiological assays are also given in this reference. Female scent glands (in CS_2) and male coremata (in CS_2 or ethyl acetate) were mailed in sealed ampoules to Cornell or Stony Brook for chemical analysis. Gas–liquid chromatography (GLC) of female extracts was conducted on a Varian 2100 instrument using FID detection and a Shimadzu C-R1A Chromatopac recorder. The following glass GLC columns and conditions were employed: Carbowax: 4-mm × 3.3-m column packed with 10% Carbowax 20 M on 60/80 Chromosorb W-AW-DMCS (column: 195°C; injector: 225°C; detector: 220°C). OV-1: 4-mm × 4.57-m column packed with 3% OV-1 on 60/80 Chromosorb W-AW-DMCS (column 250–280°C; injector: 250°C; detector: 290°C). XF-1150: 2-mm × 2.4-m column packed with 10% XF-1150 on 80/100 Chromosorb W-AW-DMCS.

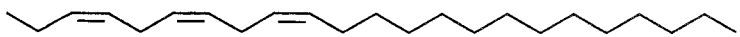
All solvents were reagent grade; however, anhydrous pyridine was also distilled from CaH_2. Alumina for column chromatography was Woelm neutral alumina of the indicated activity grade. Machery Nagel alumina and silica sheets were employed for analytical thin-layer chromatography (TLC), and R_f values on alumina were for 95:5 CH_2Cl_2–CH_3OH (v/v). TLC visualization was achieved by exposure to I_2 or ultraviolet light. Gas chromatographic–mass spectral (GC-MS) analyses were obtained by electron impact (70 eV) using a Finnigan 3300 GC-MS instrument coupled to a System Industries 150 data system. Proton magnetic resonance (NMR) spectra (300 MHz) were recorded on a Bruker WM-300 or Nicolet NT-300 spectrometer, whereas 90-MHz spectra were obtained using a Varian EM-390 spectrometer. NMR samples were dissolved in $CDCl_3$ and data are expressed in ppm (δ) downfield from tetramethylsilane, which was an internal standard. Infrared spectra were recorded on a Perkin-Elmer model 299B spectrophotometer and optical rotations were measured using a Perkin-Elmer model 141 polarimeter with a 1-dm thermostatted cell.

Analysis of Female Lure Gland Components. Female lure gland samples were further extracted with 0.1–0.2 ml of CS_2 before GLC analysis or pre-

parative fractionation, which was performed using a splitter (10% to detector and 90% to collector). Fractions were collected using 3-mm OD glass U-tubes cooled in Dry Ice. For electrophysiological assays, preparative fractions were dissolved in pentane, hexane, or CH_2Cl_2 and sent to Seewiesen in glass ampoules. NMR samples were prepared by washing collection tubes with several portions of $CDCl_3$, directly into NMR tubes. NMR (300 MHz) and mass spectral data for (Z,Z,Z)-3,6,9-heneicosatriene (XVII, Scheme 7) and (Z,Z)-2-(2,5-octadienyl)-3-undecyloxirane (II) were recorded as follows: XVII: NMR δ5.36 (m, 6H, olefinic), 2.81 (m, 4H, doubly allylic CH_2), 2.06 (m, 4H, allylic CH_2), 1.26 (m, 18H, remaining CH_2), 0.976 (t, 3H, J = 7.7 Hz, homoallylic CH_3), 0.880 (t, 3H, J = 5.9 Hz, terminal CH_3). MS *m/z* 290 (0.7), 234 (3.4), 108 (58.4), 79 (100%). II: NMR δ5.4 (bm, 4H, olefinic), 2.94 (m, 2H, epoxy CH), 2.81 (t, 2H, J = 6.6 Hz, doubly allylic CH_2), 2.4 (m, 1H, OCHC**H**CH=CH), 2.2 (m, 1H, OCHC**H**CH=CH), 2.08 (m, 2H, allylic CH_2), 1.26 (m, 20H, remaining CH_2), 0.976 (t, 3H, J = 7.4 Hz, homoallylic CH_3), 0.880 (*t*, 3H, J = 6.2 Hz, terminal CH_3). MS *m/z* 306 (0.2), 108 (52.1), 79 (100%).

Synthesis of (Z,Z,Z)-3,6,9-Tricosatriene (XVIII, Scheme 7). This synthesis was conducted in the manner described for preparation of triene XVII (Connor et al., 1980; Jain et al., 1983). To a stirred suspension of 0.19 g of $LiAlH_4$ in 10 ml of anhydrous ether was added a solution of 1.44 g of methyl linolenate (Sigma) in 5 ml of anhydrous ether. The resulting mixture was stirred under reflux for 15 min, then cooled to room temperature. Quenching with water and aq. NaOH, followed by filtration and evaporation gave 1.20 g (92%) of linolenol as a colorless, mobile oil, pure by TLC on silica gel. A solution of 1.09 g of linolenol and 0.88 g of *p*-toluenesulfonyl chloride in 15 ml of anyhdrous pyridine was held at 5–15°C for 24 hr, then poured into 90 ml of cold water. The resulting mixture was extracted with ether, and the combined extracts were dried and evaporated. The residue was chromatographed on 100 g of Woelm silica (Act. III), eluting with 0–15% ether in hexane (v/v). Thus obtained was

XVII



XVIII

SCHEME 7.

1.22 g (71%) of linolenyl tosylate as a colorless oil which was homogeneous by TLC on silica.

A solution of *n*-pentyllithium was prepared by reacting 3.02 g of *n*-pentylbromide with 0.30 g of Li wire in 15 ml of anhydrous ether at −10° to 20°C. The resulting solution was filtered, and an aliquot was titrated with 2,5-dimethoxybenzylalcohol, giving a titer of 0.76 M. A suspension of 0.21 g of anhydrous CuI (Alfa, 99.99%) in 10 ml of anhydrous ether was stirred under N_2 at −24°C as 2.9 ml of this *n*-pentyllithium solution was added. The resulting grey solution was stirred for 15 min at −24°C; then a solution of 0.42 g of linolenyl tosylate in 3 ml of anhydrous ether was added dropwise. The dark reaction mixture was stirred at −24°C for 4 hr, then 10 ml of saturated aq. NH_4Cl was added dropwise, followed by 10 ml of ether. The organic phase was washed with saturated aq. NH_4Cl, saturated aq. NaCl, dried, and evaporated. The crude product was chromatographed on Machery Nagel silica gel 60 (70–270 mesh), eluting with hexane. Thus obtained was 0.278 g (87%) of triene (XVIII) as a colorless oil. NMR (300 MHz) δ5.36 (m, 6H, olefinic), 2.80 (t, 4H, J = 5.8 Hz, doubly allylic CH_2), 2.05 (m, 4H, allylic CH_2), 1.25 (m, 22H, remaining CH_2), 0.967 (t, 3H, J = 7.5 Hz, homoallylic CH_3), 0.870 (t, 3H, J = 6.7 Hz, terminal CH_3); IR (neat) 3010m, 2960m, 2920s, 2850s, 1650w, 1460mw, cm^{-1}; MS *m/z* 318.3286 (calc. for $C_{23}H_{42}$: 318.3286; M^+, 0.3), 262 (1.2), 108 (65.2), 79 (100%).

Although samples of XVIII were pure (TLC and GLC) after column chromatography, samples for biological testing were preparatively gas chromatographed. This was carried out on a $\frac{1}{4}$-in. OD × 6-ft glass column packed with 2.7% OV-17 on Gas Chrom Q-AW-DMCS, using a Varian 920 thermal conductivity gas chromatograph. Solutions of this and other alkenes in hexane were mailed in sealed ampoules to Seewiesen for behavioral and electrophysiological testing.

Analysis of Male Coremata (Creatonotos transiens). A sample consisting of 54 coremata was extracted with 4.5 ml of $CHCl_3$. Evaporation of the extract left 18.5 mg of a yellow glassy residue, which was chromatographed on 2 g of alumina (Act. III), eluting with 0–2% CH_3OH in CH_2Cl_2 (v/v). The following fractions were obtained: (a) R_f 0.86; 3.4 mg; colorless oil; NMR (300 MHz) 5.35 (m), 5.2 (m), 4.27 (dd, J = 4.4, 11.8 Hz), 4.16 (dd, J = 6, 11.8 Hz), 2.80 (m), 2.30 (t, J = 7.5 Hz), 2.01 (m), 1.60 (m), 1.29 (lg m), 1.24 (lg m), 0.97 (t, J = 7.5 Hz), 0.87 (bt); triglyceride mixture. (b) R_f 0.63; 0.3 mg; white solid; NMR (300 MHz) 5.34 (m), 3.51 (m), 2.26 (m), 2.1-0.9 (complex), 0.997 (s), 0.902 (d, J = 6.6 Hz), 0.854 (d, J = 6.6 Hz), 0.850 (d, J = 6.6 Hz), 0.666 (s); MS *m/z* 386 (M^+), 371, 368, 353, 275. (c) R_f 0.43–0.8, mainly 0.43; 2.0 mg; mixture. (d) R_f 0.43; 4.2 mg; hydroxydanaidal; for rotation, see Table 1, entry 3.

A second sample consisting of 81 coremata was extracted with 5 ml of ethyl acetate. Evaporation of the extract left 19.3 mg of residue, which was

TABLE 1. OPTICAL ROTATIONS OF HYDROXYDANAIDAL V: SEMISYNTHETIC AND NATURAL SAMPLES

Entry	Source	Specific rotation, $[\alpha]_D^{25}$ (ethanol)	
1	Synthesis from retronecine	−140°	(c = 0.74)
2	Synthesis from heliotridine	+137°	(c = 0.73)
3	Heliotrine-fed *C. transiens*	−122° ± 20°	(c = 0.21)
4	Heliotrine-fed *C. transiens*	−170° ± 30°	(c = 0.18)
5	Heliotrine-fed *C. gangis*	−320° ± 300°	(c = 0.005)[a]
6	Monocrotaline-fed *C. gangis*	−210° ± 70°	(c = 0.016)[a]

[a]These rotations are for crude extracts and are calculated according to hydroxydanaidal assays obtained by GLC on OV-17.

chromatographed on 4 g of alumina (Act. V), eluting with 0–1% CH_3OH in CH_2Cl_2 (v/v). The higher R_f components (13.0 mg) were rapidly eluted, affording 1.8 mg of hydroxydanaidal (for rotation, see Table 1, entry 4). The higher R_f fraction was rechromatographed on 3 g of alumina (Act. III), with 0–2% CH_3OH in CH_2Cl_2 (v/v) elution. Thus obtained were 12.1 mg of triglycerides, 0.4 mg of cholesterol, and 0.7 mg of mixed fractions.

Syntheses of (R)-(−)- and (S)-(+)-Hydroxydanaidal. The enantiomers of hydroxydanaidal were prepared from retronecine-*N*-oxide and heliotridine-*N*-oxide, as described by Culvenor et al. (1970). Activated MnO_2 was prepared by the method of Attenburrow et al. (1952).

A solution of 100 mg of retronecine (mp 117.5–118.5°C; acetone) in 2 ml of absolute ethanol containing 0.2 ml of 30% aq. H_2O_2 was stirred at ambient temperature for 1 day, then the reaction was quenched by addition of MnO_2. Filtration and evaporation left 113 mg of crystalline, white *N*-oxide, which was dissolved in 4 ml of CH_3OH and oxidized with 0.9 g of freshly prepared activated MnO_2. After the reaction mixture was stirred for 3 hr at ambient temperature, it was filtered through Celite. Evaporation left 119 mg of an amber residue, which was chromatographed on 6 g of MCB aluminum oxide 90 (70–230 mesh), eluting with 0–2% CH_3OH in CH_2Cl_2 (v/v). Thus obtained was 33.4 mg of (*R*)-(−)-hydroxydanaidal as a light yellow oil; NMR (90 MHz) 9.73 (s, 1H), 6.58 (m, 2H), 5.40 (m, 1H), 4.05 (m, 3H), 2.2–3.1 (m, 2H).

Treatment of 100 mg of heliotridine (mp 114.5–115.5°C; acetone) with hydrogen peroxide in ethanol (41 hr, room temperature), followed by activated MnO_2 in CH_3OH (2 hr, room temperature) and chromatography afforded 44.8 mg of (*S*)-(+)-hydroxydanaidal. The NMR spectrum was identical to that of (*R*)-(−)-hydroxydanaidal, described above.

Sublimation at 50–60°C (0.1 mm) furnished pure (*R*)-(−)- and (*S*)-(+)-hydroxydanaidal in 28% and 37% overall yields, respectively. The rotations of these samples are given in Table 1, entries 1 and 2.

RESULTS

Chemical Characterization of Female Lure Gland Components of Creatonotos transiens *and* C. gangis. The carbon disulfide extract of 15 *Creatonotos transiens* female lure glands was initially examined by gas–liquid chromatography (GLC) on a Carbowax column, revealing two major components (Figure 1). Following the results of electroantennogram (EAG) assays (see Wunderer et al., 1986), these two GLC peaks (at 5.1 and 14.1 min), as well as the minor peaks at intermediate retention time, were selected for chemical characterization. The more volatile major component, (5.1 min) was identified as (*Z*,*Z*,*Z*)-3,6,9-heneicosatriene (XVII) by comparison of its spectroscopic (MS and [^{1}H]NMR) and GLC properties with those of synthetic samples (Connor et al., 1980; Jain et al., 1983). The longer retention time major component was identified as the chiral epoxide, (*Z*,*Z*)-2(2,5-octadienyl)-3-undecyloxirane (II), by comparison of its [^{1}H]NMR and mass spectra with data reported for natural (Hill and Roelofs, 1981; Hill et al., 1982) and synthetic samples of this epoxide (Mori and Ebata, 1981). The GLC properties of this second female component were also consistent with those of epoxide samples produced by oxidation of triene XVII by means of *m*-chloroperoxybenzoic acid.

Corresponding GLC analysis of *Creatonotos gangis* lure gland extracts revealed that the same two major components are present as in the case of *C.*

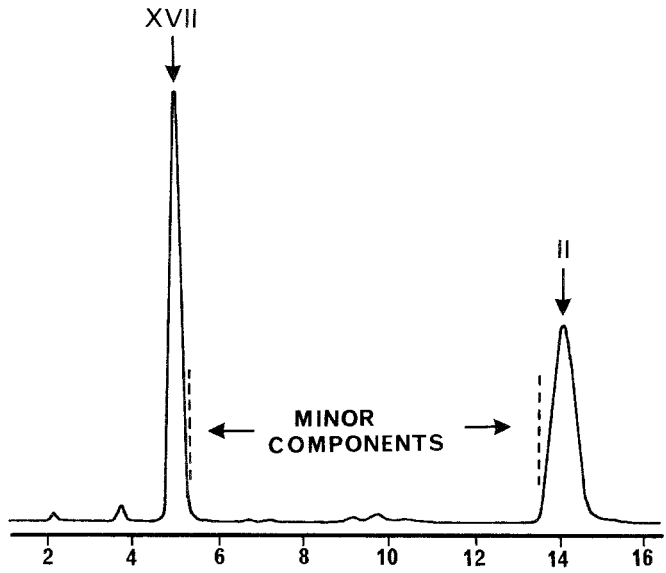


Fig. 1. Carbowax gas–liquid chromatogram of female *Creatonotos transiens* lure gland extract (retention times in min.).

transiens (see Wunderer et al., 1986). In *C. gangis* extracts, however, the ratio of epoxide II to triene XVII was found to be much larger. Thus, the ratio of II to XVII ranged from 0.9 to 2.3 for 5 *C. transiens* samples examined quantitatively, whereas this ratio ranged from 12 to 165 for 21 *C. gangis* extracts. The combined amount of volatile substances contained in each female gland was estimated to be on the order of 1–2 μg.

The minor component fraction (5.2–14 min) from the 60-gland *C. transiens* extract was further fractionated by GLC, using an OV-1 column (Figure 2). Of the four fractions produced, only the second eluted fraction, consisting of three components, showed significant EAG activity (Wunderer et al., 1986). Because this OV-1 fractionation was accompanied by substantial loss of overall activity, subsequent preparative chromatography was conducted using Carbowax.

Preparative GLC of the CS_2 extract of 82 *Creatonotos gangis* female lure glands afforded five fractions, as shown in Figure 3. The first and last fractions consisted of triene XVII and epoxide II, respectively. Of the three minor component fractions, the second (central) fraction, consisting of two components (9.5 and 10.7 min), was selected for examination. Of these two peaks, only the

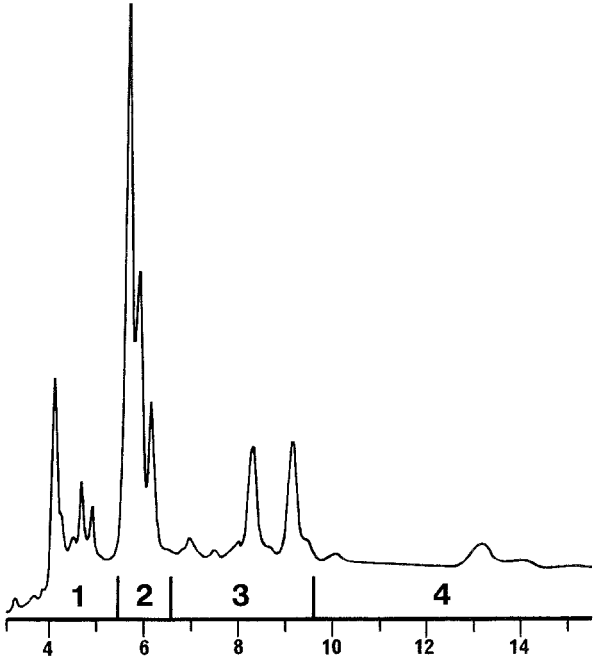


Fig. 2. OV-1 GLC fractionation of minor components obtained from Carbowax fractionation of *C. transiens* extract (retention times in min; cf. Figure 1).

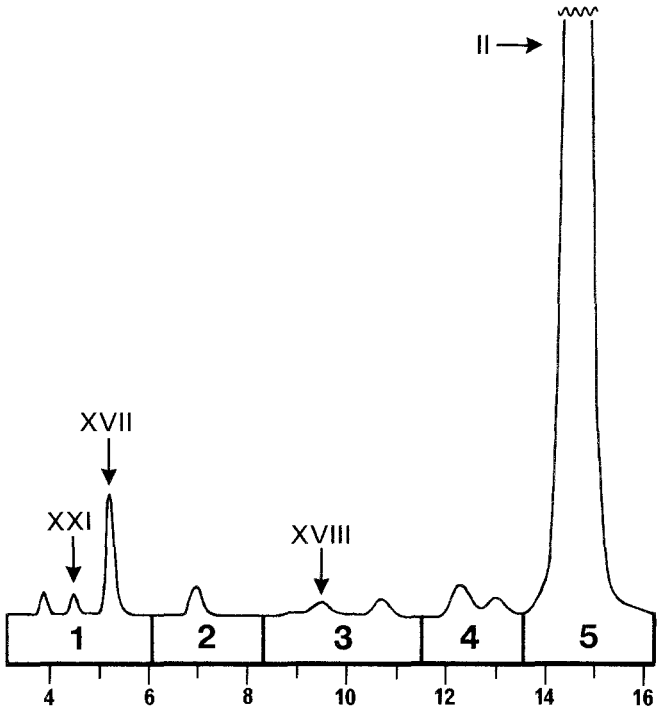


Fig. 3. Carbowax GLC fractionation of female *C. gangis* lure gland extract (retention time in min).

9.5-min peak corresponded to the active OV-1 fraction mentioned earlier (cf. Figure 2). The mass spectrum of this component (Figure 4) immediately suggested the structure (*Z,Z,Z*)-3,6,9-tricosatriene (XVIII), by comparison with the fragmentation pattern observed for triene XVII (Connor et al., 1980; Jain et al., 1983). The synthesis of triene XVIII (Figure 5) closely followed the method used to prepare XVII. Thus, XVIII was obtained in 57% overall yield from methyl linolenate, according to this three-step method. Synthetic XVIII was then shown to be identical with the 9.5-min minor component (cf. Figure 3) by GLC coinjection on Carbowax, OV-1 and XF-1150 columns and by mass spectroscopic comparison. For the relative EAG and behavioral efficacies of synthetic XVII and XVIII in *Creatonotos transiens* and *C. gangis*, see Wunderer et al. (1986).

Creatonotos female gland extracts were also examined for some of the female odor components found in other arctiid species, such as *Estigmene acrea* (Hill and Roelofs, 1981), *Hyphantria cunea* (Hill et al., 1982; Einhorn et al., 1982), and *Utetheisa ornatrix* (Connor et al., 1980; Jain et al., 1983). In particular, neither *Creatonotos* species appeared to produce (*Z,Z,Z*)-9,12,15-oc-

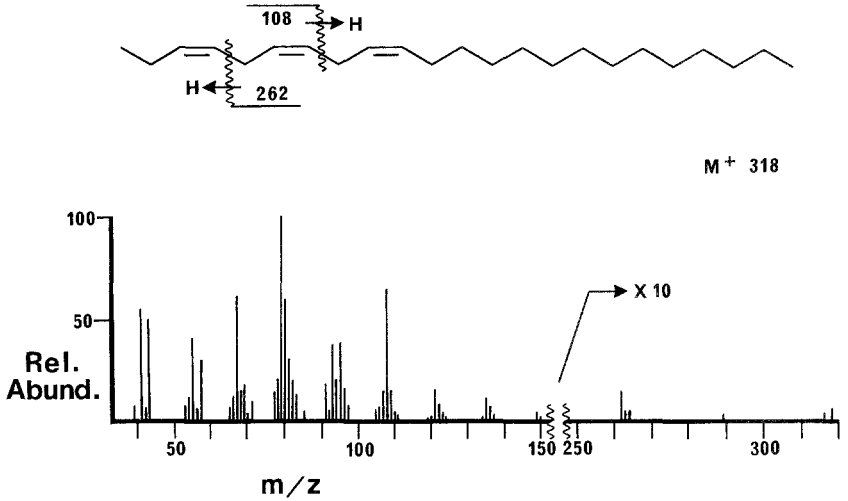
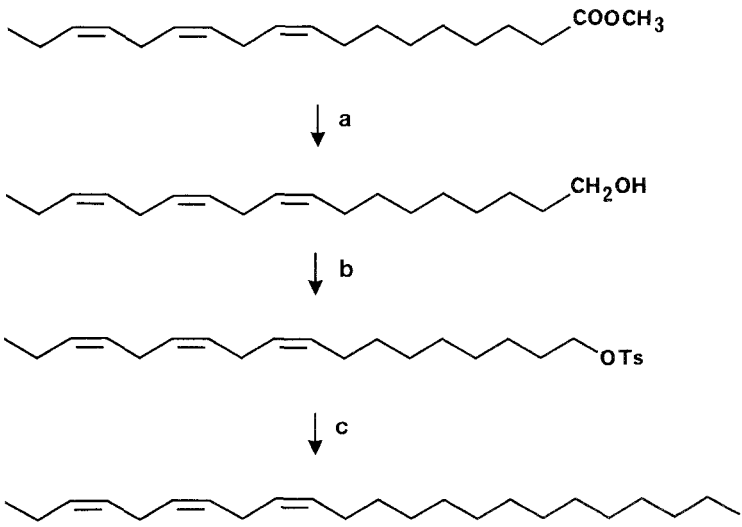


Fig. 4. Electron impact mass spectrum of 9.5-min minor component from *C. gangis* fractionation (cf. Figure 3).



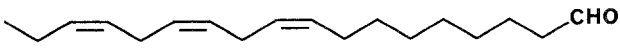
XVIII

Fig. 5. Synthesis of (*Z*, *Z*, *Z*)-3,6,9-tricosatriene (XVIII) from methyl linolenate. Reagents: (a) $LiAlH_4$/ether; (b) *p*-toluenesulfonyl chloride/pyridine; (c) $(n\text{-}C_5H_{11})_2CuLi$/ether.

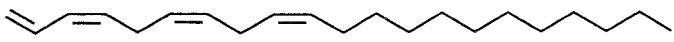
tadecatrienal (linolenal, XIX, Scheme 8), which is found in *E. acrea*, and *H. cunea* along with epoxide II. Also absent was the tetraene component of *U. ornatrix*, (*Z*,*Z*,*Z*)-1,3,6,9-heneicosatetraene (XX). All of the *Creatonotos* extracts examined, however, did show a small peak that corresponded to (*Z*,*Z*)-6,9-heneicosadiene (XXI) on Carbowax and XF-1150 GLC columns. This diene also accompanies triene XVII in *Utetheisa* (Jain et al., 1983) and has been examined for EAG activity in *Creatonotos* (Wunderer et al., 1986).

Chemical Characterization of Male Coremata Components of Creatonotos transiens *and* C. gangis. We have already reported that the development of the male scenting organs, the coremata, is controlled by access of the larvae to pyrrolizidine alkaloids, and that large coremata may contain as much as 400 μg per insect (Schneider et al., 1982). In the present study, the corematal contents were more thoroughly examined; a preliminary report of our work on the configuration of hydroxydanaidal has appeared (Bell et al., 1984). Whereas earlier studies utilized GLC and UV-monitored HPLC to examine corematal constituents, we have now employed column chromatography in order to avoid potential problems associated with GLC of materials having low volatility or thermal stability.

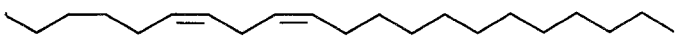
Chromatographic studies were conducted on the ethyl acetate or carbon disulfide extracts of *Creatonotos transiens* coremata, excised from individuals



XIX

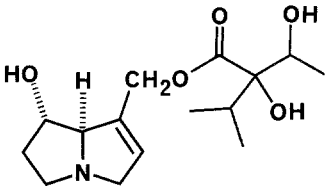


XX

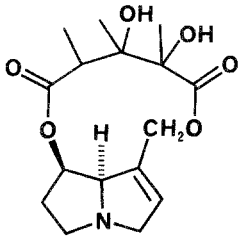


XXI

SCHEME 8.



XXII



XXIII

SCHEME 9.

which had been raised on diets containing natural heliotrine (XXII, Scheme 9) as the exclusive pyrrolizidine alkaloid. Examination of these extracts by thin-layer chromatography (TLC) showed that they consisted of three principal components: R_f 0.86 (weakly UV active), 0.63 (UV inactive), and 0.43 (UV active). Chromatography of the extracts on deactivated neutral alumina led to samples of each component that were examined by 300 MHz [^{1}H]NMR and mass spectrometry. The [^{1}H]NMR spectrum of the high R_f component (0.86) was consistent with a mixture of fatty ester triglycerides having an average of three alkene groups per glycerol unit. The next eluted component (UV inactive) was shown to be cholesterol by comparison of its mass spectrum with known fragmentations of steroids (Zaretskii, 1976) and by TLC comparison with authentic samples. The third component (UV active) proved to be (*R*)-(−)-hydroxydanaidal (V) by comparison of its physical and spectroscopic properties with material synthesized as described below. According to chromatographic recoveries, the crude extracts consisted of 18–63% glycerides, 2–8% cholesterol, and 9–23% hydroxydanaidal by weight, despite significant irreversible retention of some components: The [^{1}H]NMR spectra of crude extracts indicated only the presence of three components; UV spectra, as well as optical rotations appeared to reflect only the presence of known quantities of hydroxydanaidal (V).

Pure samples of (*R*)-(−)- and (*S*)-(+)-hydroxydanaidal (V) were required for comparison with corematal material and for electrophysiological experiments. These enantiomers were synthesized from natural retronecine and heliotridine, as shown in Figure 6. Thus, (*R*)-(−)-V was obtained from retronecine in 28% yield and (*R*)-(+)-V was obtained from heliotridine in 37%, after purification by chromatography and sublimation in both cases. The optical rotations of these semisynthetic hydroxydanaidal samples are given in Table 1, along with the data for pure and crude samples of V extracted from *Creatonotos* coremata. The purified hydroxydanaidal obtained from heliotrine-fed *C. transiens* had negative rotations (entries 3 and 4). This reflects an overall *inversion*

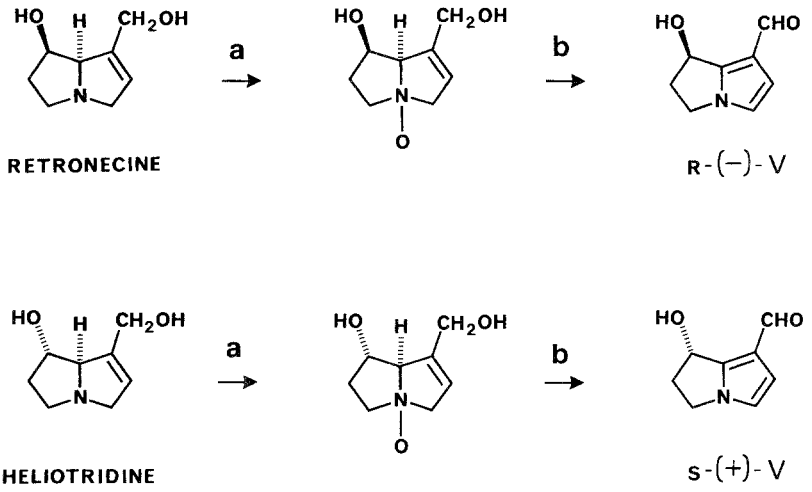


Fig. 6. Syntheses of (*R*)-(−)-hydroxydanaidal from retronecine and (*S*)-(+)-hydroxydanaidal from heliotridine. Reagents: (a) H_2O_2/ethanol; (b) MnO_2/methanol.

of configuration at the remaining asymmetric center during biosynthesis from heliotridine-derived XXII. A small number of coremata were available from groups of *C. gangis* that had been fed either heliotrine (XXII) or monocrotaline (XXIII) as the exclusive alkaloid source. In both cases, the crude extracts had weak, negative rotations (entries 5 and 6), which correlated roughly with the quantities of V they contained.

DISCUSSION

Behavioral studies of courtship in *Creatonotos* have been limited mainly to greenhouse work with *C. transiens* (Wunderer et al., 1986). These studies have shown that a dual mating and luring system is employed. Males initiate the courtship assembly by forming groups and expanding their large scenting organs, the coremata. Attracted females mate with calling males, or may at a later phase attract males by emitting their own pheromone bouquet. Trapping experiments have shown that the scent gland constituents, C_{21} triene (XVII), C_{21} epoxide (II), and C_{23} triene (XVIII), are indeed emitted by calling females. The remarkable complexity of this courtship would permit a variety of possible mechanisms for reproductive isolation between these sympatric species. In addition to potential temporal and visual factors, there are a number of possible chemical differences that could be used for species identification. Both the male and major female pheromone components are chiral, permitting differences in enantiomeric composition. Furthermore, the female pheromones of both species contain the same four components, and their ratios may be specific.

In this study we have identified four constituents of the female pheromones of *Creatonotos gangis* and *C. transiens*: C_{21} diene XXI, C_{21} triene XVII, C_{23} triene XVIII, and the chiral C_{21} epoxide II. A number of other arctiid moths are known to produce related female pheromones, as indicated in Table 2. The C_{21} polyenes are known from *Utetheisa ornatrix* and *Arctia villica*, whereas *Estigmene acrea* and *Hyphantria cunea* produce the C_{21} epoxide II as one of the female components. *Creatonotos* is unusual in that it produces both the triene XVII and the epoxide II, which may be biosynthesized by direct epoxidation of XVII (Roelofs and Bjostad, 1984). Another novel feature is the minor 23-carbon component, triene XVIII, which had not been reported previously as a lepidopteran pheromone constituent. The ratios of these three components, II, XVII, and XVIII, may carry species identifying information. Indeed, the ratio of II to XVII was consistently higher in *Creatonotos gangis* than in *C. transiens*. Electrophysiological studies indicate that the male antenna bears specialized receptor cells for II and XVII, lending a capability for recognition of this difference (Wunderer et al., 1986). Interestingly, the C_{23} triene (XVIII) has so far given EAG responses only in male antennae of *Creatonotos gangis* and not in *C. transiens*. A complete interpretation of these observations must await further investigation of: (a) behavioral effects of pheromone ratios: and (b) additional female gland constituents (see Wunderer et al., 1986). Some of the additional components are currently under identification (W. Francke, unpublished results).

The absolute configuration of C_{21} epoxide II has yet to be determined, because insufficient quantities were available for chiroptical experiments. Although a synthesis of optically active II has been reported (Mori and Ebata, 1981), an independent method is under investigation in order to provide material of extremely high optical purity (Bell and Ciaccio, unpublished results). That the chirality of II may play a role in *Creatonotos* courtship is suggested by the recent observation of enantiomer discrimination of disparlure in EAG experiments with antennae of both species (K. Hansen, personal communication). It will be particularly interesting to determine whether the enantiomer selectivities for disparlure (I) correspond to those for C_{21} epoxide II, according to the prediction of Hill and Roelofs (1981). These investigators made a straightforward correlation between the shorter epoxide substituents and the longer straight chains in the two series, predicting that (−)-(2*S*, 3*R*)-I would correspond to (+)-(2*S*, 3*R*)-II. This assumption is not necessarily valid, since the interaction of the two shorter chains with the receptor might be very different. In particular, the conformational properties of the 7-carbon, branched chain of displarlure (I) would not necessarily resemble those of the 8-carbon homoconjugated diene chain of II.

Our chromatographic examination of the extracts of male *Creatonotos transiens* coremata revealed three major constituents: lipids, (*R*)-(−)-hydroxydanaidal (V) and cholesterol. Of these, the only volatile, EAG-active compo-

TABLE 2. OCCURRENCE OF ALKENE AND EPOXIDE FEMALE PHEROMONE COMPONENTS IN ARCTIID MOTHS

Species	C_{21}				C_{23}	Reference
	Diene XXI	Triene XVII	Tetraene XX	Epoxide II	triene XVIII	
Utetheisa ornatrix	+	+	+			Connor et al., 1980; Jain et al., 1983
Arctia villica L.		+	+			Einhorn et al., 1984
Estigmene acrea				+		Hill and Roelofs, 1981
Hyphantria cunea				+		Hill et al., 1982; Einhorn et al., 1982
Creatonotos transiens	(+)	+		+	+	This work
Creatonotos gangis	(+)	+		+	+	This work

nent is hydroxydaniadal. The function of the remaining materials is unknown, although it is possible that these waxy, lipophilic substances may serve as fixatives, facilitating slow and continuous release of the male "perfume." The presence of a second volatile pheromone component was suggested by electrophysiological experiments using some coremata extracts (K. Hansen, personal communication). Chromatographic fractionation always resulted in hydroxydanaidal as the only active component. A second active component, if present, would have to be unstable to GLC and mild column chromatographic conditions employed.

We have shown that *Creatonotos transiens* males convert dietary heliotrine (XXII) to (*R*)-(−)-hydroxydanaidal (V) (cf. Table 1). It is clear from the magnitudes of the observed rotations that the hydroxydanaidal produced by *C. transiens* is at least 85% pure. A more sensitive experimental method would be required to more accurately determine the optical purity. The weak rotations of crude *C. gangis* extracts imply that this species may also produce (*R*)-(−)-V from *both* heliotrine (XXII) and monocrotaline (XXIII), requiring stereochemical inversion in one case and retention of configuration in the other (cf. below). It must be emphasized, however, that these data were obtained using crude samples that may contain optically active components other than V and that no firm conclusion may be drawn regarding the enantiometric composition of hydroxydaniadal produced by *C. gangis*.

Our initial studies of *Creatonotos* coremata (Schneider et al., 1982) revealed the chiral male pheromone, hydroxdanaidal, but did not address the optical activity of this substance. A number of possible scenarios were considered at this point. If, for example, the pheromone were to consist of a single enantiomer, then potential dietary precursors might be limited to the corresponding pyrrolizidine alkaloid series. Hence, (*R*)-(−)-hydroxydanaidal might be produced only from pyrrolizidine alkaloids derived from retronecine, e.g., monocrotaline (XXIII), having the same configuration at the residual asymmetric center (C-7). Alternately, if the insect were able to convert both retronecine- and heliotridine-derived alkaloids into the male sex pheromone, then a wider variety of plant substances could be utilized. In contrast to the African monarch butterfly, *Danaus chrysippus*, in which a pyrrolizidine precursor to the dihydropyrrolizine pheromone, daniadone, is known (Schneider, et. al., 1975), the larval food plants for *Creatonotos transiens* and *G. gangis* have not been discovered.

When *Creatonotos transiens* larvae were raised on a diet consisting of heliotridine (XXII) as the exclusive pyrrolizidine alkaloid, the hydroxydanaidal (V) isolated from the adult coremata had the (*R*)-(−) configuration. Contrary to the straightforward assumption that the stereochemistry at C-7 would be retained, biosynthesis of V led to inversion of configuration. Thus a dietary alkaloid having the "incorrect" configuration (heliotridine series) is converted to

(*R*)-(−)-V, having the retronecine stereochemistry. It would be interesting to determine whether pyrrolizidine alkaloids from both series are processed indiscriminately to V, or whether a separate set of enzymes can utilize retronecine-derived precursors without performing chemical transformations at C-7.

Another biosynthetic point of interest is the mechanism for conversion of heliotrine (XXII) into (*R*)-(−)-hydroxydanaidal (V). Two reasonable mechanisms that are consistent with the observed inversion of configuration are outlined in Figure 7. In pathway "a" C-7 is oxidized to the carbonyl oxidation level at some stage in the biosynthesis. Stereoselective reduction would then introduce the correct configuration at C-7. According to pathway "b," the leaving group ability of the C-7 hydroxyl is enhanced by protonation or conversion to a suitable derivative (X). Attack at C-7 by some oxygen nucleophile would then afford the *R* configuration by Walden inversion. Mechanism "a" requires loss of the hydrogen atom at C-7, whereas in "b" this hydrogen is retained. Isotopic labeling experiments designed to distinguish these possibilities are currently in progress (J. Meinwald et al., unpublished results).

The behavioral significance of enantioselective male pheromone production in *Creatonotos transiens*, the possibly also in *C. gangis*, has yet to be determined. The antennae of both sexes respond equally well to both the (*R*)-(−) and (*S*)-(+) enantiomers of V in EAG experiments (Wunderer et al., 1986). Interestingly, a receptor cell specialized for (*R*)-(−)-V has been discovered on male antennae, whereas a separate receptor responds to (*S*)-(+)-V and weakly even to the female pheromone components (Wunderer et al., 1986). These results indicate that at least the males may be able to detect differences in optical activity. If the male pheromone enantiomeric compositions differ between *Creatonotos* species, then this could perform the function of species identification during the formation of courtship assemblies. An alternative, or supple-

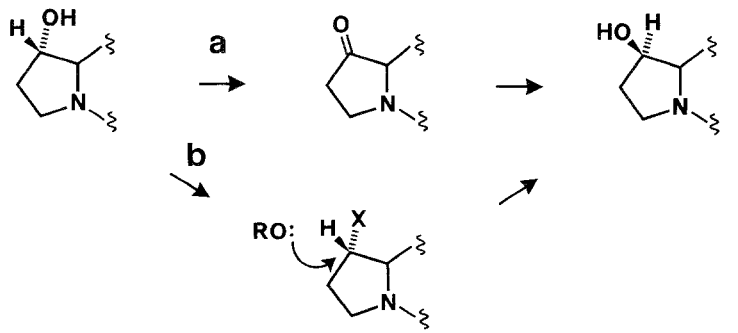


Fig. 7. Possible mechanisms for inversion of configuration at C-7 in the biosynthesis of hydroxydanaidal from heliotrine.

mental, species-specific signal might then be provided by the chiral or achiral components of the female pheromone.

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METABOLIC TRANSFORMATION OF TRITIUM-LABELED PHEROMONE BY TISSUES OF *Heliothis virescens* MOTHS

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Abstract—Unsaturated aliphatic pheromones of *H. virescens* were prepared at high specific activity (^{3}H, 58 Ci/mmol) and were employed to study tissue specificity of acetate esterase, alcohol oxidase, and aldehyde dehydrogenase in male and female *Heliothis virescens*. Thus, [9,10-$^3H_2$]Z9-14:Ac was synthesized by partial tritiation of the corresponding alkyne and was converted to the labeled Z9-14:OH and Z9-14:Al for metabolic studies. Soluble and membrane-associated enzyme activities were determined by radio-TLC assays. Esterase activity is highest in legs of both sexes, but also occurs in antennal and glandular tissues. Oxidase activity requires O_2 and is highest in female pheromone gland tissues, but it is also high in the male hairpencils. Aldehyde dehydrogenase activity was uniformly high in all tissues, but highest in antennal tissues of both males and females.

Key Words—Pheromone metabolism, tritium-labeling, acetate esterase, alcohol oxidase, aldehyde dehydrogenase, *Heliothis virescens*, Lepidoptera, Noctuidae, sensory biochemistry.

INTRODUCTION

Pheromone biosynthesis, pheromone perception, and pheromone catabolism are closely linked in terms of mate-finding behaviors of moths (review: Mayer and Mankin, 1985). In this paper, we will present experimental details on the pheromone-metabolizing enzymes of *Heliothis virescens*, using high-specific-activity, tritium-labeled Z9-14:Al (and its corresponding alcohol and acetate). First,

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however, we will survey pheromone biochemistry concisely (general review: Blomquist and Dillwith, 1983; catabolism: Ferkovich, 1981; biosynthesis: Roelofs and Bjostad, 1984). Additional recent results will be found in this symposium edition on pheromone biosynthesis (Bjostad and Roelofs, 1986; Teal and Tumlinson, 1986; Morse and Meighen, 1986) and pheromone catabolism (Lonergan, 1986; Vogt and Riddiford, 1986; Prestwich et al. 1986).

Catabolism of pheromones has been examined in only seven insects: *Bombyx mori*, *Lymantria dispar*, *Trichoplusia ni*, *Antheraea polyphemus*, *Choristoneura fumiferana*, *Heliothis virescens*, and *Reticulitermes flavipes*. The silkworm moth, *Bombyx mori*, produces bombykol (*E*10, Z12–16:OH) which is converted to "ester" and "acid" metabolites by whole male antennae exposed to labeled bombykol (Kasang, 1974). The [12,13-^{3}H]-labeled bombykol (30 Ci/mmol) and its tetrahydroisomer were prepared by partial tritiation of the 12 triple bond. At exposures of 10^{10}–10^{11} molecules/antenna, the half-life for "uptake" was 1.6 min for male and 1.0 min for female antennae. Much slower uptake was found in legs, heads, and wing scales which lack the pore tubules. Conversion from the alcohol to the corresponding labeled bombic acid and an unidentified ester, isolated by preparative TLC, showed a 4-min half-life. Chemical identifications were not performed, and no attempts to manipulate or purify the binding or catabolic proteins were reported.

The Seeweisen group also did preliminary work on disparlure biosynthesis (Kasang et al., 1974a) from the (*Z*)-[7,8-^{3}H]-2-methyl-7-octadecene (Sheads and Beroza, 1973) in female gypsy moth pupae and on racemic disparlure uptake and turnover (Kasang et al., 1974b) by male (and female) *L. dispar* antennae. In the former, aqueous ethanol suspensions of the labeled alkene were injected into female pupae two days preemergence and 34% of [^{3}H]disparlure (TLC) was recovered from female glands postemergence. There was no direct evidence of pheromone gland involvement in this conversion, however, and in view of the diel periodicity, life cycle and wild vs. lab variation of disparlure release and production (Charlton and Cardé, 1983; Richerson and Cameron, 1974), this work needs to be repeated on pheromone gland homogenates and with topical application of precursors to the gland surface. Such techniques have been used sucessfully to investigate the biosynthesis of Z11–14:Ac (Bjostad and Roelofs, 1981; Bjostad et al., 1981) in the red-banded leafroller moth and bombykol biosynthesis in silkworm moths (Yamaoka et al., 1984).

The disparlure metabolic studies are also very sketchy. At 5×10^{11} molecules/antenna of racemic [7,8-$^3H_2$]disparlure (40 Ci/mmol), about 2.5 min was required for disappearance of half the substrate and appearance of two uncharacterized polar metabolites (50%) as well as >30% of non-TLC-mobile material. Claims for specificity vs. nonspecificity of uptake and turnover are based on small differences superimposed on an exponential decay curve and are not convincing. Also, it is difficult to evaluate these data since both the attractive

(+) and inhibitory (−) enantiomers were present. It is essential to distinguish binding and catabolism of the two enantiomeric forms, since the proteins involved reside in seperate sensory cells (Hansen, 1984).

The in vivo and in vitro metabolism of *Z*7–12:Ac by *Trichoplusia ni* has been reported by Ferkovich, Mayer, and their coworkers in Gainesville (Ferkovich, 1981; Ferkovich et al., 1973; 1982a,b; Mayer et al., 1976). The acetate is converted to the corresponding alcohol by both specific and general esterases present in legs, wing scales, and antennae. The two primary roles of these esterases are to prevent sensory adaptation and to clear the body surface. The pheromone-specific catabolic proteins appear to show lower specificity for exact pheromone structures than do the receptors (measured behaviorally or by EAG or single cell), although they do show reduced susceptibility to general esterase inhibitors. This hydrolysis was demonstrated using (1) intact antennae and legs dipped in to a 10^{-3} M emulsion of pheromone in buffer; (2) in vitro homogenates of antennae, legs, cuticle, wings, and fat body; and (3) soluble proteins from the sensillum liquor of sonicated antennae. The homogenates were assayed with [^{3}H-acetate]*Z*7–12:Ac (200–800 mCi/mmol) at 10^{-6}–10^{-5} M, which is a much higher concentration (>1000×) and a much lower specific activity (300×) than for metabolic studies of bombykol, disparlure, and *E*6,*Z*11–16:Ac (see below). Nonetheless, conversion rates in the order of 6 pmol/μg protein/min in antennae were observed. Pheromone esterase activities decrease in the order of antennae > legs > wings in homogenates and wings > legs > antennae in eluates, consistent with both the postulated sensory deactivation and surface-cleaning functions.

The most thorough biochemical analysis of a pheromone binding and inactivation system is reported for *Antheraea polyphemus* (Vogt, 1984; Vogt and Riddiford, 1980, 1981, 1985; Vogt et al., 1985; Prestwich et al., 1984, 1986). Using [11, 12-$^3H_2$]*E*6,*Z*11–16:Ac (40 or 58 Ci/mmol), three electrophoretically distinct proteins were found which interact with pheromone: a nonenzymatic sensillar pheromone-binding protein (mol wt 15,000), a pheromone-hydrolyzing sensillar esterase (mol wt 55,000), and a second esterase in cuticular tissues (e.g., legs and wing scales) of males and females (Vogt and Riddiford, 1986). Oddly, an electrophoretically identical "binding protein" (mol wt 15,000) was also found in three other saturniids and a sphyngid with different pheromones, in unusually high concentrations in receptor lymph (approx. 10^5 molecules/sensillum) (Vogt and Riddiford, 1980). The sensillar esterase shows considerable specificity for acetates with *Z*-olefinic linkages and a very high V_{max} over a large concentration range.

The studies on *Choristoneura fumiferana* (Morse and Meighen, 1984a,b, 1986) show the importance of studying multiple enzyme systems in different tissues of males and females. Three activities—an acetate esterase (*E*/*Z*11–14:Ac → *E*/*Z*11–14:OH), and O_2-requiring alcohol oxidase (*E*/*Z*11–14:OH →

E/*Z*11–14:Al), and an NAD^+-requiring aldehyde dehydrogenase (*E*/*Z*11–14:Al → *E*/*Z*11–14:Acid)—were all found in glandular and other tissues using a novel bioluminescence assay (Meighen et al., 1981) and confirmed using labeled saturated analogs.

Finally, the dodecatrienol trail pheromone of *Reticulitermes* undergoes an ω-oxidation to the 1,12-diol as determined for monoene and diene analogs (Prestwich et al., 1985; G. Prestwich, C. Sack, and J.J. Brown, unpublished results). This conversion results in defluorination of (3*Z*, 6*Z*)-12-fluorododecadien-1-ol, and the monooxygenase system is currently under investigation in our laboratories.

METHODS AND MATERIALS

Animals and Enzyme Sources

Heliothis virescens were purchased from the Cotton Foundation (Memphis, Tennessee) as pupae, sexed, and reared to adult eclosion in separate 8-oz containers in a 16:8 light–dark photoperiod. At 2 hr after lights off, adults were anesthetized with CO_2 and the antennae (male and female), legs, (male and female), extruded pheromone gland (female) and extruded hairpencil–genital organ complex (male) were excised and stored in separate microfuge tubes (tissue of 5–10 animals per tube) at −90°C until required for enzyme assays.

Tissue homogenates were prepared following the procedure illustrated for antennal tissue. Twelve pairs of male (female) antennae were placed in a conical ground-glass homogenizer containing a few milligrams of glass beads (0.15 mm diameter); 100μl of pH 7.4 phosphate buffer (76 mM sodium phosphate) was added, and a motor driven pestle was used to homogenize tissue at 4°C for 5 min. Another 100 μl buffer was added, and the mixture was homogenized again. The solution and rinsings were combined in a microfuge tube to give about 12 pairs of antenna per 300 μl of buffer, and then centrifuged at 12,000 *g* for 10 min at 4°C. The soluble fraction (S) was removed and 300 μl of a detergent buffer solution (76 mM sodium phosphate, pH 7.4, 1 mM dithiothreitol (DDT), 1 mM EDTA, and 0.1% w/v Triton X-100) was added to the first pellet and sonicated for 7 min at 4°C using a Sonifer cell disrupter (model 185) operating at 50–60 W. After centrifugation at 12,000*g* for 10 min at 4°C, the supernatant (D) was removed. Finally, 300 μl of detergent buffer was added, and the pellet was resuspended by vortexing (P). Bioassays for esterase, oxidase, and aldehyde dehydrogenase were performed on each of the three protein samples (S, D, P) to account for all enzyme activity present. Protein solutions were prepared fresh for each day's experiments.

Ultrasonic hair-fracture as described by Ferkovich (1981) was also performed to obtain soluble enzymes. Thus, 50 pairs of antennae were placed in

200 μl of phosphate buffer containing 0.5 M sucrose in an ice bath and then mildly sonicated at 4°C using a Heat Systems ultrasonic cleaning bath for 60 min. These sonicates contained only soluble enzymes at 10,000*g* and were compared with homogenates for esterase and aldehyde dehydrogenase activities.

Synthesis of pheromone and metabolites

[*9,10-*$^3H_2$](*Z*)-9-*Tetradecenyl Acetate.* This was prepared by reductive tritiation of the corresponding 9-alkyne, prepared in turn by alkylation of the acetylide of 1-tetrahydropyranyloxy-8-bromooctane with 1-hexyne in THF/HMPA (Henrick, 1977). Acid hydrolysis of the THP ether (CH_3CO_2H–THF–H_2O, 4:2:1), acetylation (Ac_2O, Py), and flash chromatography provided the necessary starting material.

The tritiation was carried out at the NIH Tritiation Facility at Lawrence Berkeley Laboratory. To a sample of 10 mg of 9-tetradecyn-1-yl acetate in 5 ml of hexane was added 3 mg of 5% Pd/$BaSO_4$ and 10 μl of a 100 μg/μl hexane solution of quinoline. The reaction vessel was freeze-degassed, and the reaction was stirred 20–30 min under 1 atm of carrier-free T_2 gas. The reaction was stopped at ca. 60% conversion. The tritium and volatiles were removed in vacuo to reduce the volume by half, and the crude product mixture was centrifuged to remove the catalyst. The supernatant was concentrated under N_2 and chromatographed on 40-m ("flash") silica gel with 3% ethyl acetate–hexane and then on 20% $AgNO_3$-coated flash silica gel with 6% ethyl actate–hexane to give complete separation of the desired [9,10-$^3H_2$](*Z*)-9-tetradecenyl acetate (58 Ci/mmol) from the alkyne precursor.

[*9,10-*$^3H_2$](*Z*)-9-*Tetradecenyl Alcohol.* A solution of [9,10-$^3H_2$](*Z*)-9-tetradecenyl acetate (approx. 7 mCi) in 150 μl of 4:1 CH_3OH–3N NaOH was stirred 3 hr at 20°C. Then, 300 μl of H_2O was added, the mixture was extracted with three 200 μl portions of 1:1 ether–hexane. The extracts were dried ($MgSO_4$) and concentrated in vacuo to give radiochemically homogeneous [thin-layer chromatography (TLC) scraping, liquid scintillation counting (LSC)] alcohol (approx. 6.3 mCi, 90% yield) which was used without further purification.

[*9,10-*$^3H_2$](*Z*)-9-*Tetradecenal.* A sample of [9,10-$^3H_2$](*Z*)-9-tetradecen-1-ol of approx. 3 mCi was used for this oxidation. A total of 500 μl of CH_2Cl_2 containing the labeled alcohol was added to a solution of a small crystal of pyridinium dichromate (PDC) in 100 μl of CH_2Cl_2. The reaction mixture was stirred at room temperature under N_2 for 1–2 hr. The mixture was diluted with 5 vol of ether. This material was first chromatographed to remove the oxidant on flash silica gel with ether in a disposable pipette. After concentration in vacuo, the residue was rechromatographed on flash silica with gradually increasing percentages of ethyl acetate in hexane in a column to give radiochemically homogeneous aldehyde, using a TLC solvent system of hexane–ether–

acetic acid (90:10:2). The labeled aldehyde was particularly susceptible to chemical decomposition and was used for experiments within four days of preparation.

Enzyme Assays

Pheromone Solutions. The radiolabeled substrates used were: [9,10-$^3H_2$]Z9–14:OH for oxidase assay, [9,10-$^3H_2$]Z9–14:Al for aldehyde dehydrogenase assay, and [9,10-$^3H_2$]Z9–14:Ac for esterase assay. Hexane stock solutions were checked for purity by scraping TLC plates and counting by LSC. When the radiochemical purity was <96%, repurification by flash chromatography in a Pasteur pipet was performed. Pheromone buffer solutions were prepared fresh for a day's experiments as follows. A hexane solution (200 μl containing 30–60 μCi) of labeled substrate was evaporated under N_2 and redissolved in 500 μl of pH 7.4 phosphate buffer, which had been throughly degassed by sonication under reduced pressure followed by sparging with helium. Degassing was crucial to prevent oxidation of the labeled aldehyde to labeled acid in the pheromone buffer.

Cofactor Solutions. The oxidized cofactor nicotinamide adenine dinucleotide (oxidized form, NAD^+) (19.9 mg) was dissolved in 10 ml of pH 7.4 phosphate buffer to give 3 mM NAD^+ for use in the oxidase and dehydrogenase assays. Prior experimentation demonstrated a requirement for an oxidized cofactor as well as little difference between NAD^+ to $NADP^+$ (also at 3 mM) as the cofactor for this pheromone dehydrogenase.

Aldehyde Dehydrogenase (ALDH) Assay. Fifty microliters of [^{3}H]Z9–14:Al buffer (9×10^{-8} M) and 50 μl of 3 mM NAD^+ were added to each of eight 10 × 75-mm borosilicate tubes. At 2-min intervals, 50 μl of protein solutions (S, D, or P from different tissues) or 50 μl buffer (blank) was added. Each incubation was for 1 hr at 20°C. At $t = 1$ hr, 150 μl of ethyl acetate was added and the tube was vortexed vigorously for 15 sec. A 3-μl aliquot of the upper layer was removed to determine recovery (by LSC) and another 6 μl aliquot was spotted on 4 × 8-cm Machery-Nagel Polygram Sil G/UV 254 plates. Plates were divided into four vertical stripes and were prespotted with unlabeled Z9–14:Al, Z9–14:OH, and Z9–14:Acid. The plates were developed in hexane–ether–acetic acid (90:10:2). Spots were visualized with I_2, the TLC plate was cut into 1-cm sections, the sections were placed into 7-ml scintillation vials, and the sections counted in 4 ml of Aquasol (New England Nuclear) or Scintiverse II (Fisher). Scintillation counting was performed on a Packard TriCarb Model 3300 instrument operating at 37–45% efficiency for tritium, as determined by automatic external standardization.

Alcohol Oxidase Assay. Using [^{3}H]Z9–14:OH as substrate, the same procedure for the dehydrogenase was followed. To test for the dependence of the alcohol oxidase on the presence of oxygen, a modified assay was performed as

follows. Male legs (22 pairs) were homogenized in pH 7.4 phosphate buffer to get 2 ml of the soluble (S) fraction. An aqueous solution of [^{3}H]Z9–14:OH was made by evaporation of 100 μl (~0.15 mCi) of the hexane solution of labeled alcohol under N_2 and redissolving the residue in 2 ml of pH 7.4 phosphate buffer. Two sets of ten duplicates were prepared: one set of ten was degassed and held under N_2, and one set was exposed to air. Into each test tube, 100 μl of [^{3}H]Z9–14:OH buffer solution and 100 μl of protein solution were added at 4°C. The tubes were transferred to 30°C bath, shaken vigorously for 30 sec, and then incubated for periods of 5, 10, 15, 20, 25, 30, 35, 40, and 45 min. After 15 min, those tubes which had been flushed with N_2 were exposed to air, vortexed vigorously, and then incubation was continued. At each interval, duplicates of each set were quenched with 200 μl of ethyl acetate. A 6-μl aliquot of upper layer was spotted on TLC, developed, cut, and counted as described above.

Acetate Esterase Assay. The assays were the same as described above for the aldehyde dehydrogenase (ALDH), but using [^{3}H]Z9–14:Ac as substrate and replacing the 50 μl of NAD^+ buffer with phosphate buffer.

Autoradiography. TLC plates spotted with 6 μl of ethyl acetate layers of blank, S, D, and P enzyme incubations were sprayed with En^{3}Hance (New England Nuclear) spray, placed in contact with preflashed Kodak X-Omat XAR-5 X-ray film for 4 hr to 4 days at −80°C, and developed as usual with Kodak GBX chemicals.

Protein Concentrations. These were determined by the dye-binding method (Bradford, 1976) using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Pheromone and derivatives (Figure 1) were synthesized at high specific activity by partial reductive tritiation of 9-tetradecyn-1-yl acetate on poisoned $Pd/BaSO_4$, affording the [9,10-$^3H_2$](Z)-9-tetradecen-1-yl acetate ([^{3}H]Z9–14:Ac) at a nominal specific activity of 58 Ci/mmol. The location and extent of the tritium labels is assumed, although [^{3}H]NMR examination is in progress. Hydrolysis with aqueous methanolic sodium hydroxide on a micromole scale gave corresponding [^{3}H]Z9–14:OH. Oxidation of this alcohol to [^{3}H]Z9–14:Al was generally performed on a 3-mCi (52-nmol) scale and required careful monitoring to avoid overoxidation or conversion to other products. Several attempts with pyridinium chlorochromate (PCC) in dichloromethane led to undesired labeled materials intermediate in TLC polarity between the alcohol and aldehyde. It was not readily controlled by decreasing the amount of oxidant. However, use of pyridinium dichromate (PDC) in CH_2Cl_2 under N_2, even in >100-fold excess, gave a controlled conversion to the labeled aldehyde in 2 hr at 20°C.

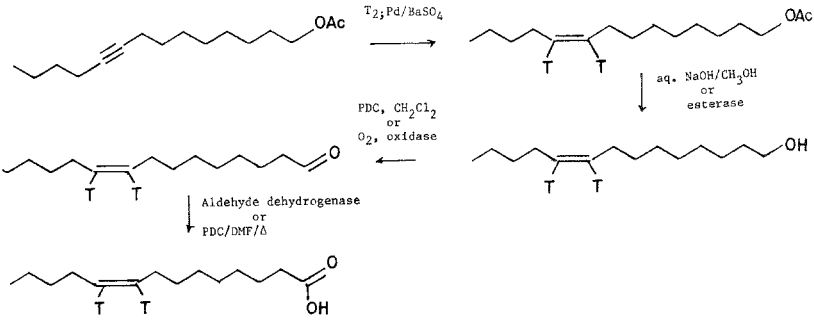


FIG. 1. Synthesis of labeled substrates and enzymic interconversions (T = ^3H).

All labeled materials were purified by flash chromatography over silica gel in a disposable pipet using hexane–ethyl acetate eluents. The labeled aldehyde was never more than one week old, and never more than 4 hr in aqueous working solutions. Failure to degas aqueous buffers used for pheromone stocks or incubations led to high "background," i.e., nonenzymic conversion to the carboxylic acid.

Tissue homogenates were prepared fresh each day from tissues stored at −90°C and used completely. This eliminated problems due to using enzyme solutions of variable age and subject to freeze–thaw sequences. The tissues were homogenized first in a phosphate buffer lacking additives, and the pellet was rehomogenized and ultrasonicated in a detergent buffer containing EDTA, dithiothreitol (DDT), and Triton X-100 to solubilize membrane-associated proteins. As discussed below, from the tissue distributions of esterase, oxidase, and dehydrogenase activities, it appears that essentially all enzyme activities are readily solubilized in buffer or in a very mild nonionic detergent. Only a small (but reproducible) residual activity of aldehyde dehydrogenase is found in the pellet fraction from female antennae.

All enzyme assays were performed with no-carrier-added substrate solutions at 10^{-8}–10^{-9} M, i.e., using a physiologically meaningful concentration of substrate. At least three repetitions per enzyme assay have been performed with separate tissue preparations and reproducible results have been obtained. The data presented in the tables and figures often illustrate a representative result as the average of duplicate subsamples for a given tissue preparation. Using the TLC assay, we could readily detect differences of 1–2% conversion (100–200 cpm out of 10,000 cpm).

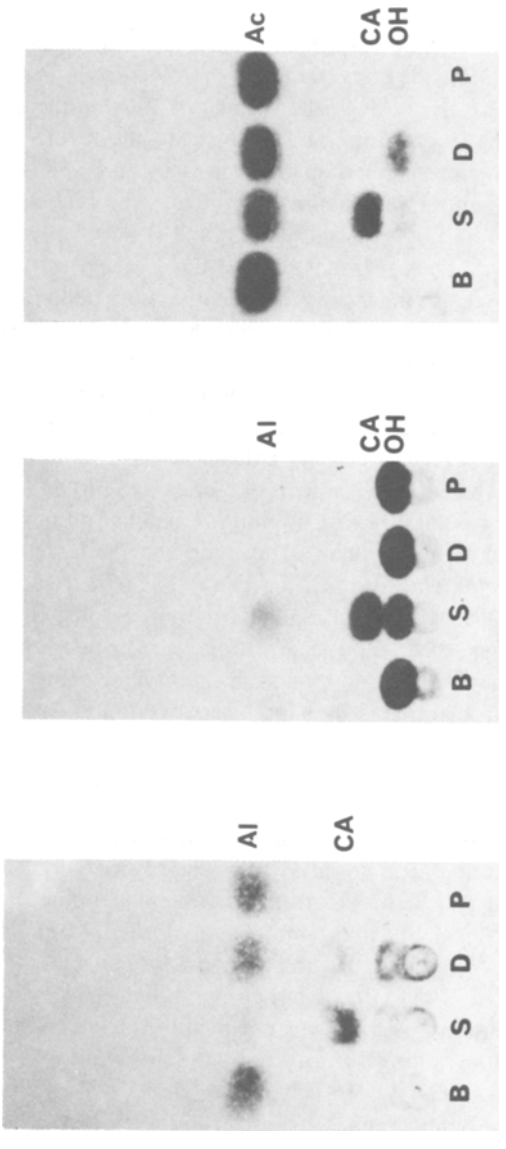
Figure 2 shows a set of autoradiograms of TLC plates to allow visualization of three enzyme activities for each of three extraction methods from both male and female leg tissue. Autoradiograms for antennal and glandular tissues appear quite similar and are omitted here; the quantative data for enzyme activ-

ity appear in Figure 3 below. The autoradiograms provide evidence for: (1) conversion of [^{3}H]*Z*9–14:Al to [^{3}H]*Z*9–14:Acid by the aldehyde dehydrogenase (ALDH), (2) conversion of [^{3}H]*Z*9–14:OH to the aldehyde by the alcohol oxidase (OX) with further transformation to the carboxylic acid by ALDH, and (3) hydrolysis of [^{3}H]*Z*9–14:Ac to the labeled alcohol by the esterase (EST) with subsequent transformations by OX and ALDH to the labeled acid. The autoradiograms demonstrate clearly the high level of radiochemical purity for each starting substrate (see blank B lanes). It also appears that the soluble aldehyde dehydrogenase is the most active enzyme in both sexes (documented below), giving nearly quantitative conversion of aldehyde to carboxylic acid. This occurs whether the aldehyde is added as substrate or is produced in vitro from oxidase on *Z*9–14:OH or combined esterase–oxidase action on *Z*9–14:Ac. Only in the detergent fraction for the esterase assay do we observe simple conversion of *Z*9–14:Ac to *Z*9–14:OH, since the OX activity has been removed and the ALDH activity is low.

As seen in Table 1, the enzymatic hydrolysis of [^{3}H]*Z*9–14:Ac to the alcohol is rapidly lost by dilution below 100 antennal equivalents (AE) per milliliter of homogenate. However, the aldehyde dehydrogenase activity was still detectable as low as 0.8 AE/ml. The oxidase activity appeared to be lower than the aldehyde dehydrogenase activity. Thus, in both male and female antennal tissues, the aldehyde dehydrogenase is the most active enzyme, consistent with its postulated importance in pheromone removal. Any aldehyde produced via oxidase action is thus rapidly converted to carboxylic acid as seen in Figure 2.

Comparison of the homogenization method and hair-fracture sonic bath method (Ferkovich, 1981) for obtaining antennal enzymes (Table 2) showed that essentially all the esterase and, indeed, a greater level of aldehyde dehydrogenase could be obtained by this milder treatment. Moreover, the quantity of cuticular material is considerably reduced. Mechanical fracture of lyophilized sensory hairs from the antennal branches used in collecting the larger (100–300 μm long) sensilla of *Antheraea polyphemus* gave protein solutions free of hemolymph and antennal branch–cuticle contamination (Vogt and Riddiford, 1981; Prestwich et al., 1986). However, this mechanical fracture/hair separation method was not readily transferred to the filiform antennae with 10 to 30-μm-long sensory hairs, such as those of male and female *H. virescens* moths. Thus, our assay results show total enzyme activity present in cuticular, hemolymph, and sensillar proteins of the antennae. The use of leg tissue is meant to control for the presence of general enzyme activities found in the cuticle and hemolymph.

Figure 3 illustrates the distribution of enzyme activities present in *Heliothis* moth tissues. The key features are : (1) Aldehyde dehydrogenase is the primary enzyme activity found in both leg and antennal tissues of males and females. (2) Oxidase activity is low in antennae, but higher in male legs and glandular



MALE

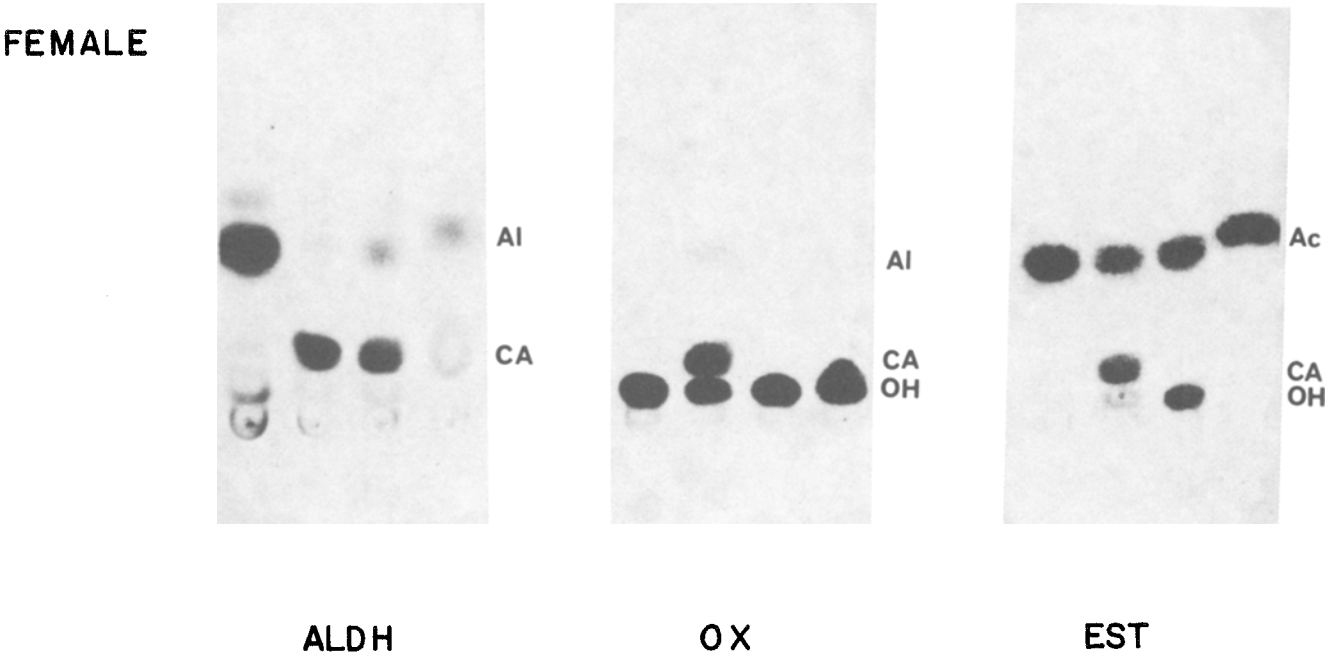


FIG. 2. Autoradiograms of TLC plates illustrating interconversions of [^{3}H]Z9–14:Ac (Ac) and the corresponding alcohol (OH), aldehyde (Al), and carboxylic acid (CA) by male (top) and female (bottom) leg tissues. The letters B, S, D, and P denote blanks, soluble, detergent, and pellet activities, respectively (see text for details); ALDH = aldehyde dehydrogenase, OX = alcohol oxidase, EST = acetate esterase.

TABLE 1. DILUTION SERIES FOR SOLUBLE ANTENNAL ENZYMES[a]

Antennal equivalent per milliliter solution[b]	Esterase (%)[c]		Aldehyde dehydrogenase (%)[d]	
	Male	Female	Male	Female
360	27	30	—	—
120	10	5	—	—
80	0	0	62	53
26.67	—	—	50	33
8.0	—	—	50	33
2.67	—	—	31	15
0.8	—	—	10	15
0.267	—	—	0	0

[a] Percent conversion to product, expressed as means of duplicate assays.
[b] A homogenate of male or female antennae of 350 AE/ml has 0.34 mg/ml total protein for the esterase. For the 80 AE/ml used for aldehyde dehydrogenase assays, males had 0.087 mg/ml and females 0.074 mg/ml total protein.
[c] One-hour incubation.
[d] Fifty-minute incubation.

tissues of both sexes. (3) Esterase activity is low in antennae, higher in legs, and highest glandular tissue of both sexes. (4) Males and females show all three enzymic activities in all tissues. Striking male–female differences include (a) a pellet-associated aldehyde dehydrogenase in female antennae; (b) relatively lower oxidase activity in female legs and glandular tissue, and (c) relatively higher esterase and dehydrogenase activity in female glandular tissues.

Figure 4 illustrates the results of the experiment which demonstrated that the oxidase from male legs (the highest activity source) is an O_2-requiring en-

TABLE 2. COMPARISON OF HOMOGENIZATION AND ULTRASONIC AGITATION FOR OBTAINING ENZYMES[a]

	Esterase[b]		Aldehyde dehydrogenase[c]	
	Male	Female	Male	Female
Homogenate	28	30	22	35
Ultrasonicate	33	35	59	61

[a] Conversions expressed as percent product from duplicate assays.
[b] Fifty pair of antennae to get 200 μl of soluble fraction. Protein concentrations for homogenate: males, 0.39 mg/ml; females, 0.45 mg/ml. Protein concentrations for ultrasonicate: males, 0.47 mg/ml; females, 0.47 mg/ml.
[c] Twelve pair of antennae to get 300 μl of soluble fraction. Protein concentrations: males, 0.022 mg/ml; females, 0.035 mg/ml.

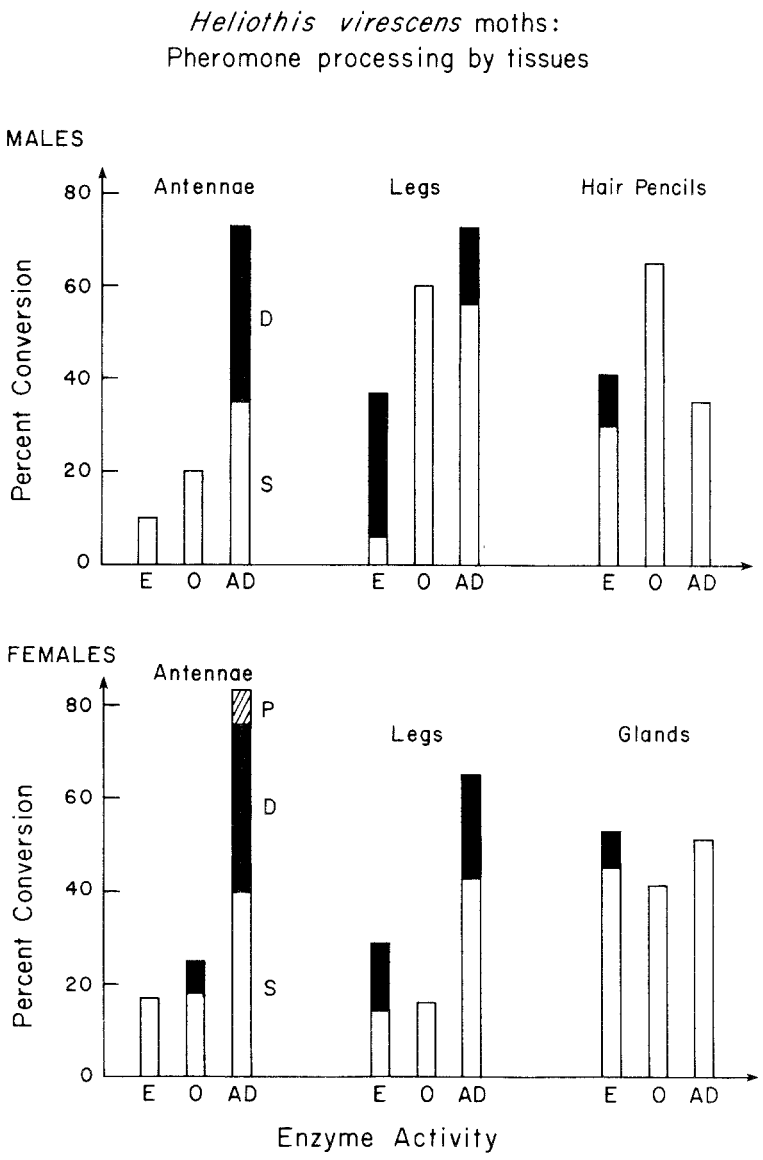


FIG. 3. Morphological distribution of acetate esterase (E), alcohol oxidase (O), and aldehyde dehydrogenase (AD) activities in adult moths (*Heliothis virescens*). All incubations were run for 1 hr at 20°C, and values shown are means of percent conversion for duplicates. Homogenates of two pairs of antennae and legs per 50 μl were used and two pheromone glands or hairpencils per 50 μl were used. Enzyme activities are shown for soluble (S, open bars), detergent-solubilized (D, solid bars), and insoluble pellet (P, cross-hatched) fractions.

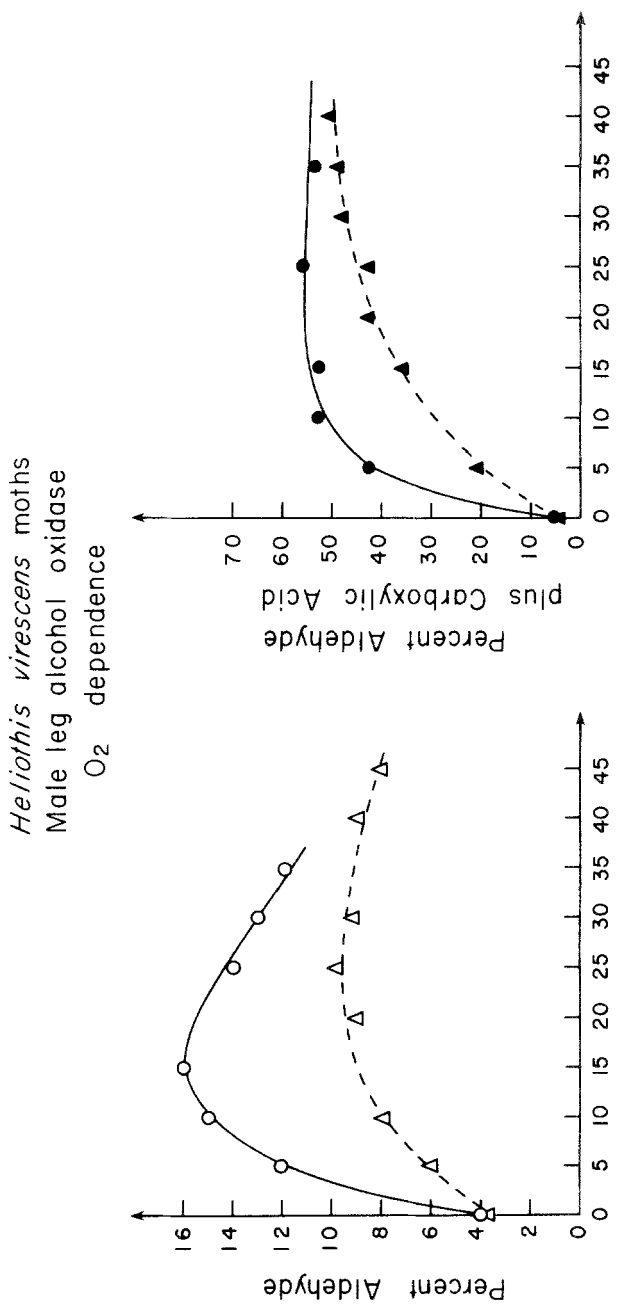


FIG. 4. Dependence of alcohol oxidase of legs of male *Heliothis virescens* moths on the presence of oxygen. Samples were incubated in air (circles) or under N_2 for 15 min followed by air (triangles). The left panel shows only conversion of [^{3}H]Z9-14:OH to the aldehyde, while the right panel shows conversion to the aldehyde plus carboxylic acid.

zyme, as opposed to being an $NAD(P)^+$-requiring alcohol dehydrogenase. This assay was conducted as described by Morse and Meighen (1984a) for *Choristoneura fumiferana* tissues. An initially lower rate of conversion of the alcohol to aldehyde (and then acid) is seen in the N_2-saturated condition, although it is difficult to reduce this rate to zero by purging the last few nanomoles of oxygen from solution. A modest increase in oxidation to the aldehyde (which is then rapidly converted to the carboxylic acid by the aldehyde dehydrogenase) is seen when air is readmitted to a N_2-saturated incubation. Failure to see a more dramatic increase may also reflect a negative effect of anoxia on the enzyme system. Cofactor deletion experiments were unsuccessful due to endogenous $NAD(P)^+$ levels.

It is important to consider the present experimental results in terms of the related metabolic conversions in *Antheraea polyphemus*, *Trichoplusia ni*, and *Choristoneura fumiferana*. For the wild silkmoth *Antheraea polyphemus*, Vogt and coworkers have described in detail the tissue localization (Vogt and Riddiford, 1981), kinetic properties (Vogt et al., 1985), and substrate specificity (Prestwich et al., 1986) of the male-specific sensillar esterase. All of these studies used freshly chromatographed, stoichiometrically labeled [11,12-$^3H_2$]-6*E*, 11*Z*-16:Ac at 58 Ci/mmol which allowed routine monitoring in the 10^{-7}–10^{-9} M range. This esterase, mol wt 55,000, shows a K_m of 2.2×10^{-6} M and V_{max} of 5.4×10^{-12} M/sec[1] for the conversion of 6*E*, 11*Z*-16:Ac to the corresponding alcohol, and the calculated half-life for pheromone under natural conditions is estimated to be 15 msec over a wide concentration range. Furthermore, the sensillar esterase showed high specificity for *Z*-unsaturated aliphatic acetates, e.g., the 6*E*,11*Z*-16:diazoacetate and saturated 16:Ac were poor substrates, while the 9*Z*-14:Ac was a good substrate. Only unsaturated 14- and 16-carbon acetates with *Z* olefinic linkages appeared to act as effective competitive inhibitors of the 6*E*, 11*Z*-16:Ac hydrolysis (Prestwich et al., 1986). Potent nanomolar inhibition was observed for 1,1,1-trifluoro-2-tetradecanone, an aliphatic trifluoromethyl ketone transition-state analog for serine esterases (Hammock et al., 1982), but not for traditional esterase inhibitors (Vogt et al., 1985). Finally, a cuticle-bound wing-scale esterase was also described for *A. polyphemus* (Vogt and Riddiford, 1986).

The key findings for the cabbage looper moth, *Trichoplusia ni*, were summarized in the Introduction. To reiterate, buffer-elutable surface esterases were found in wings > legs > antennae which degrade *Z*7–12:Ac labeled at 0.4 Ci/mmol in the acetate moiety and using concentrations in the 2×10^{-6} to 2×10^{-5} M range. These were quite reasonably postulated to serve a general cleaning role, i.e., preventing every emitting or receiving insect from becoming a pheromone source. Esterase activity in the homogenates decreased in the order antennae > legs > wings, implicating a specific receptor-clearing role for the antennal esterase in both sexes. Using unlabeled aqueous suspensions of do-

decenyl acetates at 4×10^{-3} M and monitoring hydrolysis by GC, moderate specificity for the hydrolysis of the *Z*7 isomer was found (cf. Ferkovich, 1981). In none of these experiments was it possible to follow both reactant and product at physiologically meaningful concentrations.

The spruce budworm (*Choristoneura fumiferana*) pheromone consists of >98% of a mixture of two aldehydes, *E*11–14:Al and *Z*11–14:Al in a 96:4 ratio. In an important first paper, Morse and Meighen (1984a) used novel luminescence assays to show three enzyme activities in tissues of female moths: (1) an esterase which hydrolyzes both *E* and *Z*11–14:Ac to the alcohol, (2) an O_2-dependent oxidase which converts fatty alcohols to aldehydes, and (3) an NAD^+-dependent aldehyde dehydrogenase which yields fatty acids as products. They found highest specific activity for all three enzymes in the pheromone gland and proposed the acetate as the storage form for the aldehyde pheromone. This was confirmed in later studies with ^3H- and ^{14}C-labeled saturated fatty acids, alcohols, and esters in vivo (Morse and Meighen, 1984b). In this volume, they describe in detail a new synthesis of enzymology and pheromone chemistry in which the interconversion of functional groups accounts for differences in pheromone blends used by closely related species (Morse and Meighen, 1986).

Our results with the tobacco budworm moth, *Heliothis virescens*, show also that acetate esterase, alcohol oxidase, and aldehyde dehydrogenase activities are present in all tissues of both sexes. As with the silkmoth, the substrates used in this study were all derived from [9,10-$^3H_2$]*Z*9–14:Ac, are stoichiometrically labeled, and allow studies at "physiological" nanomolar concentrations. It is also important to note that our studies used the minor aldehyde component of the ca. 93:7 blend of *Z*11–16:Al to *Z*9–14:Al which constitutes the major stimulatory cue for upwind flight. Not surprisingly, we found that the aldehyde dehydrogenase activity is by far the greatest, and it dominates the pheromone processing in both male and female antennae. Indeed, in all tissues one can readily observe (cf. Figure 2 for leg tissue) that in a 1-hr incubation, virtually all of the labeled alcohol or aldehyde resulting from esterase or oxidase activity is rapidly converted to the carboxylic acid.

An analogous result is reported in this volume by Teal and Tumlinson (1986), who show that exogenously applied "irrelevant" alcohols are converted in vivo to aldehydes by glands of female *H. virescens* and *H. zea*. This oxidase showed little specificity for alcohol chain length or unsaturation, but it did not oxidize secondary alcohols to ketones. The rapid dehydrogenase activity was not taken into account in their experiments, and this could well lead to underestimation of the real aldehyde output. They concluded from biosynthetic rates that the oxidase occurs in a cuticular-bound form in the surface of the pheromone gland. Our results, however, are more consistent with a readily soluble oxidase present in all tissues. It it is indeed cuticular, then it is readily removed by simple homogenization and is not membrane-bound.

A diel periodicity in the rise of alcohol (e.g., Z11-16:OH) and aldehyde titer occurrence (cf. Teal and Tumlinson, 1986) is triggered by the release of a brain factor in response to the onset of scotophase (Raina and Klun, 1984). On the basis of this information, the lack of acetate esters in the gland, and the abundance of Z11-16:OH in immediately precalling females, Teal and Tumlinson (1986) invoke a direct route from Z11-16:Acid (as storage form) to Z11-16:OH (as precursor) to act as a substrate for the aldehyde released during calling. This contrasts with the absence of a fatty acid reductase in gland tissues of *Choristoneura* (Morse and Meighen, 1986). Clearly, an explanation is still required for the high level of esterase found in our work in glandular tissues of males and females. The esterase level of *Heliothis* is similar to that found in *Choristoneura* (Morse and Meighen,, 1984a,b, 1986) and could be consistent with acetates as pheromones or precursors in *Heliothis* as well. However, despite careful analysis by several groups (cf. Teal and Tumlinson, 1986), acetates have not been detected in either *H. zea* or *H. virescens* female pheromone glands.

It remains to be determined what role pheromone processing enzymes play in the real sex lives of moths. Research in the area of pheromone biochemistry by enzymologists, chemists, behaviorists, and physiologists promises to provide new basic insights and potentially novel control strategies.

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SEX PHEROMONE BIOSYNTHESIS IN THE RED-BANDED LEAFROLLER MOTH, STUDIED BY MASS-LABELING WITH STABLE ISOTOPES AND ANALYSIS WITH MASS SPECTROMETRY

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Abstract—A technique for mass-labeling was developed to study sex pheromone biosynthesis in the red-banded leafroller moth, *Argyrotaenia velutinana*. With this technique, the pheromone components and all fatty acyl groups in the pheromone gland were analyzed for incorporation of label in the same analytic run with gas chromatography–mass spectrometry, using chemical ionization and selected ion monitoring (GC-SIM-CI-MS). Sex pheromone glands were incubated with fatty acids or triacylglycerols labeled with at least three deuterium atoms or carbon-13 atoms. The results of these incubations support an interpretation in which hexadecanoate is chain shortened to tetradecanoate, which is desaturated to produce (*E*)- and (*Z*)-11-tetradecenoate precursors for the sex pheromone components (*E*)- and (*Z*)-11-tetradecen-1-yl acetate. Labeled (*E*)- and (*Z*)-11-tetradecenoyl groups in synthetic triacylglycerols were not incorporated into the sex pheromone components, perhaps indicating that this lipid class is not a donor of the immediate fatty acyl precursors in sex pheromone biosynthesis.

Key Words—*Argyrotaenia velutinana*, Lepidoptera, Tortricidae, red-banded leafroller, biosynthesis, sex pheromone, mass-labeling, deuterium labeling, carbon-13 labeling.

INTRODUCTION

Extensive studies over the last few years on sex pheromone biosynthesis in the red-banded leafroller moth *Argyrotaenia velutinana* have provided a complete picture of the sequence of fatty acyl intermediates involved. The pathway was established principally on the basis of experiments in which sex pheromone

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glands were incubated in vivo and in vitro with an array of radiolabeled fatty acids. The steps in the pathway include biosynthesis of hexadecanoate from acetate, production of tetradecanoate from hexadecanoate by chain shortening (probably by the microperoxisomes in the gland), delta-11 desaturation of tetradecanoate to generate (*E*)- and (*Z*)-11-tetradecenoate, and production of the main pheromone components (*E*)- and (*Z*)-11-tetradecenyl acetate, respectively, by reduction and acetylation of these fatty acyl intermediates. A review of this work has appeared recently (Roelofs and Bjostad, 1984).

A problem in these studies was the opportunity for radiolabeled fatty acids to be broken down in the pheromone gland and give misleading results (see Bjostad and Roelofs, 1981). For example, if sex pheromone glands are incubated with radiolabeled tetradecanoic acid, it is tempting to conclude that the appearance of radiolabel in (*Z*)-11-tetradecenoate is good evidence for delta-11 desaturation. Unfortunately, the possibility remains that the radiolabeled tetradecanoate may simply have undergone mitochondrial degradation, generating radiolabeled acetyl CoA that may have been incorporated into (*Z*)-11-tetradecenoate by some other route.

We have now analyzed this pathway with a completely different set of techniques based on the use of stable isotopes to label the fatty acyl intermediates. Incorporation of label was determined by selected ion monitoring (SIM) in conjunction with chemical ionization (CI) in gas chromatography–mass spectrometry (GC-MS). Compounds containing at least three deuterium atoms or ^{13}C atoms were synthesized or purchased for use in the present study. A similar approach has been used in studying pheromone biosynthesis in bark beetles (Fish et al., 1979; Hendry et al., 1980), and in the rice stem borer moth *Chilo suppressalis* (Arai et al., 1984).

A consequence of introducing several stable isotopes into a compound is that it becomes "mass-labeled." A convenient way to analyze a series of compounds in a GC trace for incorporation of a mass-labeled compound is to use a GC-MS program that specifies a set of mass filters in the following way. The masses and GC retention times of all compounds of interest must be known. Each GC peak can consist of a mixture of mass-labeled and unlabeled isotopes of a compound, and prior to emergence of the GC peak into the source of the mass spectrometer, the GC-MS program is changed to scan only for the masses of the (chemically ionized) unlabeled and mass-labeled compounds expected in the GC peak. Two mass-filtered GC traces are generated for each injected sample, one showing unlabeled compounds and one showing mass-labeled compounds (for the specified increment in mass). By using one of the unlabeled compounds in the sample as an internal standard, the pair of mass-filtered GC traces from pheromone glands incubated with a mass-labeled compound can be compared with the pair of traces from unincubated control glands, and degree of incorporation can readily be determined.

The recycling problem mentioned above for radiolabeled compounds is much less troublesome when multiple-labeled stable isotopomers are used instead. For example, although mitochondrial degradation of the multiple-labeled precursor [1,2,3,4-$^{13}C_4$]tetradecanoic acid would produce [1,2-$^{13}C_2$]acetyl CoA, incorporation of only one of these labeled acetyl CoA molecules would produce a molecule of [1,2-$^{13}C_2$](*Z*)-11-tetradecenoate, which would not be detected as a compound with four additional mass units (it has two additional mass units). Exactly two [1,2-$^{13}C_2$]acetyl CoA molecules would have to be incorporated in order to mislead the investigator, but the probability that two would enter the same (*Z*)-11-tetradecenoate is very tiny (the square of the probability that one would enter). It is also quite easy to detect recycling if it occurs, because the (*Z*)-11-tetradecenoate can be analyzed for incorporation of two additional mass units as well as for incorporation of four additional mass units.

Triacylglycerols comprise the most abundant lipid class in sex pheromone glands of *A. velutinana* (and of all other moth species that have been analyzed, see Roelofs and Bjostad, 1984) and contain most of the (*E*)- and (*Z*)-11-tetradecenoyl groups in the gland. The role of the triacylglycerols in sex pheromone biosynthesis is not known in any moth species. We prepared synthetic triacylglycerols with mass-labeled fatty acids at defined glyceryl positions, incubated pheromone glands with these synthetic triacylglycerols, and determined incorporation of the mass-labeled fatty acyl groups into pheromone components and into the fatty acyl constituents of all the lipid classes in the gland. These lipid classes include triacylglycerols, diacylglycerols, choline phosphatides, and ethanolamine phosphatides (Bjostad et al., 1981).

METHODS AND MATERIALS

Isotopically Labeled Compounds. [16,16,16-$^{2}H_3$]Hexadecanoic acid and [^{13}C]KCN were obtained from Stohler Isotope Chemicals (Waltham, Massachusetts). Purity of [16,16,16-$^{2}H_3$]hexadecanoic acid was greater than 99%, as indicated by GC-MS analysis of the methyl ester prepared with diazomethane (generated from Diazald, Aldrich Chem. Co., Milwaukee, Wisconsin).

[1,2,3-$^{13}C_3$]Tetradecanoic acid was synthesized by stepwise addition of [^{13}C]KCN, as follows. [1-^{13}C]Dodecanonitrile was prepared by addition of [^{13}C]KCN (10 mg) to undecyl bromide in dimethyl sulfoxide (0.5 ml) in a 4-ml vial with a Teflon-lined screw cap. After 1 hr at 25°C, water (2 ml) was added and the mixture was extracted twice with hexane (1 ml each). The nitrile was purified by Florisil chromatography in a Pasteur pipet and hydrolyzed with NaOH to form the carboxylic acid, as described by Bjostad and Roelofs (1981). [1-^{13}C]Dodecanoic acid was converted to the alcohol by adding Red-Al (3.4 M, 0.5 ml) to the neat acid in a 4-ml vial. Excess Red-Al was destroyed by

adding 2 M NaOH (2 ml) until the mild fizzing ceased, and [1-^{13}C]dodecanol was extracted with hexane (twice with 1 ml). The hexane solution was washed twice with water in a 4-ml vial (1 ml each) and evaporated to apparent dryness with a nitrogen stream.

[1-^{13}C]Bromododecane was prepared from the alcohol with triphenylphosphine dibromide, as described in Bjostad and Roelofs (1981). Further [^{13}C]KCN addition and hydrolysis produced [1,2-$^{13}C_2$]tridecanoic acid, and a final round of reduction, bromination, [^{13}C]KCN addition, and hydrolysis produced [1,2,3-$^{13}C_3$]tetradecanoic acid. Yields were nearly quantitative for the four synthetic procedures; no starting material was observed by GLC analysis at the end of any reaction.

[1,2,3-$^{13}C_3$]Tetradecanoic acid, [1,2,3,4-$^{13}C_4$]tetradecanoic acid, [1,2,3,4-$^{13}C_4$](*E*)-11-tetradecenoic acid, and [1,2,3,4-$^{13}C_4$](*Z*)-11-tetradecanoic acid were all synthesized with the same set of reactions. The latter three compounds were synthesized, respectively, from bromodecane, (*E*)-7-decenol, and (*Z*)-7-decenol prepared by the acetylenic route described by Henrick (1977). It was possible to carry out the syntheses of the four compounds in parallel, adding aliquots of a given reagent to each of four vials at a particular step. Because the sequence of reactions was rapid and easy to work up in 4-ml vials, the average working time was 2 hr for each successive addition of a ^{13}C atom to a compound.

Two incubation experiments were conducted with triacylglycerols. For the first of these experiments, a 50:50 mixture of *sn*-1-oleoyl, *sn*-2-[1,2,3,4-$^{13}C_4$](*Z*)-11-tetradecenoyl, *sn*-3-oleoyl-glycerol; and *sn*-1-oleoyl, *sn*-2-[1,2,3,4-$^{13}C_4$](*E*)-11-tetradecenoyl, *sn*-3-oleoyl-glycerol was synthesized by incubating a 50:50 mixture of the labeled fatty acids with *sn*-1,3-dioleoyl-glycerol in a solution of dicyclohexylcarbodiimide and 4-pyrrolidinopyridine (Hassner and Alexanian, 1978) for 4 hr at 25°C. The triacylglycerols were purified by Florisil chromatography in a Pasteur pipet (Bjostad and Roelofs, 1981), eluting successively with hexane (4 ml), 1:3 dichloromethane–hexane (4 ml), 1:1 dichloromethane–hexane (4 ml), dichloromethane (4 ml), and 1:3 ether–dichloromethane (4 ml). The triacylglycerols eluted in the dichloromethane fraction, as indicated by TLC with solvent system II (see below). Base-catalyzed methanolysis of the synthetic triacylglycerol blend was conducted, and the products were analyzed by GC-MS (see below). The expected proportions of methyl oleate, methyl [1,2,3,4-$^{13}C_4$](*Z*)-11-tetradecenoate, and methyl [1,2,3,4-$^{13}C_4$](*E*)-11-tetradecenoate were observed (4:1:1, respectively). For the second triacylglycerol experiment, *sn*-palmitoyl, *sn*-2-[1,2,3,4-$^{13}C_4$](*Z*)-11-tetradecenoyl, *sn*-3-palmitoyl-glycerol was synthesized and purified by similar methods.

Insect Handling. Insects were reared on a semisynthetic medium (Shorey and Hale, 1965), and sexes were separated as pupae and maintained on a 16:8

light–dark cycle. Adult females (48–72 hr posteclosion) were anesthetized with carbon dioxide gas for 5 sec, their glands were everted by squeezing the tip of the abdomen with a small clip, and a droplet (0.2 μl) of a labeled compound in dimethyl sulfoxide was applied to each gland with a 10-μl syringe mounted on a micromanipulator (Bjostad and Roelofs, 1984). The droplets were completely absorbed into the glands after about an hour, and 4 hr after the droplets were applied, the glands were excised by plucking them from the ovipositors with fine forceps. Lipids were extracted from the glands by allowing them to stand in 1 ml chloroform–methanol (2 : 1) at 4°C for 12–16 hr in a 4-ml vial with a Teflon-lined screw cap.

Chemical Derivatives. Fatty acyl groups of lipids were converted to methyl esters by base-catalyzed methanolysis. A sample in a 4-ml vial was evaporated to apparent dryness with a nitrogen stream, and a 1 M KOH in methanol (0.5 ml) was added. After 30 min, 1 M HCl (1 ml) was added, and the mixture was extracted twice with hexane (1 ml each). The hexane extract was washed once with water and evaporated to apparent dryness in a 4-ml vial with a nitrogen stream. The pheromone components are acetates, and methanolysis converts them to the corresponding alcohols. Acetyl chloride (100 μl) was added to convert the alcohols back to acetates, and after 30 min, the acetyl chloride was evaporated to apparent dryness with a nitrogen stream. The products were taken up in 1 ml hexane. Acetyl chloride does not react with the methyl esters (Bjostad et al., 1981).

Separation of Lipid Classes. Lipid classes were separated by thin-layer chromatography. Precoated 20 × 20-cm Whatman K5 thin-layer chromatographic plates (Whatman, Inc., Clifton, New Jersey) were broken into small plates (2 × 9 cm) and cleaned before use by soaking 12–16 hr in methanol in a Coplin staining jar. Plates were dried for 1 hr at 100°C in an oven, allowed to cool for 10 min, and used immediately for the separation. Solvent systems used were (I) 62 : 34 : 4 chloroform–methanol–water and (II) 80 : 20 : 2 hexane–ether–acetic acid.

Lipids on TLC plates were visualized by brief exposure to iodine vapor. Spots were lightly marked with a probe, and the iodine was allowed to evaporate. When color was no longer visible (ca. 30 min), fractions were scraped into 4-ml glass vials with Teflon-lined caps and extracted for 12–16 hr with 2 : 1 chloroform–methanol. Extracts were filtered through a small plug of glass wool in a Pasteur pipet and evaporated to apparent dryness with a nitrogen stream. Base-catalyzed methanolysis and treatment with acetyl chloride were performed as described above.

Gas Chromatography–Mass Spectrometry. Compounds were separated with a Supelcowax 10 gas chromatograph column (30 m × 0.25 mm, Supelco Inc., Bellefonte, Pennsylvania), interfaced with a Hewlett-Packard 5985 mass spectrometer. The separation was performed with temperature programming

from 40°C to 250°C at 6°C/min (3 min delay). Nitrogen was used as a carrier gas.

An essential feature of the project was the use of chemical ionization in conjunction with selected ion monitoring. Chemical ionization was performed with isobutane as a reagent gas. Because chemical ionization is much gentler than electron ionization, most of the ionized molecules of a compound remain intact, and the mass spectra obtained are very simple. The chemical ionization mass spectrum for a methyl ester consisted of a prominent M+1 peak, a much smaller M+2 peak (about 10% the height of M+1), and even smaller peaks at higher masses. The spectrum of an acetate was similarly dominated by the M+1 peak, but a peak at M−59 (loss of acetate) was also present and typically about half the height of the M+1 peak. The lack of further fragmentation also means a great increase in sensitivity in detecting the compounds of interest.

Selected ion monitoring is a technique that allows an additional increase in sensitivity, by programming the mass spectrometer to analyze for only one or a few masses, unlike the complete-spectrum mode of operation in which the mass spectrometer sequentially evaluates the abundance of each of a few hundred masses. When a complete spectrum is being obtained, the time spent analyzing for a particular mass is usually less than one percent of the total analysis time, and the sensitivity of the instrument for any one mass is reduced accordingly.

Analysis of Mass-Labeled Compounds. The mass spectrometer was programmed to scan initially for the M+1 peaks of only two compounds, the labeled and unlabeled isomers of methyl tetradecanoate (the first compound of interest to emerge from the column). For example, in glands incubated with [16,16,16-$^2H_3$]hexadecanoic acid, chain shortening would produce [14,14,14-$^2H_3$]tetradecanoic acid. The methyl ester of this labeled isomer (mol wt 243) has a mass three units greater than that of unlabeled methyl tetradecanoate (mol wt 240). The mass spectrometer was therefore initially programmed to scan for the masses 244 and 241, corresonding to the M+1 values for the two compounds (alternately scanning for mass 244 for 0.25 sec, then for mass 241 for 0.25 sec). The labeled and unlabeled isomers of methyl tetradecanoate have almost identical retention times, appearing in the GC trace as a single peak.

The first GC peak was well resolved from the second peak that emerged from the GC column, which consisted of a mixture of the labeled and unlabeled isomers of methyl (*E*)-11-tetradecenoate. Just before this second GC peak began to emerge into the source of the mass spectrometer, the program changed to scan for the masses 242 and 239, corresponding, respectively, to the M+1 values for labeled and unlabeled methyl (*E*)-11-tetradecenoate. The program changed in this way just before the emergence of each GC peak to scan for the labeled and unlabeled isomers of 12 compounds in all, which emerged from the GC column in the following order: methyl tetradecanoate, methyl (*E*)-11-tetradecenoate, methyl (*Z*)-11-tetradecenoate, 1-tetradecyl acetate, (*E*)-11-tetra-

decen-1-yl acetate, (Z)-11-tetradecen-1-yl acetate, methyl hexadecanoate, methyl (Z)-9-hexadecenoate, methyl octadecanoate, methyl (Z)-9-octadecenoate, methyl (Z,Z)-9,12-octadecadienoate, and methyl (Z,Z,Z)-9,12,15-octadecatrienoate.

For glands incubated with [16,16,16-$^2H_3$C]hexadecanoic acid, incorporation into any of the 12 compounds of interest would result in an isotopomer with three additional mass units. Sequential plotting of the most abundant ion (M+1) of the most abundant isotopomer (MAI) of each of the 12 compounds of interest allows a GC trace of the unlabeled native compounds in the glands to be generated. Alternatively, scanning for MAI+3 generates a GC trace allowing detection of mass-labeled compounds resulting from incorporation of [16,16,16-$^2H_2$]hexadecanoic acid. By alternating scanning for MAI and MAI+3 every 0.25 sec, it is possible, within a single GC run, to detect the unlabeled native compounds in the gland and also to detect any mass-labeled isotopomers of those compounds that might be formed.

It is not sufficient to demonstrate the existence of GC peaks at the correct retention times in the MAI+3 trace in order to conclude that incorporation of [16,16,16-$^2H_3$]hexadecanoic acid has taken place in glands incubated with this mass-labeled compound. In order to make a meaningful assessment of incorporation of mass-labeled compounds, it is important to compare the MAI and MAI+3 GC traces for unincubated control glands with corresponding traces for glands incubated with [16,16,16-$^2H_3$]hexadecanoic acid. Even for control glands that have not been incubated with a mass-labeled precursor, the chemical ionization–mass spectra of the 12 compounds of interest include tiny MAI+3 mass spectral peaks.

Ideally, each gland extract would be expected to contain the same overall amounts of the compounds of interest, because 20 glands were used to make each extract. In practice, handling losses (and individual female variation) can cause the overall amounts to vary. Use of an internal standard is the best way to control for this variation, a technique in which a known amount of a compound not occurring in the sample is added, so that the area of the peak for this compound in the resulting GC trace can be compared to the areas of the sample peaks and allow them to be quantified. A variation of this technique was used to allow comparison of the MAI and MAI+3 GC traces from an extract of unincubated control glands with the MAI and MAI+3 GC traces from an extract of glands incubated with [16,16,16-$^2H_3$]hexadecanoic acid. The most abundant of the 12 compounds derived from the sex pheromone glands is methyl oleate (methyl (Z)-9-octadecenoate), and this compound was a convenient internal standard to use in quantifying the MAI and MAI+3 compounds in a given extract. The amount of oleate in the gland is reasonably constant with respect to the other fatty acyl groups in the gland (for examples, see the four chromatograms in the right-hand column of Figure 2, or the three chromato-

grams in the top row of Figure 3). By representing the amounts of the MAI and MAI+3 compounds in the gland in proportion to the amount of MAI methyl oleate, the MAI and MAI+3 traces for unincubated control glands and for glands incubated with a mass-labeled precursor could be directly compared with one another.

RESULTS

Sex pheromone glands incubated with [1,2,3,4-^{13}C]tetradecanoic acid were able to incorporate this compound into the three pheromone components 1-tetradecyl acetate, (*E*)-11-tetradecen-1-yl acetate, and (*Z*)-11-tetradecen-1-yl acetate (Figure 1, peaks D, E, and F, respectively). This incorporation is apparent in comparison of the MAI+4 GC trace for control glands with that for glands incubated with [1,2,3,4-^{13}C]tetradecanoic acid; the MAI GC traces for the two extracts were nearly identical. Incorporation into the three fatty acyl biosynthetic precursors of the pheromone components, tetradecanoate, (*E*)-11-tetradecenoate, and (*Z*)-11-tetradecenoate, was also observed (peaks A, B, and C, respectively). A small amount of incorporation into the saturated fatty acyl groups hexadecanoate (peak G) and octadecanoate (peak I) was observed, presumably due to chain lengthening. None of the unsaturated fatty acyl groups in the gland other than (*E*)-11-tetradecenoate and (*Z*)-11-tetradecenoate incorporated [1,2,3,4-^{13}C]tetradecanoic acid.

Sex pheromone glands were incubated with different amounts of [1,2,3-^{13}C]tetradecanoic acid (Figure 2). Comparison of Figures 1 and 2 illustrates that the GC peaks were smaller in the MAI+4 control trace for [1,2,3,4-^{13}C]tetradecanoic acid than in the MAI+3 control trace for [1,2,3-^{13}C]tetradecanoic acid. For this reason, it is apparent, in comparison of these two figures that compounds labeled with four additional mass units allow a better assessment of incorporation than compounds labeled only with three additional mass units. As glands were incubated with increasing amounts of [1,2,3-^{13}C]tetradecanoic acid, the amount of labeled (*E*)- and (*Z*)-11-tetradecenoate increased slightly, but the amount of labeled tetradecanoate increased greatly, in direct proportion to the amount added initially. This indicates that biosynthesis of (*E*)- and (*Z*)-11-tetradecenoate was near its maximum even at 100 ng labeled tetradecanoate per gland. An unexpected observation was that the ratio of *Z* to *E* increased as the initial amount of labeled tetradecanoate increased. The amounts of the pheromone components produced were approximately the same for all three amounts of topically applied labeled tetradecanoate, indicating that the rates of biosynthesis of these compounds were also near maximum.

The products of glands incubated with [1,2,3-^{13}C]tetradecanoic acid or with [1,2,3,4-^{13}C]tetradecanoic acid were analyzed with a series of mass-selective filters to verify that incorporation was apparent only in the expected mass window (Figure 3). Glands incubated with [1,2,3-^{13}C]tetradecanoic acid dif-

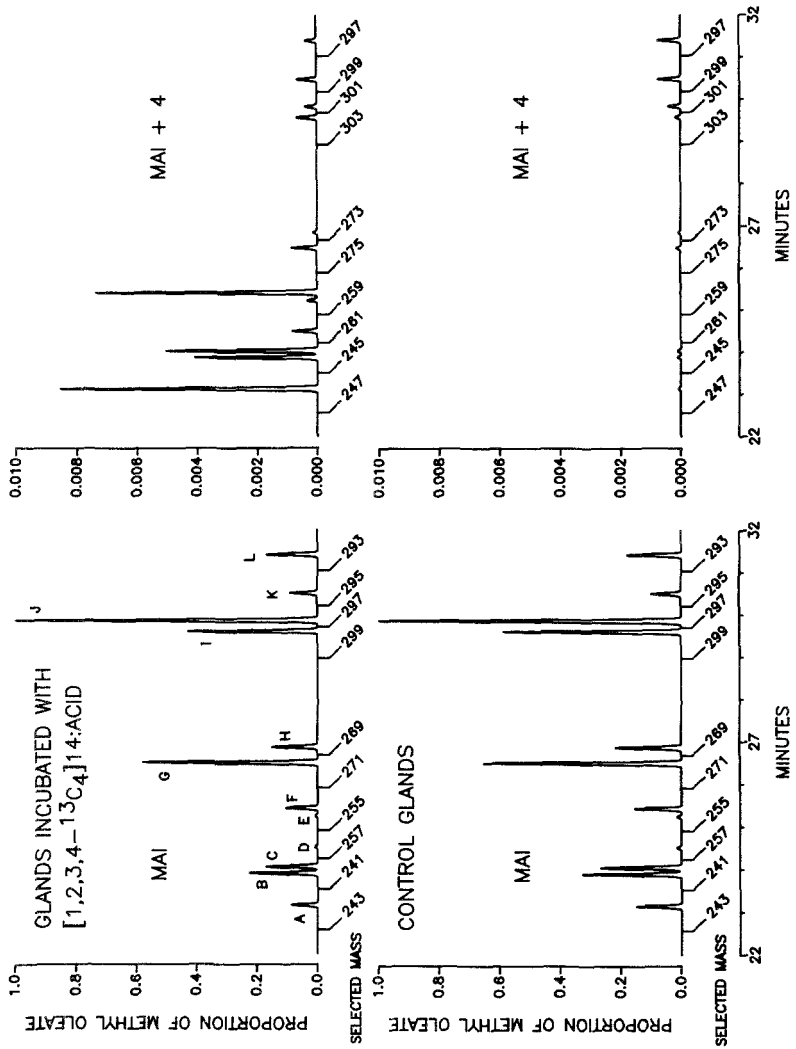

FIG. 1. Incorporation of [1,2,3,4,-$^{13}C_4$]tetradecanoic acid into pheromone components and fatty acyl groups in sex pheromone glands of *A. velutinana*. A = methyl tetradecanoate, B = methyl (*E*)-11-tetradecenoate, C = methyl (Z)-11-tetradecenoate, D = 1-tetradecyl acetate, E = (*E*)-11-tetradecen-1-yl acetate, F = (Z)-11-tetradecen-1-yl acetate, G = methyl hexadecanoate, H = methyl (Z)-9-hexadecenoate, I = methyl octadecanoate, J = methyl (Z)-9-octadecenoate, K = methyl (Z,Z)-9,12-octadecadienoate, L = methyl (Z,Z,Z)-9,12,15-octadecatrienoate (oleate). MAI = most abundant ion of most abundant isotopomer.

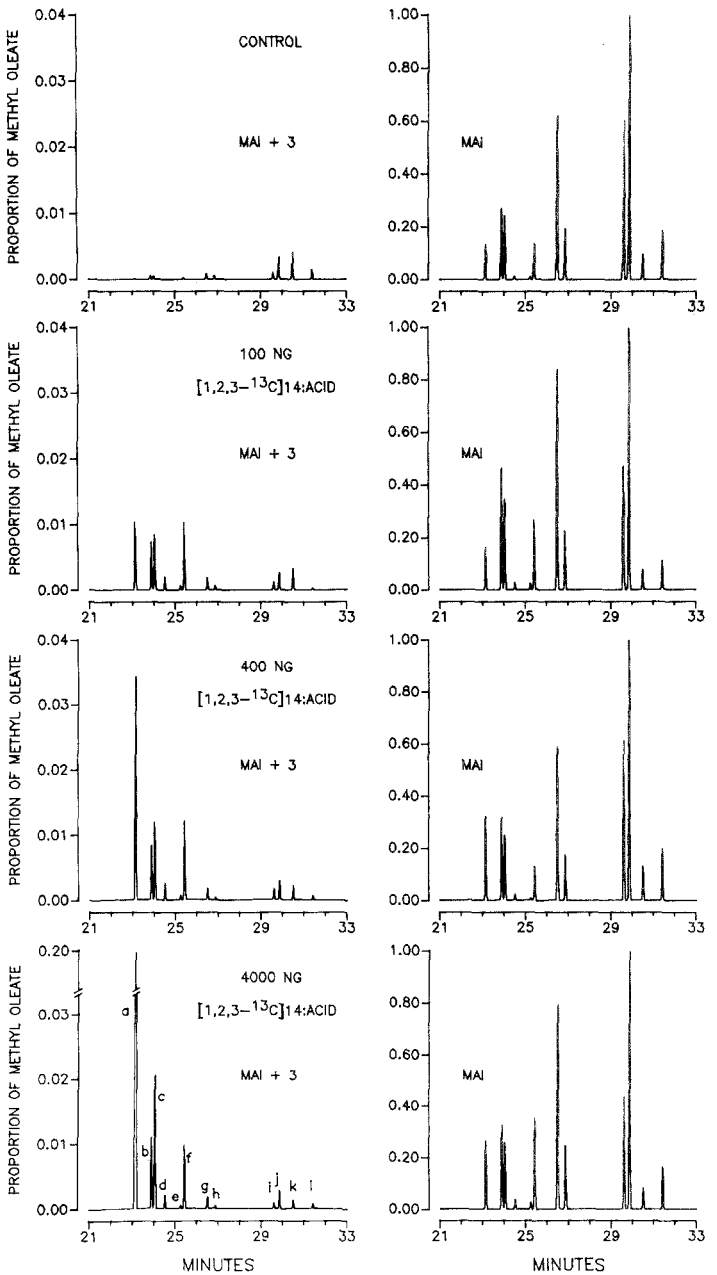

FIG. 2. Incorporation of different amounts of [1,2,3-$^{13}C_3$]tetradecanoic acid into pheromone components and fatty acyl groups in sex pheromone glands of *A. velutinana*. Abbreviations as in legend for Figure 1.

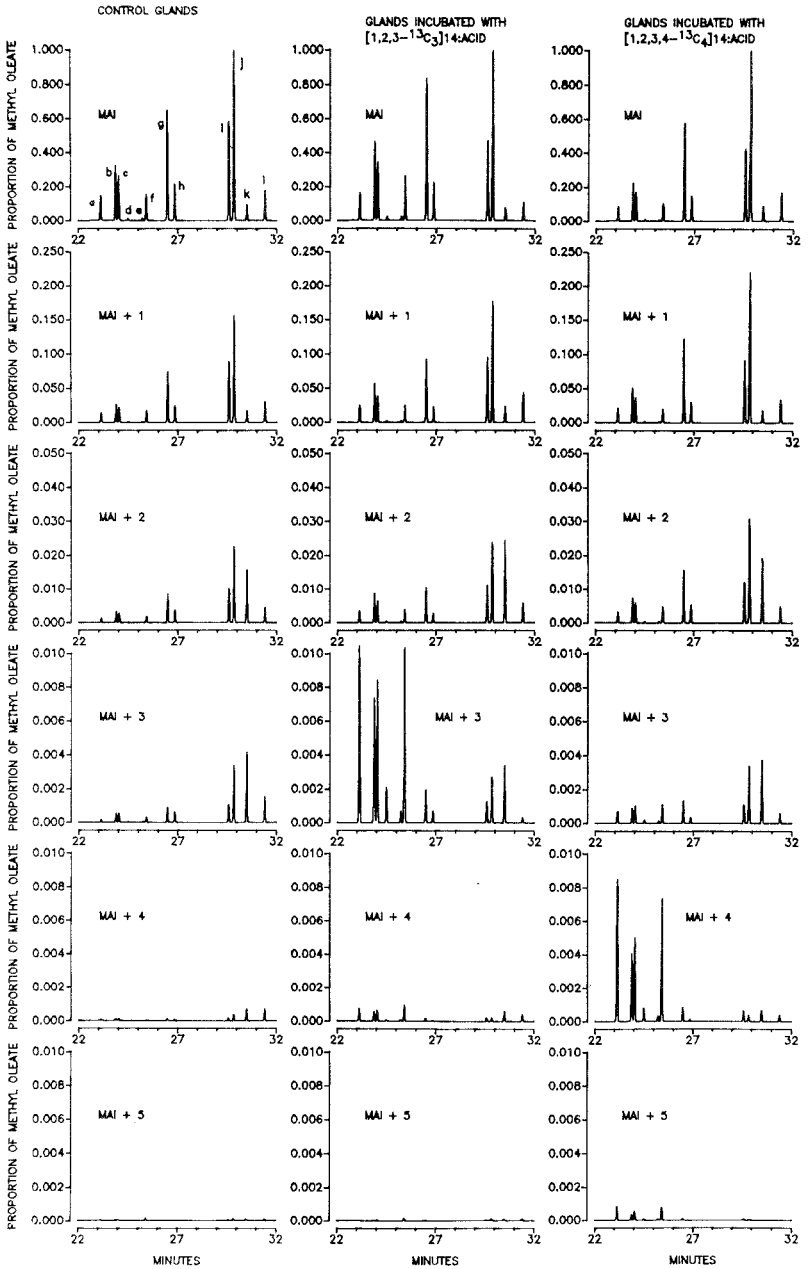

FIG. 3. Analysis with different mass filters of glands incubated with [1,2,3,4-$^{13}C_4$]tetradecanoic acid, glands incubated with [1,2,3-$^{13}C_3$]tetradecanoic acid, and control glands. Abbreviations as in legend for Figure 1.

fered from control glands almost exclusively in the MAI+3 window (but very slightly in the MAI+4 window). Glands incubated with [1,2,3,4-^{13}C]-tetradecanoic acid differed from control glands almost exclusively in the MAI+4 window (but very slightly in the MAI+5 window). The slight differences in the mass windows one unit higher are due to the small M+2 peaks (about 10% the amount of the M+1 peaks) in the chemical ionization mass spectra of the compounds of interest.

Glands incubated with [1,2,3,4-^{13}C](*Z*)-11-tetradecenoic acid were analyzed with a filter for MAI+4 compounds, and incorporation into the expected product [1,2,3,4-^{13}C](*Z*)-11-tetradecen-1-yl acetate was observed, but into no other compounds in the gland (Figure 4). Similarly, glands incubated with [1,2,3,4-^{13}C](*E*)-11-tetradecenoic acid showed incorporation into (*E*)-11-tetradecen-1-yl acetate, but into no other compounds in the gland (Figure 5).

Incubation of sex pheromone glands with [16,16,16-$^{2}H_3$]hexadecanoic acid

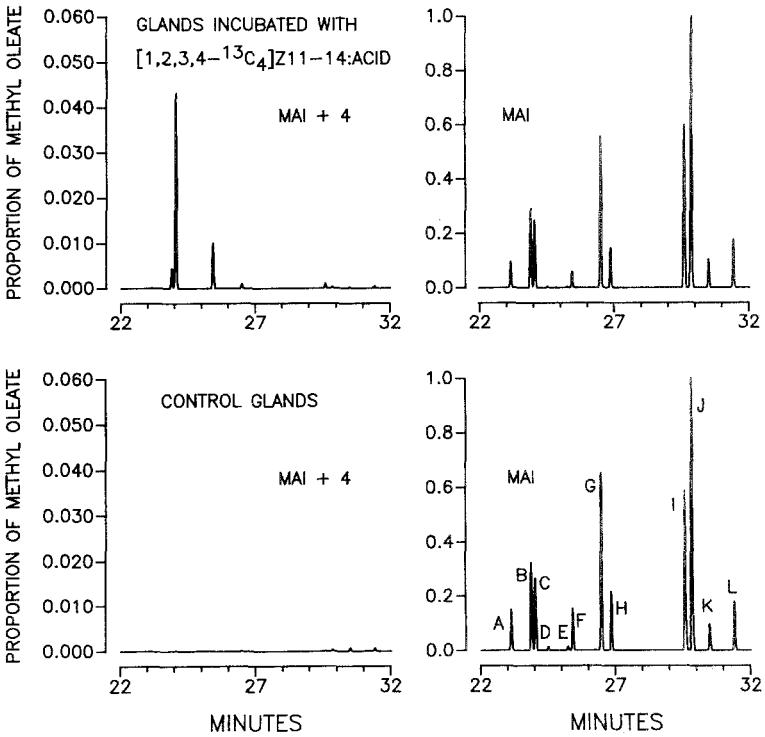

FIG. 4. Incorporation of [1,2,3,4-$^{13}C_4$](*Z*)-11-tetradecenoic acid into pheromone components and fatty acyl groups in sex pheromone glands of *A. velutinana*. Abbreviations as in legend for Figure 1.

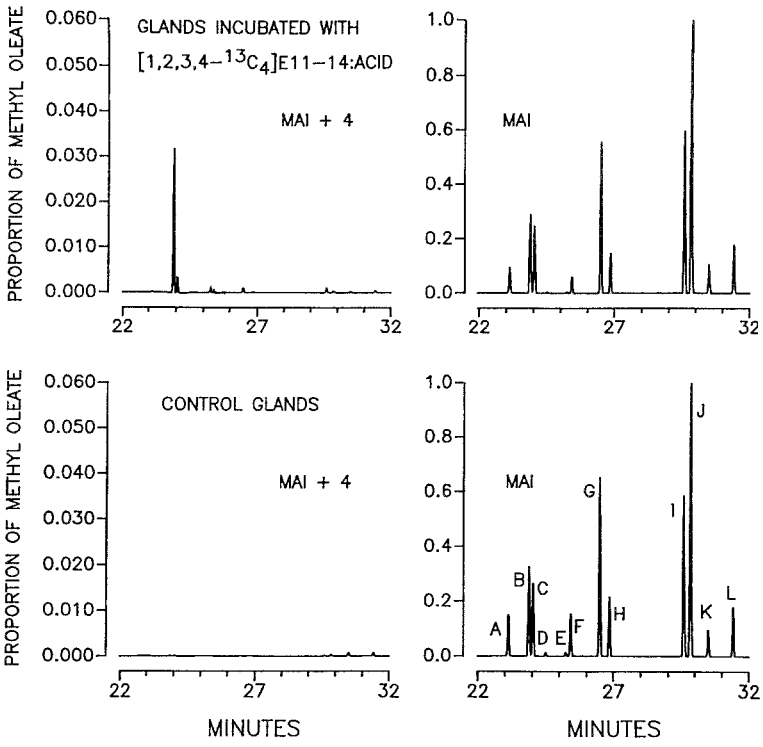

FIG. 5. Incorporation of [1,2,3,4-$^{13}C_4$](*E*)-11-tetradecenoic acid into pheromone components and fatty acyl groups in sex pheromone glands of *A. velutinana*. Abbreviations as in legend for Figure 1.

(Figure 6) resulted in the appearance of mass-labeled pheromone components (peaks D, E, and F) and the mass-labeled immediate fatty acyl precursors (peaks A, B, and C). A small amount of incorporation in octadecanoate was also observed, presumably due to chain-lengthening. Incorporation into unsaturated fatty acyl compounds other than (*E*)-11-tetradecenoic acid and (*Z*)-11-tetradecenoic acid was not observed.

Glands incubated with synthetic triacylglycerols did not incorporate the mass-labeled (*Z*)- or (*E*)-11-tetradecenoyl groups into the sex pheromone components, nor were these fatty acyl groups incorporated into choline phosphatides or ethanolamine phosphatides. Results for the 50:50 mixture of *sn*-1-oleoyl, *sn*-2-[1,2,3,4-$^{13}C_4$](*Z*)-11-tetradecenoyl, *sn*-3-oleoyl-glycerol; and *sn*-1-oleoyl, *sn*-2-[1,2,3,4-$^{13}C_4$](*E*)-11-tetradecenoyl, *sn*-3-oleoyl-glycerol are shown in Figure 7. The experiment with *sn*-1-palmitoyl, *sn*-2-[1,2,3,4-$^{13}C_4$](*Z*)-11-tetradecenoyl, *sn*-3-palmitoyl-glycerol gave similar results.

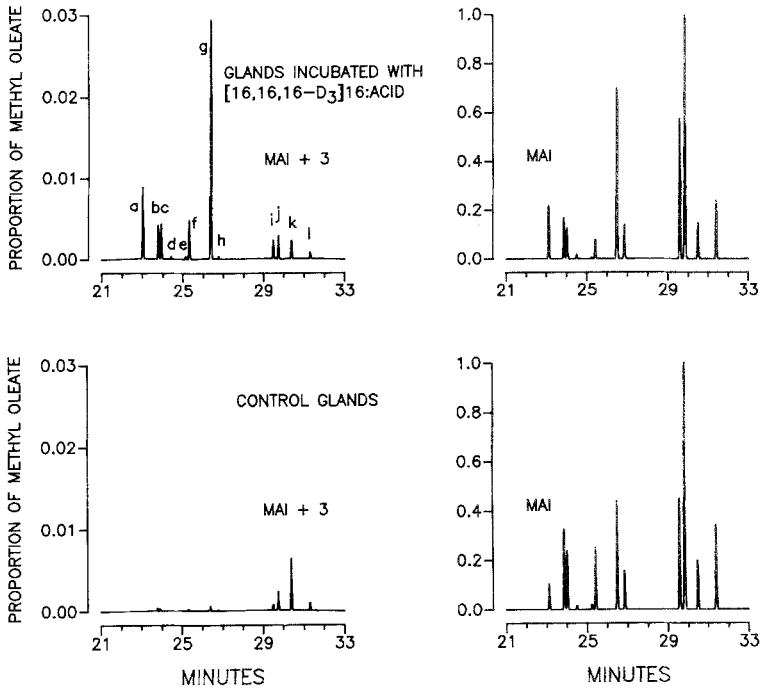

FIG. 6. Incorporation of different amounts of [16,16,16-$^2H_3$]hexadecanoic acid into pheromone components and fatty acyl groups in sex pheromone glands of *A. velutinana*. Abbreviations as in legend for Figure 1.

DISCUSSION

Incubation of sex pheromone glands of *A. velutinana* with fatty acids mass-labeled with several atoms of deuterium or ^{13}C in the present set of experiments produced results completely consistent with our previous work with radiolabeled fatty acids (Bjostad and Roelofs 1981). We concluded previously that hexadecanoate was the first fatty acyl intermediate in the biosynthesis of the sex pheromone components. Incubation with [16,16,16-$^2H_3$]hexadecanoic acid (an MAI+3 mass-labeled compound) in the present study resulted in the production of MAI+3 mass-labeled pheromone components and of the structurally analogous fatty acyl intermediates, but fatty acyl groups that were found not to be intermediates in the biosynthesis of the sex pheromone components in previous studies with radiolabel (Bjostad and Roelofs, 1981; Bjostad and Roelofs 1984) were found not to incorporate three extra mass units in the present experiment (peaks H, I, J, K, L, Figure 6). Biosynthesis of these other fatty acyl groups takes place entirely before adult emergence, and only the fatty acyl intermediates involved in pheromone production are biosynthesized in the adult female (Bjostad and Roelofs, 1981).

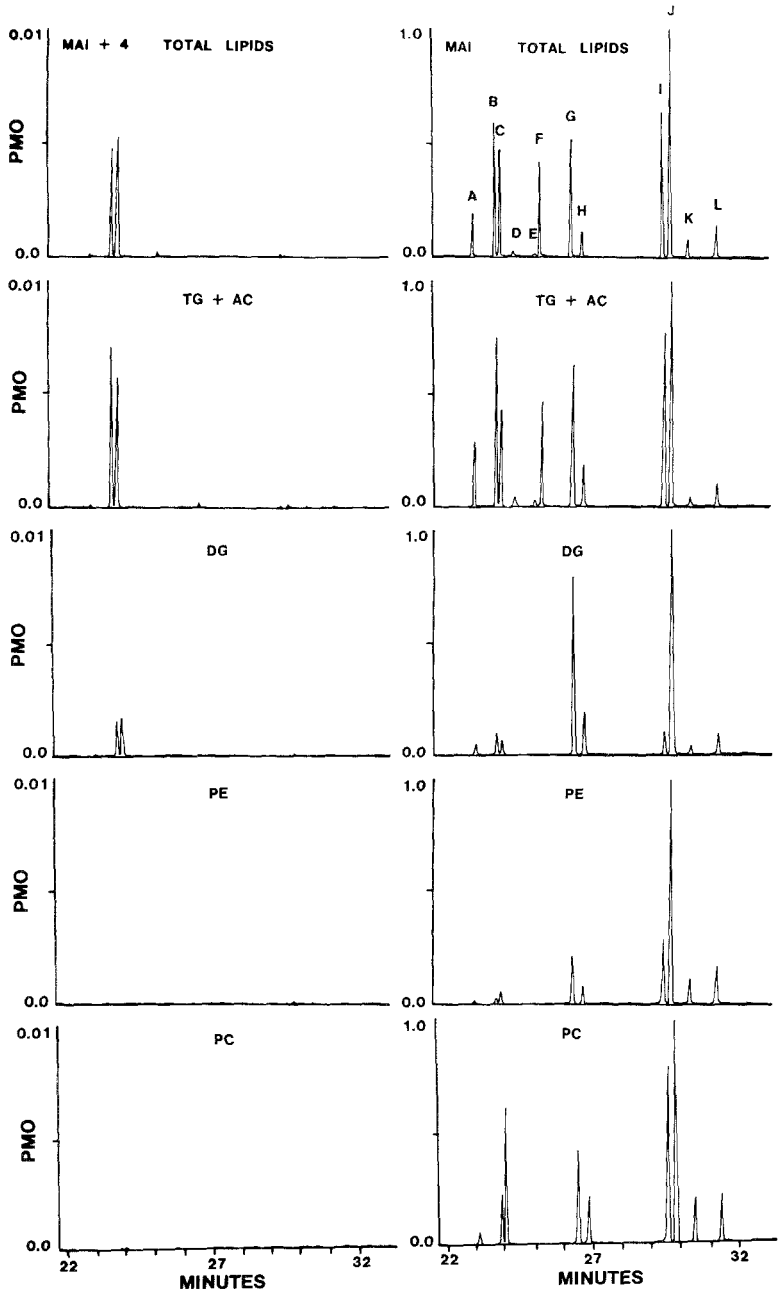

FIG. 7. Incorporation of a 50:50 mixture of *sn*-1-oleoyl, *sn*-2-[1,2,3,4-$^{13}C_4$](*Z*)-11-tetradecenoyl, and *sn*-3-oleoyl-glycerol; and *sn*-1-oleoyl, *sn*-2-[1,2,3,4-$^{13}C_4$](*E*)-11-tetradecenoyl, and *sn*-3-oleoyl-glycerol into pheromone components and fatty acyl groups in sex pheromone glands of *A. velutinana*. TG = triacylglycerols, AC = acetates, DG = diacylglycerols, PE = ethanolamine phosphatides, PC = choline phosphatides, PMO = proportion of methyl oleate in the control glands. Other abbreviations as in legend for Figure 1.

Incubation of glands with MAI+3 or MAI+4 mass-labeled tetradecanoic acid resulted in mass-labeled pheromone components and their structurally analogous fatty acyl precursors, but did not result in mass-labeled hexadecanoate. This is consistent with our previous conclusion that tetradecanoate is the second fatty acyl intermediate in the pathway.

Glands incorporated MAI+4 mass-labeled (*E*)- or (*Z*)-11-tetradecenoic acid only into the corresponding pheromone component and not into the other geometric isomer. This supports our previous conclusion that geometric isomerases do not occur in the gland. The incorporation of the *E* isomer was only 9% that of the *Z* isomer, despite the fact that glands were incubated with the same amount of each precursor, indicating that selection of the *Z* precursor for reduction and acetylation to the analogous pheromone component occurs at 11 times the rate of conversion of the *E* precursor. The lack of incorporation into tetradecanoate or hexadecanoate is consistent with our previous conclusion that each of the 11-tetradecenoates is a third fatty acyl intermediate in the pathway to each of the respective pheromone components.

Incubation with different amounts of [1,2,3-$^{13}C_3$]tetradecanoic acid showed that the rate of pheromone production was near maximum even for the smallest amount tested (100 ng/gland). Of the MAI+3 label in the gland after incubation with this amount, most was in the pheromone components and their immediate fatty acyl precursors, implying excellent conversion of MAI+3 mass-labeled tetradecanoate to mass-labeled (*E*)- and (*Z*)-11-tetradecenoate and subsequent conversion to the pheromone components. This is puzzling in light of the overall loss of [1,2,3-$^{13}C_3$]tetradecanoic acid that must have taken place, because 100 ng of methyl [1,2,3-$^{13}C_3$]tetradecanoate would produce a GC peak approximately 0.4 units in height on the scale shown (proportion of methyl oleate). The MAI+3 mass-labeled compounds only account for a total of about 0.04 units, indicating 10% recovery of mass-labeled material. The remaining 90% was presumably degraded in the gland or was eliminated from the gland.

The relative abundance of peaks K and L (methyl linoleate and methyl linolenate, respectively) was surprisingly high in mass-filtered chromatograms for MAI+2, MAI+3, and higher mass compounds, even for control glands (for example, see Figure 3). We initially suspected that compounds other than methyl linoleate and methyl linolenate isotopomers may be present with the same retention times, but higher molecular weights. If this were true, the increased relative abundance of peaks K and L should be apparent only in one of the higher mass-filtered chromatograms, but the increase is actually seen in chromatograms for MAI+2, MAI+3, MAI+4, and MAI+5 compounds (higher masses were not examined). An alternative possibility involves differential incorporation of isotopes of carbon. Insects can biosynthesize some fatty acids themselves, but rely on dietary sources of linoleate and linolenate (Downer, 1978), which was of plant origin for the insects used in this study. Insects can either deplete or enrich their body tissues in ^{13}C with respect to the

^{13}C content of their food (DeNiro and Epstein, 1978). If the insects used in this study were able to biosynthesize fatty acids that were depleted in ^{13}C relative to their dietary linoleate and linolenate, this carbon isotope fractionation may account for the increased relative abundance of linoleate and linolenate in several of the higher mass chromatograms rather than one alone.

The use of multiple-labeled stable isotopes as markers for biosynthetic studies has some advantages over the use of radiolabeled compounds. First, the likelihood of misleading results because of recycling of acetyl CoA from mitochondrial degradation is greatly reduced, as mentioned earlier in the introduction. A second advantage of mass-labeled compounds is the ability to distinguish alternative biosynthetic pathways in the same experiment. For example, chain-lengthening of (*Z*)-9-dodecenoate can be postulated as an alternative path to (*Z*)-11-tetradecenoate. The relative importance of this route could be tested by incubating sex pheromone glands with a mixture of [1,2,3,4-$^{13}C_4$](*Z*)-9-dodecenoic acid and [1,2,3,4,5,6-$^{13}C_6$]tetradecenoic acid at the same time. Mass spectral analysis for MAI+4 incorporation and for MAI+6 incorporation would establish the contributions of each pathway. It is advisable to use mass-labeled compounds at least two mass units apart; the CI mass spectrum of a compound labeled with four extra mass units (MAI+4) includes a small peak at MAI+5 (about 10% the abundance of the MAI+4 peak).

The (*Z*)- and (*E*)-11-tetradecenoate groups in the gland occur in four lipid classes: triacylglycerols, diacylglycerols, choline phosphatides, and ethanolamine phosphatides. Although hexadecanoate, tetradecanoate, and the (*Z*)- and (*E*)-11-tetradecenoates are known to be successive fatty acyl intermediates in the biosynthesis of the pheromone components (*Z*)- and (*E*)-11-tetradecenyl acetate, the role of each of the glycerolipid classes that carry these fatty acyl groups is not yet known. Most of the (*Z*)- and (*E*)-11-tetradecenoate groups in the gland occur in the triacylglycerols (Bjostad et al., 1981). Surprisingly, the pheromone components did not incorporate any labeled (*Z*)- or (*E*)-11-tetradecenoate when glands were incubated with the synthetic triacylglycerols tested in the present study.

These results contradict our earlier conclusion that the triacylglycerols are a source of (*Z*)- and (*E*)-11-tetradecenoate for the biosynthesis of the corresponding pheromone components. We found previously that in glands incubated with radiolabeled acetate for different time intervals, there were significant changes in the triacylglycerols in the relative proportions of radiolabel in tetradecanoate, (*Z*)-11-tetradecenoate, and (*E*)-11-tetradecenoate (Bjostad and Roelofs, 1984). Because the relative proportion of radiolabeled tetradecanoate decreased with time of incubation, we concluded that triacylglyceryl tetradecanoate was being used to make triacylglyceryl (*Z*)- and (*E*)-11-tetradecenoate. We further concluded that the increase in the relative proportion of radiolabeled (*E*)-11-tetradecenoate with time of incubation could be explained in terms of a 10:1 difference in the respective rates of conversion of triacylgly-

ceryl (*Z*)- and (*E*)-11-tetradecenoate to the corresponding pheromone components.

The incubations of glands with synthetic triacylglycerols in the present study were an attempt to demonstrate more directly that the triacylglycerols were acyl donors for the biosynthesis of the sex pheromone components. The complete lack of incorporation that we observed has several possible explanations. One possibility is that the results are an artifact and that the synthetic triacylglycerols were simply unable to enter the pheromone gland cells despite the fact that labeled fatty acids were readily incorporated. The triacylglycerols are larger and less polar than the fatty acids, and this may have resulted in such exclusion.

If the results are not an artifact, then they indicate that triacylglycerols are not acyl donors for pheromone biosynthesis and that there is a different explanation for the temporal change we observed previously in the relative proportions of radiolabeled tetradecanoate, (*Z*)-11-tetradecenoate, and (*E*)-11-tetradecenoate (Bjostad and Roelofs, 1984). The explanation must also take into account the related observation in the present study (Figure 2) that incubation of glands with increasing amounts of [1,2,3-$^{13}C_3$]tetradecanoic acid resulted in an increase in the amounts of (*Z*)- and (*E*)-11-tetradecenoate produced and also an increase in the proportion of *Z* to *E*.

One such explanation can be constructed in terms of the following scenario. Delta-11 desaturation of tetradecanoate may initially produce twice as much (*Z*)-11-tetradecenoate as (*E*)-11-tetradecenoate. This is the ratio we have observed in the choline phosphatides and in the ethanolamine phosphatides (Figure 7), and there is evidence that desaturation actually takes place on fatty acyl groups of phospholipids (Pugh and Cates, 1979). As (*Z*)-11-tetradecenoate is produced, some is converted to the pheromone component (*Z*)-11-tetradecenyl acetate and some is incorporated into triacylglycerols that may be a metabolic dead end. Only a tiny proportion of the (*E*)-11-tetradecenoate is converted to the corresponding pheromone component, and most of this isomer is incorporated into triacylglycerols. A fresh supply of (*Z*)- and (*E*)-11-tetradecenoate in a 2 : 1 ratio is continually made available to the enzymes that produce (*Z*)- and (*E*)-11-tetradecenyl acetate, and disposal of the unused portion is accomplished by incorporating it into triacylglycerols. In this interpretation, the triacylglycerols are not acyl donors for the pheromone components and may serve instead as a "dumping ground" for tetradecanoate, (*Z*)-11-tetradecenoate, and (*E*)-11-tetradecenoate that are not used in pheromone biosynthesis. If this is true, a reasonable alternative explanation for the observed temporal change in proportion of these three fatty acyl groups in the triacylglycerols can be proposed in terms of differences in the rate of pheromone biosynthesis at different times of day. The rates of biosynthesis of the pheromone components relative to those of their fatty acyl precursors may be higher in the latter part

of the dark period. Because the amount of (*E*)-11-tetradecenoate used for pheromone biosynthesis is only one tenth that of the *Z* isomer, the proportion of unused (*E*)-11-tetradecenoate relative to that of the *Z* isomer would be higher in the latter part of the dark period.

This scheme may also account for the results in the present study involving incubations of glands with different amounts of [1,2,3-$^{13}C_3$]tetradecanoic acid (Figure 2). Approximately equal amounts of labeled (*E*)-11-tetradecenoate, (*Z*)-11-tetradecenoate, and (*Z*)-11-tetradecen-1-yl acetate were observed in glands incubated with 100 ng [1,2,3-$^{13}C_3$]tetradecanoic acid. The rates of biosynthesis of labeled pheromone components and 11-tetradecenoates were near maximum even at this lowest amount tested. By greatly increasing the amount with which glands were incubated, presumably it was possible to increase slightly the amounts of (*Z*)- and (*E*)-11-tetradecenoate produced by desaturation (in a 2 : 1 ratio). Because the rates of biosynthesis of the corresponding pheromone components remained about the same (and in a 9 : 1 ratio of *Z* to *E* isomers), unused (*Z*)-11-tetradecenoate may have accumulated at an increased rate with respect to unused (*E*)-11-tetradecenoate. Incorporation of these unused *Z* and *E* isomers into triacylglycerols would result in an increase in the *Z* to *E* ratio.

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INSECT OLFACTORY RECEPTOR RESPONSES TO COMPONENTS OF PHEROMONE BLENDS

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Abstract—Multicomponent pheromone systems are common in many insect species. As our knowledge about the number of different chemical compounds actually involved in a particular communication system increases, so too does the need for an efficient neural mechanism for the encoding of behaviorially relevant odor compounds. Here we consider the electrical activity of olfactory receptor neurons in a subset of the individual pheromone-sensitive sensilla on the antennae of male cabbage looper moths (*Trichoplusia ni*). Responses to single- and multiple-component stimuli, drawn from seven behaviorally active compounds, were obtained at several different intensities. Some blends elicited electrical responses which were not readily predicted from a knowledge of the receptor neuron's response to individual components.

Key Words—Cabbage looper, *Trichoplusia ni*, Lepidoptera, Noctuidae, insect attractant, electrophysiology, olfactory receptor, pheromone blend.

INTRODUCTION

The chemical communication system of *Trichoplusia ni*, the cabbage looper moth (CL), is now known to involve at least seven behaviorally relevant odor compounds, six of which are produced by the female (Bjostad et al., 1984). As has been the case in a wide range of species, the number of components identified in the female gland of *T. ni* has increased over the years, keeping pace with technical developments in modern chemistry. In particular, the recent advances in microanalytical procedures have made it possible to monitor the composition of the effluvia from a single behaviorally active female (Baker et al., 1981; Guerin et al., 1981; Löfstedt et al., 1982). This contrasts with the sac-

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rifice of 500,000 virgin females required in Butenandt's original identification of bombykol in the domestic silkworm (Butenandt, 1963).

As our awareness of the behavioral and chemical complexity of an insect's pheromonal communication system expands, so too should our appreciation for the demands made upon its nervous system (Cardé and Baker, 1984; Dethier, 1971). For example, it would be reasonable to postulate that the insect olfactory system would be characterized by: extreme specificity at the molecular level, so that exceedingly small changes in the chemical structure of pheromone compounds can be discriminated; a reasonable amount of neural plasticity, so that the molecular diversity that is to be processed can continue to increase as other organisms, including organic chemists, synthesize new behaviorally relevant compounds; enormous gain, so that very dilute, but evolutionarily important, chemical signals, like those emanating from willing but remote sexual partners, can be detected; considerable resolving power, so that these signals, which are likely to be buried in a sea of other nonsignificant compounds, can be discriminated; wide dynamic range, so that small differences in local concentration gradients may be used to locate the sources of distant chemical signals; and, finally, all of these requirements must be packed within the space allocated to the nervous system and fueled within the limited energy budget imposed by the overall metabolic cost of existence.

The sensory processing of complex chemical signals by the insect olfactory system has often been postulated to involve a set of narrowly tuned, highly specific olfactory receptor neurons, one for each of the behaviorally relevant component compounds in the pheromone blend (Priesner, 1979a,b, 1980, 1983). In experiments where individual components of a pheromone blend are used as stimuli for single olfactory receptor neurons, many of the behaviorally relevant components do seem to have their own, narrowly tuned specialized receptor neuron type, each apparently dedicated to detecting and signaling its presence (Löfstedt et al., 1982). Even in those instances where several additional compounds may stimulate the same single-receptor neuron, the concentrations required for threshold electrophysiological responses are often so large that a behaving animal would likely never encounter them in sufficient amounts to elicit meaningful neural activity. Thus, each behaviorally relevant component of a complex pheromone blend is thought to have its own private channel of communication with the central nervous system. There the composition of a particular stimulus mixture may be unambiguously decoded by simply noting which of the separate input channels are activated.

From a theoretical point of view, this type of system is an efficient neural encoder, only in those cases where the pheromone blend consists of only a few compounds. That is, as the number of compounds in the communication system increases, so too does the requirement for additional private input channels (O'Connell, 1975, 1981). Moreover, in any system where all of the primary receptor neurons are very narrowly tuned, the ability to respond to small amounts

of other compounds, including those produced by potential competitors, is severely restricted and can include only those chemicals with nearly identical structural and chemical properties to those which are already in the stimulus set. Thus, a system which relies on highly specialized, narrowly tuned receptors to encode information about pheromones is less adaptive than other arrangements in the sense that it imposes a form of odor blindness on the nervous system which restricts its ability to process the pheromonal signals emanating from other sources.

In contrast, efficient encoding of a very large number of different odor compounds can be accomplished, on theoretical grounds, by a relatively small number of different olfactory receptor neuron types if each group is uniquely responsive to a range of different odors (Erickson, 1982; O'Connell, 1981). The identity of a particular stimulus may then be unambiguously decoded by the central nervous system if it evaluates in parallel the relative amounts of neural activity elicited across the whole population of receptor neuron types. It is important to note that the relative breadth of tuning among the different classes of olfactory receptor neurons need not be constant in this type of encoding mechanism. Thus, some fraction of the available input channels could be more narrowly tuned than others.

Although highly specific olfactory receptor neurons, apparently tuned to individual behaviorally relevant pheromone components, are observed in many species, it is common to find systems in which behaviorally relevant compounds are processed in the absence of such tuned receptor neurons (Löfstedt et al., 1982; O'Connell, 1972, 1975). There are also insects in which olfactory receptor neurons exist in males that are apparently tuned to compounds that are not produced by the species female (Löfstedt et al., 1982; O'Connell, 1985c; Priesner, 1979a,b). Given the chemical complexity of many pheromone systems and the well founded desire to use them as ecologically sound methods of insect pest control, it is absolutely necessary that the neural mechanisms underlying, and responsible for, pheromone perception become the objects of intensive study.

One of the basic problems to be evaluated may be stated quite simply: given that the olfactory communication systems of insects are, in nearly all cases, sophisticated (with multiple chemical signals conveying a variety of interrelated messages each of which ultimately results in a series of integrated, context-specific behavioral responses), how does the insect nervous system detect, encode, and process these messages unambiguously and still cope with the range of theoretical restraints enumerated earlier? An initial step toward answering this admittedly global question involves a consideration of the specific response properties of particular classes of insect olfactory receptors when they are each presented with behaviorally relevant components of the pheromone blend.

The earliest component identified from female CL (Ignoffo et al., 1963;

Berger, 1966), and the major volatile component of the gland is (Z)-7-dodecenyl acetate (Z7–12 : Ac). This compound is essential for flight initiation in males and was originally thought to be entirely responsible for the biological activity obtained with female gland extracts. Subsequently, (Z)-7-dodecenol (Z7–12 : OH) was shown to significantly arrest upwind flight in males exposed to either calling females or to sources of synthetic Z7–12 : Ac (Toba et al., 1970; Tumlinson et al., 1972; McLaughlin et al., 1974). Although this compound has occasionally been found in gland extracts, it is generally considered to arise artificially in the process of chemical separation or analysis (Linn et al., 1984). It is thus classified in common with other behaviorally active compounds which are not components of a female's pheromone blend, as an interspecific compound, perhaps one involved in some speciation mechanism which includes the CL (Fletcher-Howell et al., 1983; Leppla 1983).

A saturated 12-carbon acetate, dodecyl acetate (12 : Ac) was next identified in female glands and was shown to modulate several of the close-range search behaviors of males, including landing frequency and total time spent on the pheromone source (Bjostad et al., 1980). Recently four additional compounds were found in the female gland. They have been identified chemically and implicated behaviorally as important components of the pheromone blend (Bjostad et al., 1984). These compounds include: (Z)-5-dodecenyl acetate (Z5–12 : Ac); 11-dodecenyl acetate (11–12 : Ac); (Z)-7-tetradecenyl acetate (Z7–14 : Ac); and (Z)-9-tetradecenyl acetate (Z9–14 : Ac). Although the exact roles of these latter four compounds in modulating normal male behavior is still being evaluated, there is little doubt that, in wind-tunnel assays, the two previously identified CL female components elicit only about 25% of the close-to-source behaviors observed when animals are exposed to the total complement of the six female-produced components (Bjostad et al., 1984; Linn et al., 1984). Moreover the more complex six-component synthetic blend elicits behavioral responses which are quantitatively equivalent to those elicited by excised virgin female pheromone glands, both in terms of the number of males responding and the amount of time required for the full behavioral sequence.

The experiments and discussion reported here continue our attempts to unravel blend perception in the peripheral olfactory system of the cabbage looper moth. We had previously focused on the three original compounds whose behavioral import was known (O'Connell, 1985b,c). There we focused especially on the quantitative response characteristics of a selected subset of pheromone-receptor neurons (HS sensilla) which had earlier been shown to be very responsive to Z7–12 : Ac and Z7–12 : OH (O'Connell et al., 1983). Here we expand these studies in HS sensilla by considering the alterations in neural discharge engendered in these receptor neurons by the addition of the four recently identified pheromone components to the various blends previously considered. The effect of adding the behavioral inhibitor Z7–12 : OH to some of these mixtures

was also examined. The number of stimulus combinations was kept manageable by fixing the intensity and composition ratios of the various components at levels near those found in female gland extracts. Since we have explored only a limited portion of the available behaviorally relevant stimulus domain, in only one of the two recognized classes of pheromone-sensitive sensilla, it is not yet possible to provide a thorough description of potential coding mechanisms. However, we hope to show here additional instances where olfactory receptor neurons are responsive, in novel ways, to multiple-component stimuli even in those cases where an individual behaviorally relevant pheromone component is not processed by a separate unique class of receptor neuron.

METHODS AND MATERIALS

Animals. All of the adult moths used in this study were derived from the laboratory-reared stock maintained by the Insect Attractants, Behavior and Basic Biology Research Laboratory, USDA, Gainesville, Florida. Eggs were collected from filter papers placed in laying cages, washed in dilute sodium hypochlorite solution, and rinsed with distilled water. After hatching, larvae were supplied with ad libitum Velvetbean Caterpillar Diet (#9795, Bio-Serve Inc., Frenchtown, New Jersey 08825). Newly pupated animals were sexed, and experimental males were isolated from further contact with females. Virgin adult males had access to 5% sucrose solution and were used as subjects when 2–5 days of age. All animals were maintained at 23°C, 70% relative humidity, and exposed to a 14 : 10-h light–dark cycle.

Recordings. Animal preparation, tungsten microelectrode construction, extracellular recording conditions, odor cartridge placement, and their control were as described elsewhere (O'Connell, 1975, 1985c; O'Connell et al., 1983). Briefly, males were restrained and fixed in a recumbent position on a Plexiglas holder. One of their antennae was held in place with transparent adhesive tape and then viewed at 600 power with the long working distance objectives of a light microscope. Transillumination of the antennae revealed the shafts and bases of individual olfactory sensilla and allowed the positioning of electrolytically sharpened recording microelectrodes. In general, we found that stable recordings were routinely obtained with these techniques and that odor-induced responses could be monitored for considerable time periods, apparently limited only by the gradual dehydration of the animal over a 24- to 96-hr interval.

The electrical signals generated by the two olfactory receptor neurons found in each sensillum were processed with an AC-coupled amplifier (bandpass 0.3–3.0 kHz), displayed on an oscilloscope, and then sorted and timed by an online digital computer (O'Connell et al., 1973). For each stimulus application the computer produced: (1) an event–time histogram; (2) a reconstructed spike

record; and (3) the total number of action potentials elicited in the prestimulus, stimulus, and poststimulus intervals for both receptor neurons in a sensillum. All the data were obtained from sensilla whose morphological and physiological properties classified them as HS sensilla (O'Connell et al., 1983).

Stimulation. All the compounds used here are found in the female cabbage looper pheromone gland or are known to modulate male behavior. Both Z7-12:Ac and Z7-12:OH were obtained from Farchan Chemical Co., Columbus, Ohio. The saturated compound, 12:Ac was a gift from Dr. W. Roelofs, New York State Agricultural Experiment Station, Geneva, New York. The purity of these materials was evaluated prior to use by Albany International, Controlled Release Division, Needham Heights, Massachusetts. Based upon gas chromatographic retention times on polar (SP-2340) and nonpolar (SPB-1) columns (60M, WCOT), each calibrated against authentic standards, the level of cross-contamination among these three compounds was negligible (detection limit, <0.1%). Absolute purity was 87% for Z7-12:OH (+8% *E*7-12:OH), 93% for Z7-12:Ac (+5% *E*7-12:Ac), and 98% for 12:Ac. The remaining female produced compounds, 11-12:Ac; Z5-12:Ac; Z7-14:Ac, and Z9-14:Ac, were a gift from Dr. M.S. Mayer, Insect Attractants, Behavior and Basic Biology Research Laboratory, USDA, Gainesville, Florida.

A range of stimulus intensities for each compound was produced by serial dilution with light mineral oil (Aldrich Chemical Co., Milwaukee, Wisconsin). The dilution series varied in decade steps from 0.1 μg/μl to 0.0001 μg/μl and were known to span the range of physiologically effective intensities typical for HS sensilla. The relative amount of each compound present in a synthetic mixture was matched to those observed analytically in extracts of female glands except for Z7-12:OH which, although behaviorally active, seems not to be a natural glandular constituent. This latter compound was evaluated in mixtures at a proportion equal to that used with mixtures containing Z7-12:Ac. The various single and multicomponent stimuli were produced by spotting, in turn, 0.5 μl of the appropriate individual dilution series onto a filter paper (160 mm^2) held in individual glass odor cartridges. Make-up mineral oil, where required to equalize the evaporative surface area, was added to each filter paper so that total liquid volume for each cartridge was 1.5 μl.

This method of stimulus preparation results in single cartridges that evoke responses equivalent to those that are obtained when the individual components of a blend are kept in separate cartridges and mixed in the air space surrounding a single sensillum. Thus, within our ability to measure differences between these two modes of stimulation, a single-odor cartridge delivers multiple-component stimuli with individual release rates equivalent to those obtained with cartridges containing only a single pheromone component. Most mixtures were evaluated at a single concentration and composition ratio. The concentration level normally selected was one which would, on average, result in a half-

maximal action potential discharge. This allowed us to detect both increases and decreases in the neural activity evoked by mixtures. For the principal component of the female pheromone blend, Z7–12 : Ac, the 0.005 μg/cartridge was routinely used. The six female-produced compounds were made up as a single stock solution at the composition ratios observed in extracts of female glands (Table 1) to minimize the total volume involved when these compounds were evaluated.

Completed odor cartridges were sealed with Teflon plugs and caps and stored in individual Teflon-capped glass vials which were, in turn, stored in an opaque container continuously purged with dry nitrogen (1.6 liters/min). Between experiments this container was purged and stored at 4°C. These are necessary precautions for pheromone storage and handling and are designed to prevent cross-contamination between individual samples.

Individual stimuli were 2 sec in duration and applied, under computer control, by passing purified, oxygen-free nitrogen (60 ml/min) over the filter paper contained in each glass odor cartridge. The outlet of the odor cartridge was positioned 8 mm from the antennal surface. In the interstimulus interval (usually 5 min), the antenna was bathed by an opposed, humidified pure air stream (Ultra Zero grade; Matheson, Gas Products, East Rutherford, New Jersey), at a flow rate of 120 ml/min. Each stimulus was presented in duplicate to each olfactory receptor neuron. The order of stimulus presentation proceeded from cartridges containing only single compounds through to those containing all seven compounds. All the responses reported here were obtained with a single set of odor cartridges. Control odor cartridges, which contained only 1.5 μl of mineral oil on filter paper, were interspersed with odor stimuli on a random basis to monitor for inadvertent contamination of the odor line connections. In the few cases where contamination was revealed by measurable responses to control car-

TABLE 1. COMPOSITION OF SIX-COMPONENT *Trichoplusia ni* PHEROMONE BLEND

Compound	Relative proportion[a]	Composition ratio (%)	Amount in the 0.005-μg dilution
Z7–12 : Ac	100.0	81.6	0.00408
Z5–12 : Ac	9.1	7.4	0.00037
12 : Ac	8.2	6.7	0.00034
11–12 : Ac	2.8	2.3	0.00012
Z7–14 : Ac	1.5	1.2	0.00006
Z9–14 : Ac	0.9	0.7	0.00004

[a] Average proportion (relative to Z7–12 : Ac) measured in the gland extracts of six individual females (Bjostad et al., 1984).

tridges, replacement of the relevant Teflon fittings eliminated subsequent responses to controls. In these cases, all of the response data since the preceding control cartridge presentation were discarded and the individual stimuli were repeated. Thus, there was no need to correct any of the response measures reported here for background responses associated with the mechanics of stimulus application.

Data Analysis. The response measure reported here was derived by subtracting the number of action potentials produced by a particular receptor neuron during the 10 sec immediately preceding the 2-sec stimulus pulse from the number of action potentials which occurred in the succeeding 10 sec. The duplicate responses obtained with each odor cartridge were averaged to provide the data set for individual neurons. Because of the large number of possible stimulus combinations among the seven relevant compounds, the range of effective stimulus concentrations likely to be appropriate for each, and the range of potentially interesting composition ratios among the constituents of a particular mixture, the number of individual stimuli evaluated in a single recording session (2 hr) was necessarily only a small fraction of the available stimulus set. In each case, the responses to multicomponent stimuli were compared to the algebraic sum of the responses elicited in the same receptor neurons by the appropriate intensities of their single components. The resulting pairs of expected and observed values were compared statistically with the Wilcoxon matched-pairs signed-ranks test or with the sign test (Siegel, 1956).

RESULTS

Response Characteristics. Action potentials were recorded extracellularly from sensilla (HS) on the male CL antenna which contains receptor neurons that are highly sensitive to the major pheromone component, Z7-12:Ac. The neurophysiological and morphological characteristics of this class of sensilla have been described and contrasted with the properties of the other class of pheromone-sensitive sensilla on the antenna (O'Connell et al., 1983; Grant and O'Connell, 1985). Each HS sensillum is innervated by two spontaneously active receptor neurons that produce action potentials which can be reliably differentiated from each other by their amplitudes and waveforms. By convention, the receptor neuron producing the larger amplitude action potential is designated the A neuron and that producing the smaller is designated the B neuron. The mean number of spontaneous action potentials ($\pm$ SEM) in this sample of 26 individual sensilla was 0.28 $\pm$ 0.05 impulses/sec for the A receptor neurons and 0.33 $\pm$ 0.06 impulses/sec for the B receptor neurons. In addition to this typical range of spontaneous activity, individual HS sensilla were also characterized by their length, shape, and responsiveness to low doses (0.0005–0.005 μg) of Z7-12:Ac and Z7-12:OH.

Responses to Individual Components. Five of the seven behaviorally relevant compounds have been evaulated as individual stimuli. Their intensities were adjusted to approximate those measured in female pheromone glands (Table 1), except for Z7–12:OH which is not produced by the female and thus was evaluated at an intensity equal to that used for Z7–12:Ac. As demonstrated in previous studies (Grant and O'Connell, 1985; O'Connell, 1985a–c; O'Connell et al., 1983) and illustrated here in Figure 1 (taken from O'Connell, 1985c), the average A receptor neuron in HS sensilla is reliably responsive to low doses (0.005 μg) of Z7–12:Ac whereas the average B receptor neuron is responsive to comparable amounts of Z7–12:OH. Neither receptor neuron is particularly responsive to 12:Ac, even when larger intensities are evaluated (Figure 1). Similarly, we show in this sample of olfactory receptor neurons that neither Z5–12:Ac (0.0005 μg) nor Z7–14:Ac (0.00005 μg) alone, elicited consistent amounts of electrical activity in either receptor neuron (Figure 2).

Responses to Mixtures. The average magnitude of the electrical response obtained from individual receptor neurons to stimulation with the various mixtures is displayed in Figure 2. The response obtained in each receptor neuron to stimulation with a particular mixture was compared to the response expected if the responses to individual components were additive. The observed and expected response values for each receptor neuron were then compared to each other with the Wilcoxon matched-pairs signed-ranks test. In general, the direction of the statistically significant trends observed in the responses elicited by

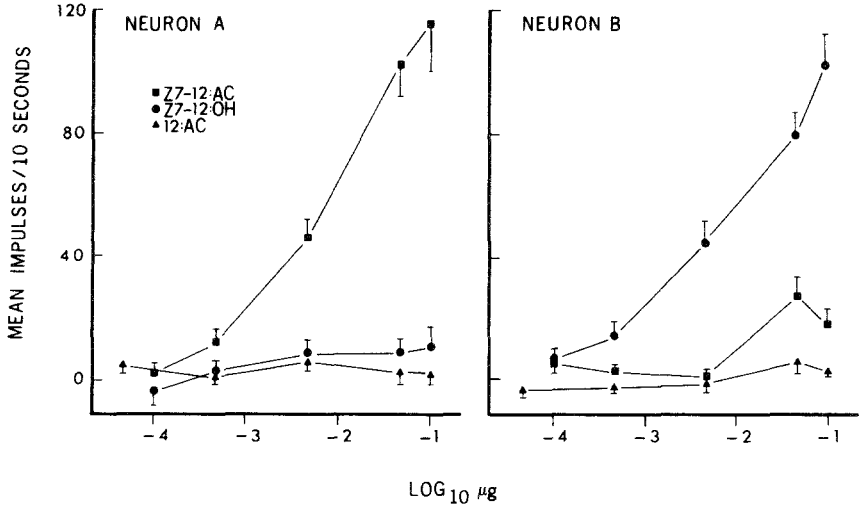

FIG. 1. The average (mean ± SEM) dose–response functions for both A (left) and B (right) receptor neurons ($N = 35$) in response to stimulation with graded doses of each of three behaviorally relevant compounds (from O'Connell, 1985c).

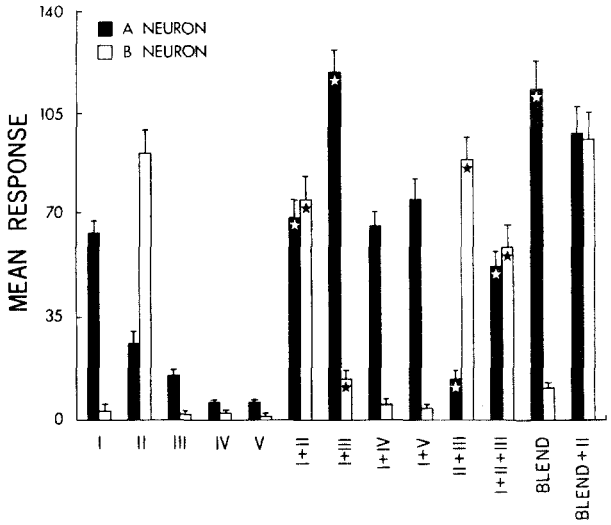

FIG. 2. The average (mean ± SEM) response (in impulses/10 sec) to the indicated single- and multiple-component stimuli. In all cases, a minimum of 12 pairs of A and B receptor neurons contributed to each mean (range 12–26). The compounds and their doses are: I = Z7-12:Ac (0.005 μg); II = Z7-12:OH (0.005 μg); III = 12:Ac (0.0005 μg); IV = Z5-12:Ac (0.0005 μg); V = Z7-14:Ac (0.00005 μg); blend = all of the compounds listed in Table 1. Statistically significant differences ($P < 0.02$, two-tailed, Wilcoxon matched-pairs signed-ranks test) between the observed and expected responses to multicomponent stimuli are starred. Cartridges containing mineral oil were on average ineffective stimuli for both A (1 ± 1 impulses/10 sec) and B (2 ± 1) receptor neurons.

mixtures was similar in both the average A and B receptor neuron. Of the five binary mixtures evaluated in this sample of HS sensilla, three elicited responses which were significantly different from the expectations of the additive model (starred bars in Figure 2). We previously demonstrated in another sample of 47 sensilla that 12:Ac has a significant synergistic effect on the magnitude of the responses elicited by Z7-12:Ac in both the A and B receptor neuron of the average HS sensillum (O'Connell, 1985b,c). We show in this sample of sensilla, similar levels of synergy and note that 12:Ac seems unique in this regard because the other two minor pheromone components examined (Z5-12:Ac and Z7-14:Ac) did not significantly alter the responses elicited in either receptor neuron when they were individually combined with Z7-12:Ac. The responses elicited in both receptor neurons, by a blend containing all six of the identified female-produced compounds, were, on average, indistinguishable from those elicited by the binary mixture containing only Z7-12:Ac and 12:Ac. In addition, the average magnitude of the A receptor neuron discharge elicited by this blend is significantly larger than would be expected from the additive model.

The responses elicited by mixtures which contain Z7–12 : OH are, on average, reliably smaller than those predicted by summing the responses obtained with the individual components of the mixture. The three-component blend (I + II + III, see Figure 2 legend) elicited responses which were, on average, smaller than those obtained with the most effective single component. The one exception observed here involved the mixture containing all seven behaviorally active compounds. In this case both receptor neurons produced responses to this complex blend which were, on average, equal to those expected from the additive model (Figure 2). The alterations in average discharge magnitude evoked in the receptor neurons by addition of Z7–12 : OH to stimuli containing various components of the pheromone blend were examined in more detail by computing a series of average response histograms. These histograms were obtained by averaging the response histograms obtained from 12 individual sensilla, each stimulated with the indicated compounds. They display the average number of impulses which occurred in successive 100-msec bins for the 5 sec immediately following stimulus onset and provide a convenient summary of the average pattern of discharge evoked by a particular stimulus.

On the left of Figure 3 we show the average responses evoked by several single and multicomponent stimuli in this sample of twelve A receptor neurons. On the right are the responses obtained in the same set of neurons for these mixtures, now with the inclusion of 0.005 μg of Z7–12 : OH. Although these response histograms are averages of the activity elicited in these 12 neurons by the indicated components, it is clear that they retain characteristic differences in both the amount of neural activity evoked and its temporal distribution from one stimulus to another. In part, these observations are in agreement with those of Grant and his colleagues who showed that individual receptor cells tend to have unique temporal patterns of discharge. Some receptor neurons respond to stimulation with a fixed dose of a selected pheromone component by producing a reasonably steady tonic level of discharge which may persist for the duration of the stimulus exposure. Others will respond to the same stimulus with an initial phasic burst of activity followed by a more prolonged tonic level of discharge even in situations where it is known that rectangular pulses of pheromone are being provided (Grant et al., 1985).

The histograms of Figure 3 also reinforce our earlier observation that the addition of 10% 12 : Ac to Z7–12 : Ac (Figure 3B) greatly enhances the amount of neural activity elicited in A receptor neurons especially when compared to the amount of activity ascribable to the presence of Z7–12 : Ac alone (Figure 3A). This increase is even more impressive in light of the relatively narrow tuning of A receptor neurons previously demonstrated in this species and the general failure of 12 : Ac to be an effective single stimulus for the two receptor neurons in HS sensilla (O'Connell et al., 1983). This ability to synergize the responses elicited in A receptor neurons is unique to 12 : Ac since the addition

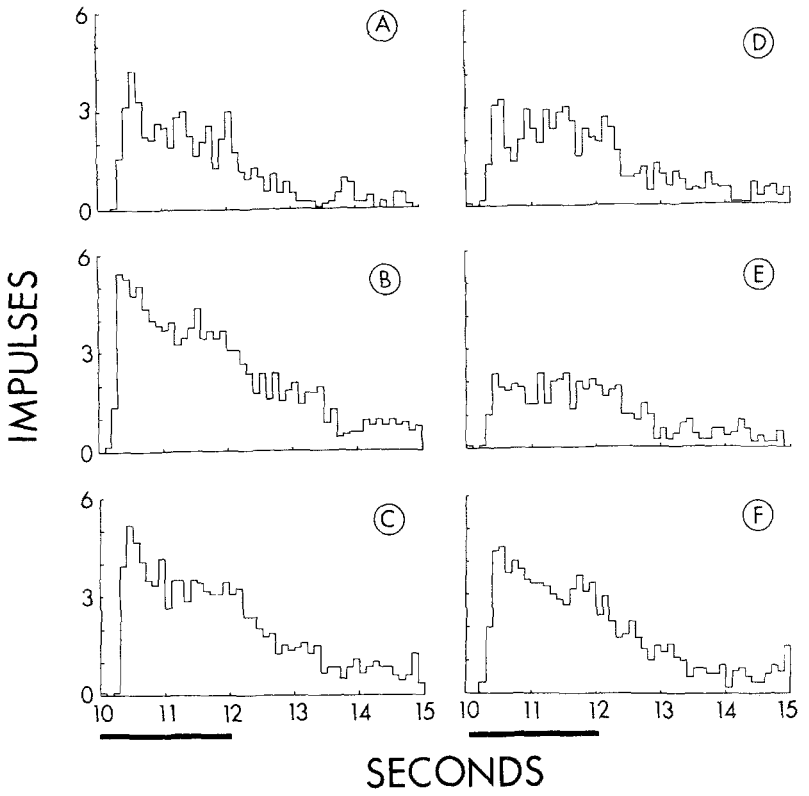

FIG. 3. Response histograms averaged across 12 individual A receptor neurons. The 2-sec stimulus period is indicated by the bars at the bottom. The stimuli evaluated include: (A) Z7-12:Ac; (B) Z7-12:Ac + 12:Ac; (C) blend (in Table 1); (D) Z7-12:Ac + Z7-12:OH; (E) Z7-12:Ac + 12:Ac + Z7-12:OH; (F) blend + Z7-12:OH.

of the remaining four female-produced compounds to the blend (Figure 3C) did not elicit an additional increase in the amount of discharge obtained when compared to that evoked by stimulation with the binary mixture (Figure 3B). However, these additional materials are not without impact on neural activity because they clearly prevent the significant reduction in discharge expected when Z7-12:OH is added to the total blend (Figure 3F). That is, the addition of Z7-12:OH to the binary mixture containing Z7-12:Ac and 12:Ac normally results in a net reduction in the response evoked by the trinary mixture (Figure 3B and E). However, the response elicited by stimulation with the total blend plus Z7-12:OH (Figure 3F) remains equivalent to that obtained with the total blend alone (Figure 3C).

The effects of these alterations can be more easily appreciated by subtract-

ing the appropriate average response histograms from each other to produce difference histograms as shown in Figure 4. Again we see that there are two significant interactions, one involving the increase elicited by the addition of 12:Ac to Z7–12:Ac (Figure 4B) and a second involving the large reduction elicited by the addition of Z7–12:OH to this binary mixture (Figure 4E). In both cases the total amount of neural discharge and its temporal pattern have been substantially altered. There is also a tendency for the alterations observed to be larger in the earlier portions of the neural discharge. It is also clear that the newly identified female-produced pheromone components do not add substantially to the response obtained with the binary mixture (Figure 4C), yet they do prevent the reduction in response expected subsequent to the addition of Z7–12:OH to the female blend (Figure 4F).

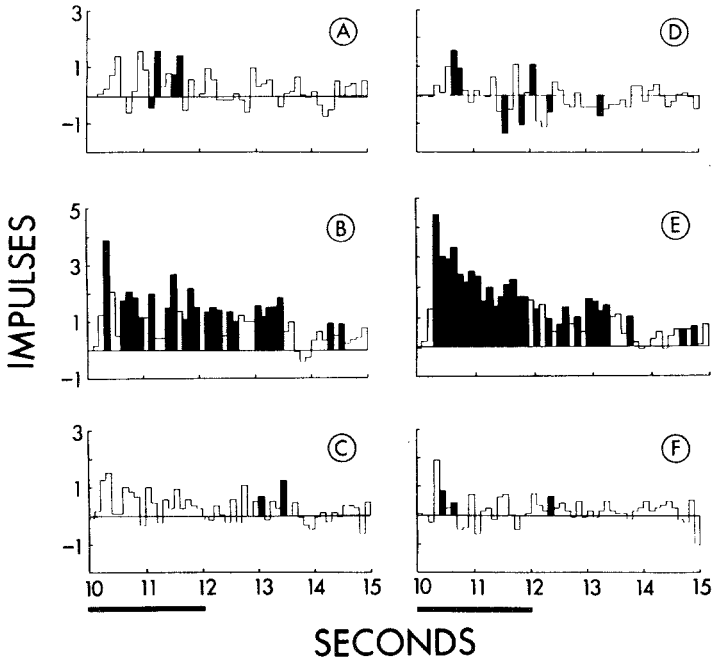

FIG. 4. Difference histograms which plot in 100-msec bins the number of action potentials remaining after two average response histograms (shown in Figure 3) have been subtracted from each other. Each of the bins with statistically significant difference scores ($P < 0.05$, one-tailed, sign test) are indicated in black. The response histograms subtracted are: (A) Z7–12:Ac + Z7–12:OH − (Z7–12:Ac + Z7–12:OH + 12:Ac); (B) Z7–12:Ac + 12:Ac − (Z7–12:Ac); (C) Z7–12:Ac + 12:Ac − (blend); (D) Z7–12:Ac − (Z7–12:Ac + Z7–12:OH); (E) Z7–12:Ac + 12:Ac − (Z7–12:Ac + 12:Ac + Z7–12:OH); (F) blend − (blend + Z7–12:OH).

DISCUSSION

Given the high level of specificity encountered historically in individual insect pheromone-receptor neuron studies, most researchers seem to assume that an *N* component pheromone blend is processed by *N* different specialized receptor neurons. Although the interactive aspects of blend perception have not often been considered in detail, multicomponent stimuli, when they have been used, usually fail to evoke unique responses in single receptor neurons beyond those which can be accounted for from a knowledge of their responses to individual components of a blend. A potential contributing factor to this failure to observe interactions among multicomponent stimuli may be the natural tendency to examine only those single-pheromone compounds which are clearly responsible for a well-defined component of male sexual behavior, are known to produce a large summated neural discharge (EAG), or are processed by specialized olfactory receptor neurons. Nearly every species so far examined produces a large number of pheromone-like compounds whose collective behavioral and electrophysiological properties are not explored in the context of a multicomponent pheromone blend because they individually fail to elicit specific behavioral responses or discriminable units of electrical activity from peripheral olfactory receptor neurons.

The four pheromone components, recently described in the female cabbage looper gland, provide a good object lesson which demonstrates the rapid increases in the chemical, behavioral, and electrophysiological complexity of pheromone communication systems. As individual stimuli, these compounds fail to elicit any of the normal components of male sexual behavior (Linn et al., 1984). They are also completely inactive when mixed together at intensities comparable to their abundance in extracts of the female pheromone gland. However, addition of the originally identified pheromone component Z7–12 : Ac to these compounds results in a five-part blend which is fully competitive with calling virgin females. Addition of the sixth female-produced compound 12 : Ac, does not elicit further significant increases in the amount of male behavior observed. If individual single components are removed from the total blend, only Z7–12 : Ac can be associated with a specific component of male behavior. As the complexity of the blend is further reduced, decrements in male behavior begin to occur in a graded fashion. The close-to-source behaviors are more affected than are those further downwind. However, it is still not possible to ascribe the absence or reduction of a particular behavioral sequence with the removal of a specific compound (Linn et al., 1984).

It is possible that the behavioral results obtained by Linn and his colleagues (1984) in the CL may represent an isolated case in which the pheromone communication system is unusually complex. However, the ever-growing list of compounds identified in individual female pheromone glands suggests that their

results may be the norm (Baker and Cardé, 1979; Baker et al., 1976; Bjostad and Roelofs, 1983). Although we have only begun to evaluate the physiological responses elicited in one of the two subsets of pheromone-sensitive sensilla by stimulation with only a few of the possible blends among the seven behaviorally active compounds, six of which are female-produced pheromone components, it is clear that our conceptualization of the neural basis for pheromone discrimination must be greatly expanded. Mixture interactions of the sort described here, at the level of primary olfactory receptor neurons, provides appropriate grist for a system in which more complex coding mechanisms may occur.

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SCALE ESTERASE: A Pheromone-Degrading Enzyme from Scales of Silk Moth *Antheraea polyphemus*

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Abstract—Body scales of the silk moth *Antheraea polyphemus* contain an esterase which can degrade the female sex pheromone of this species. This esterase, which appears to be stabilized to the scale cuticle, is present in both sexes, but is species specific. The enzyme may play a significant role in the behaviors associated with sex-pheromone attraction, helping to filter out stimulus noise by degrading adsorbed pheromone, thus preventing adsoptive body surfaces from becoming uncontrolled pheromone sources.

Key Words—Insect, pheromone reception, sex pheromone, molecular behavior, cuticular proteins, *Antherea polyphemus*, Lepidoptera, Saturniidae.

INTRODUCTION

During their sexual flight, male moths display rapid (< 1 sec) behavioral responses to fluctuations in the ambient pheromone concentration (Kramer, 1975; Marsh et al., 1981). This temporal sensitivity suggests the presence of processes acting to maintain an overall low level of background stimulus noise. Such a process exists within the lumen of the pheromone-sensitive sensory hairs of the silk moth *Antheraea polyphemus*, where there is a potent pheromone-degrading enzyme, the sensillar esterase (Vogt and Riddiford, 1981a,b, 1985; Vogt et al., 1985). This enzyme rapidly metabolizes residual pheromone within the sensory hair, ensuring that the neuron is stimulated predominately by new, incoming pheromone.

Lipophilic pheromone molecules adsorb to the general body surface as well

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as to the sensory hairs (Kasang, 1971; Mayer, 1975). Several studies have suggested the presence of enzymatic processes near the surfaces of moth bodies, which might degrade adsorbed pheromone, thus preventing the moth's body from becoming an uncontrolled pheromone source (Kasang, 1971; Mayer, 1975; Vogt and Riddiford, 1981b; Ferkovich, et al., 1982). However, none of these studies was successful in isolating the source of this "surface" enzymatic activity.

We now report that the scales covering the body surface of *A. polyphemus* possess a potent pheromone-degrading enzyme and that this enzyme can function to inactivate adsorbed pheromone. The process is present in both male and female *A. polyphemus*, but is species specific. This enzyme exists in such a manner that it may contribute significantly to preventing an individual male moth from becoming his own pheromone source or a source for other males, and it may function in the female in the control of pheromone release.

METHODS AND MATERIALS

Animals. Antheraea polyphemus, Actias luna, Hyalophora cecropia, Samia cynthia (all Fam. Saturniidae, subfam. Saturniinae), and *Automeris io* (Fam. Saturniidae, subfam. Hemileucinae) were purchased as pupae, stored in diapause at 4°C, and allowed to develop to adults at 25°C on a 18:6 light–dark cycle. *Manduca sexta* (Fam. Sphingidae, subfam. Sphinginae) were taken from our colony.

Scale Collection and Preparation. Scales were removed, using forceps, from appropriate body surfaces of recently emerged moths (1–3 days old). Care was taken not to remove underlying tissues. Scales were weighed and, for aqueous assays, suspended in detergent buffer (10 mM Tris HCl, pH 7.2, 0.1% Triton X-100). Inclusion of detergent was required to wet the otherwise highly hydrophobic scales. In general, amounts of scales processed per batch ranged from 0.5 to 20 mg, depending on species and surface of origin. To remove any soluble contaminants, scales were washed with several milliliters of detergent buffer by first suspending in 1 ml of buffer, then collecting scales on a 0.22-μm Millipore filter. Scales were rinsed with at least 2 ml of additional detergent buffer, and subsequently scraped from the filter and stored in detergent buffer in sealed vials. Scales were either stored frozen, or unfrozen at 0°C, as indicated.

Preparation of Substrates and Inhibitors. Substrates were either [11,12-^{3}H]-6*E*,11*Z*-hexadecadienyl acetate (58 Ci/mmol; prepared by Prestwich according to Prestwich et al., 1984), the major component of the *A. polyphemus* sex pheromone (Kochansky et al., 1975) or alpha-naphthyl acetate (alpha-NA). [^{3}H]Pheromone was stored in hexane at −70°C. When purity, as assessed by TLC, was <98%, the pheromone was repurified by flash chromatography (Still

et al., 1978). The pheromone was prepared for use by drying an aliquot of hexane solution under N_2 and redissolving in EtOH. An aqueous assay solution of 3×10^{-8} M pheromone was prepared by diluting 10 μl of EtOH stock (3×10^{-6} M pheromone) into 1 ml of detergent buffer using a vortex mixer. An aqueous assay solution of alpha-NA was prepared by diluting 100 μl of EtOH stock (10^{-1} M alpha-NA) into 10 ml of detergent buffer, using a vortex mixer.

Esterase inhibitors, 1,1,1-trifluoro-2-tetradecanone (TFT) (Hammock et al., 1982; Vogt et al., 1985) and *O*-ethyl-*S*-phenylphosphoramidothiolate (EPPAT) (Sparks and Hammock, 1980; Vogt et al., 1985) were supplied by Dr. T. Sparks in EtOH stock solutions (10^{-1} M inhibitor). Aqueous assay solutions were prepared by diluting 30 μl of EtOH stock into 5 ml of detergent buffer using a vortex mixer (6×10^{-4} M inhibitor).

Enzyme Assay: Pheromone. Pheromone metabolism by isolated scales was studied either in aqueous suspension (detergent buffer) or in air. Scale concentrations were as indicated in figure legends. For all assays, pheromone concentration was 10^{-8} M.

For aqueous assays utilizing endpoint kinetics (Figure 3), 100 μl of scale suspension (0.54 mg/ml) was incubated with 50 μl of aqueous [^{3}H]pheromone. Incubations were performed in 10 × 75-mm borosilicate tubes at 23°C on a rotary mixer. Four incubations were run together: three with scales and the fourth with buffer (control). At 1-min intervals, 50 μl of pheromone was added to each tube, and reactions were quenched after 5 min by addition of 150 μl of ethyl acetate (EA), mixing the contents of each using a vortex mixer for 10 sec. One EA aliquot (3 μl) was assayed for radioactivity, and two aliquots (3 μl) were chromatographed (Macherey-Nagel, Polygram SIL G/UV254, 4 × 8 cm, coated plastic). TLC plates were developed using 25% EA in hexane. Plates were loaded with unlabeled pheromone (6*E*,11*Z*-16:OAc) and pheromone product (6*E*,11*Z*-16:OH) and, after development, spots were made visible using iodine vapor (R_f 0.61 and 0.33, respectively). Spots were cut out and associated radioactivity was measured in Aquasol in a Beckman LS230 scintillation counter. Controls routinely showed $< 1\%$ radioactivity at product R_f. Velocities were determined by multiplying the alcohol/(alcohol + acetate) ratios (from TLC) by the substrate concentration. To study the effect of boiling, scales were boiled 30 min, then stored on ice for 24 hr prior to the assay.

For the aqueous assays examining pheromone degradation over time (Figure 1), the above assay was modified such that each of three borosilicate tubes initially contained 1.5 ml of scale preparation in detergent buffer and one contained detergent buffer alone (control). At $t = 0$, 0.75 ml pheromone was added. At 5, 13, 21, 31, 38, and 46 min respectively, 100 μl from each tube was transferred to 200 μl of EA and mixing was performed using a vortex mixer. One 3-μl aliquot of EA was assayed for total radioactivity and two aliquots were chromatographed as described above. At 13 min, the reaction was slowed by transferring 1.5 ml of incubation mixture to a microfuge tube and centrifug-

ing 3 min. The relatively scale-free supernatant was assayed at 21 and 31 min, respectively. At 31 min, the scale pellet amd supernatant were remixed using a vortex mixer, and the reaction was continued at full strength in the microfuge tube. The volume of the pellet was approximately 10% of the total incubation volume, such that the pellet could account for the majority of degradation which occurred during this separation period.

To study degradation of pheromone adsorbed onto wing scales from air (Table 1), scales were packed into cartridges made from the protective tips of 1 ml disposable syringes, with a pin hole at the end of each tip. A filter paper odor source containing approximately 10^{-8} g of pheromone (4 μCi) was inserted into a 1-ml syringe connected to bottled air. Cartridges containing scales and empty control cartridges were sequentially attached to the syringe for 5 min, with airflow maintained at 300 ml/min. Evaporated pheromone passing through the cartridge could adsorb either onto the scales or the walls of the cartridge. At the end of pheromone application, each cartridge was washed with 50 μl of toluene, and this wash was analyzed via TLC as described above.

Enzyme Assay: alpha-NA. Degradation of alpha-NA (Figure 2) was monitored spectrophotometrically (Varian Superscan II) at 321.5 nm (product, alpha-naphthol). Two-hundred microliters of scale suspension (0.54 mg scales) were preincubated (1 hr) in a microfuge tube (1.5 ml) with 1.2 ml of inhibitor (TFT or EPPAT, 10^{-4} M) in detergent buffer or with detergent buffer alone. The suspension was centrifuged (Beckman microfuge), and 1 ml of supernatant removed. At $t = 0$, 1 ml of alpha-NA (containing inhibitor as indicated) was added, and the contents were mixed using a vortex mixer. The final scale concentrations were 0.38 mg/ml, the initial alpha-NA concentrations were 7×10^{-4} M, and the total incubation volume was 1.4 ml. Incubations were done at 23°C on a rotary mixer. At indicated times, tubes were microfuged 2 min, and 1 ml of supernatant was transferred to a quartz cuvette and the amount of product present was measured. After measurement, the supernatant was transferred back to the microfuge tube, and scales were resuspended by vortexing. The time between initiation of a centrifugation and resuspension of scales was 12 min. At 60 min, the reaction mixture was microfuged for 4 min, the supernatant was removed and assayed, and held isolated from scales through the 90-min reading. To study the effect of freezing, scales were frozen on Dry Ice and thawed just prior to the experiment. To study the effect of boiling, scales were boiled 30 min, just prior to the experiment.

Gel Electrophoresis. Scales (1.84 mg) were washed as described above, and homogenized (BOLAB ground glass homogenizer, 0°C) in 400 μl detergent buffer. Branches of six male antennae (10.3 mg) were homogenized (200 μl). Pieces of male forewing (33.02 mg) were homogenized (400 μl). All homogenates were microfuged for 2 min, and 30-μl aliquots of the supernatants as well as of the first 1 ml of scale wash were subjected to electrophoresis on a

nondenaturing polyacrylamide slab gel (Figure 4) (0.7 mm thick, 5% stacking, 10% running, 4°C, 20 mA constant current) (Vogt and Riddiford, 1981a). After electrophoresis, the gel was reacted with alpha-NA and beta-NA in the presence of fast blue RR (Shaw and Prasad, 1970; Vogt and Riddiford, 1981a) to reveal esterase bands.

Histochemistry. Unfixed, frozen sections (16 μm) of male wings were dried (1 hr, 50°C) and reacted with the same enzyme stain used to identify esterase bands from electrophoresis. Sections were preincubated for 10 min in phosphate buffer (0.1 M, pH 7.2) or in the presence of esterase inhibitor (TFT, 2×10^{-4} M phosphate buffer). Sections were then reacted (1 hr) with the same esterase stain described above for electrophoresis, in the absence (Figure 5A) or presence (Figure 5B) of TFT (2×10^{-4} M). After staining, sections were transferred to a drop of distilled water on a microscope slide, allowed to air dry, and mounted in glycerol–gelatin. Sections were photographed under identical exposure settings on a Leitz photomicroscope using tungsten-balanced Ektachrome slide film. Figures (Figure 5) were taken from black-and-white negatives made from these slides.

RESULTS

Degradation of Air-Adsorbed Pheromone. When pheromone was blown through a cartridge of *A. polyphemus* scales (male, dorsal forewing), about 25% of the adsorbed pheromone was metabolized to the alcohol in 5 min (Table 1). In both the control cartridges and those containing scales, the residual amount of undegraded pheromone was similar (Table 1), suggesting that most of the undegraded pheromone was adsorbed to the walls of the cartridge, the airflow

TABLE 1. DEGRADATION OF AIR-ADSORBED PHEROMONE BY WING SCALES[a]

Experimental conditions	Weight scales (mg)	Total dpm recovered	% converted to alcohol
No scales	0	3956	0
	0	3190	0
	0	3700	0
Scales	0.67	5232	22
	0.81	4249	24
	0.84	5742	31

[a] Cartridges containing either scales or no scales were exposed to an airstream blown over a filter paper source containing 4 μCi of pheromone (approx. 10^{-8} mol) for 5 min, then a toluene wash of the cartridge was analyzed by TLC.

having channeled between the scale plug and the cartridge wall. Therefore, the rate of metabolism of pheromone adsorbed to the scales was probably much higher.

Degradation of Solubilized Pheromone. When *A. polyphemus* scales (male, dorsal fore-wing) were suspended in detergent buffer (0.1% Triton X-100), they were able to degrade both pheromone (Figure 1) and alpha-NA (Figure 2) to their corresponding alcohols (Vogt et al., 1985). Initial studies utilizing alpha-NA as well as beta-NA as substrates demonstrated a substrate preference for alpha-NA. Incubation of scales (0.38 mg/ml) with 10^{-4} M beta-NA (upper solubility limit) showed no degradation over 1 hr. Under otherwise identical conditions, incubations with alpha-NA at concentrations exceeding 5×10^{-5} M were required to observe degradation over 1 hr. Therefore, the highest obtainable concentration of 7×10^{-4} M alpha-NA was chosen for these assays.

This degradative process occurred within an insoluble fraction and was strongly associated with the scales, as there was a substantial reduction in activity when scales were removed from the substrate by centrifugation (Figures 1 and 2). Degradative activity was unaffected by a single freezing and thawing

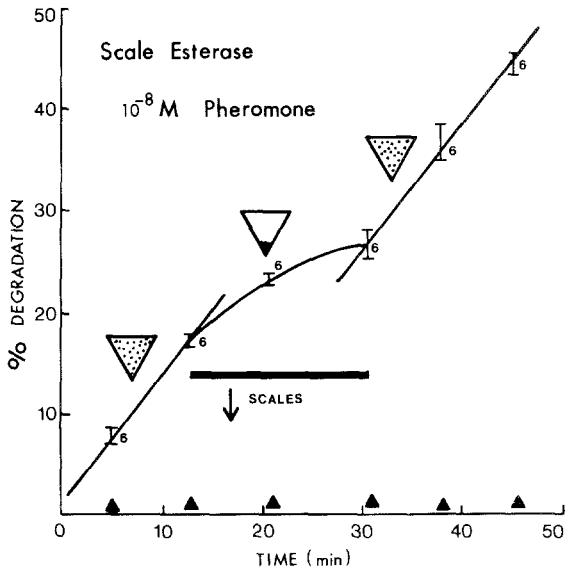

FIG. 1. Degradation of pheromone (10^{-8} M) by scales (0.9 mg/ml) of the dorsal fore-wing of male *A. polyphemus* (percentage degradation as alcohol/alcohol + acetate). At 13 min, scales were centrifuged to pellets in the bottoms of respective incubation vessels and remixed with supernatant at 31 min. The large open triangles indicate the state of dispersion of scales in the microfuge tubes during the three phases of the experiment. The small filled triangles represent control levels of radioactivity at the alcohol R_f. Data bars indicate range of data values at each time point. Each data bar represents six assays.

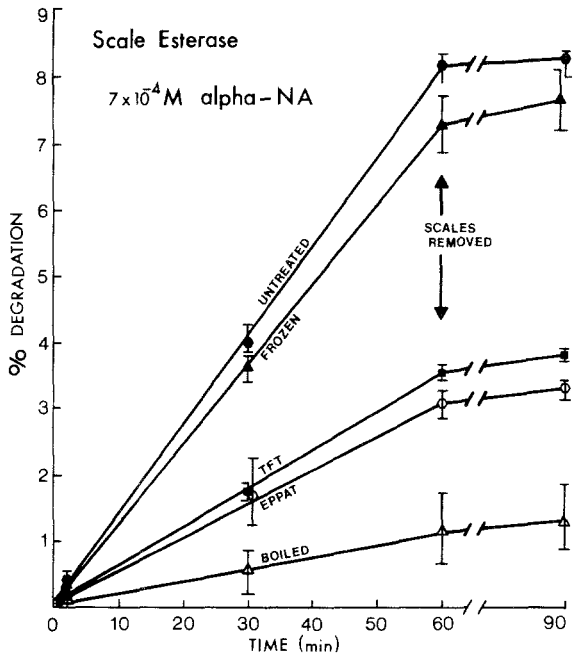

FIG. 2. Degradation of alpha-NA (7×10^{-4} M) by scales (0.36 mg/ml) of dorsal hindwing of male *A. polyphemus*. Untreated: scales were fresh, receiving no pretreatment. Frozen: scales were frozen at −20°C and thawed at room temperature prior to the assay. TFT and EPPAT: scales were preincubated in these inhibitors (10^{-4} M) for 1 hr before the assay. Boiled: scales were boiled 30 min prior to the assay. In all assays, after 60 min incubation, scales were removed from substrate by centrifugation, and the supernatant was then assayed at 90 min. The data time points are not corrected for the time required (12 min) for each set of spectrophotometric readings. Data bars indicate spread of data values at each time point. Each data bar represents the range of three assays.

(Figure 2), but was reduced by two known esterase inhibitors, TFT and EPPAT (Figure 2). Activity was also reduced by boiling for 30 min, although residual activity persisted through this treatment (Figure 2), returning to about 50% after 24 hr at 0°C (Figure 3).

Source Specificity. Degradative activity was present in scales collected from a variety of body surfaces of *A. polyphemus* (Figure 3), with the long scales of the dorsal thorax having the most activity and those of the ventral abdomen having the least. Activity was not sex specific in *A. polyphemus* (Figure 3), but did show a distinctly species-specific pattern (Figure 3). No observable esterase activity was detected from the scales of *Hyalophora cecropia*, *Actias luna*, or *Manduca sexta*, although low levels of activity (approximately 10% that of *A. polyphemus*) were detected from the scales of *Samia cynthia* and *Automeris io* (Figure 3).

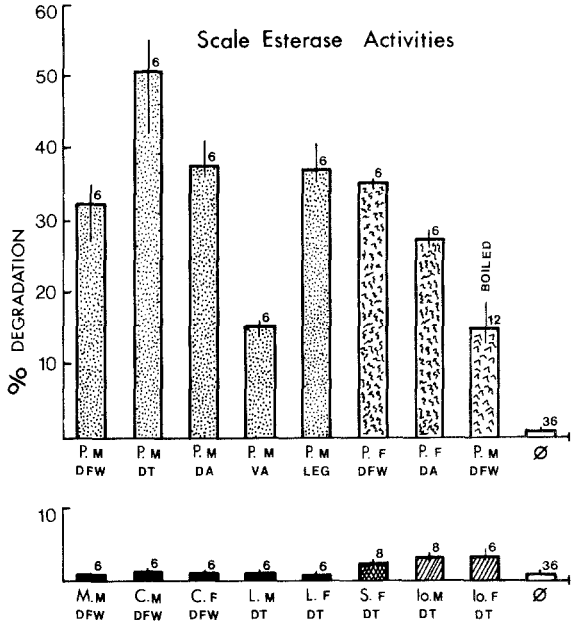

FIG. 3. Comparison of pheromone (10^{-8} M) degradation by scales (0.36 mg/ml) collected from various sources [percentage degradation over 5 min as alcohol/(alcohol + acetate)]. Species sources: P = *A. polyphemus*; M = *Manduca sexta*; C = *Hyalophora cecropia*; L = *Actius luna*; S = *Samia cynthia*; Io = *Automeris io*; "Ø" = controls (no scales). Sex is indicated by M (male) or F (female). Scale sources: DFW = dorsal forewing; DT = dorsal thorax; DA = dorsal abdomen; VA = ventral abdomen; LEG = leg. Data bars indicate range of data with accompanying numbers indicating the number of assays per data bar. The histograms represent the mean of the data. BOILED scales were previously frozen, were boiled 30 min, then stored unfrozen at 0°C for 24 hr prior to assay.

Histochemical Studies. Vogt and Riddiford (1981a) had previously reported an integumental esterase, suggesting that it might be present to degrade pheromone adsorbing to the body surface. This integumental esterase was abundant in all cuticularized tissues, including antennae and wings (Figure 4) (Vogt and Riddiford, 1981a). It was the major soluble esterase present in wings (Figure 4), degrading beta-NA and alpha-NA with comparable vigor (Vogt and Riddiford, 1981a; Vogt, unpublished). No soluble esterases from scale washes or scale homogenates were detected by gel electrophoresis (Figure 4), suggesting that the integumental esterase was not responsible for the scale-associated pheromone metabolism.

In an attempt to localize this soluble integumental esterase within the wing tissue, we reacted frozen sections of male adult wing (Figure 5) with the same

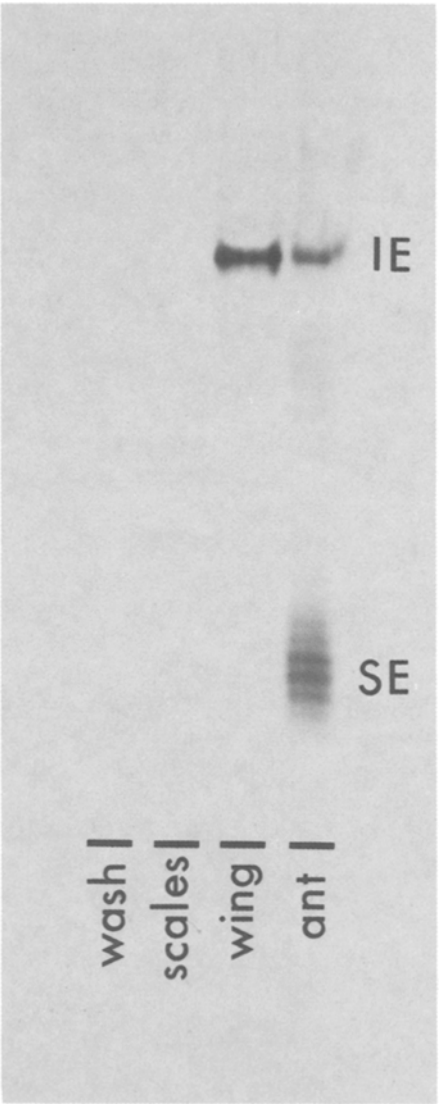

FIG. 4. Electrophoretic gel of soluble esterases released from homogenates of antennal branches (ant), forewings (wing), scales of dorsal forewing (scales), and an initial wash of these scales, all from male *A. poplyphemus*. Tissues were homogenized in detergent buffer at 0°C, and then microfuged to remove particulate matter. IE indicates integumental esterase, and SE indicates sensillar esterase (Vogt and Riddiford, 1981a). Tissue amounts per lane were: ant, 1.54 mg; wing, 2.47 mg; scales, 0.138 mg; wash, 30 μl of a 1-ml detergent wash of 1.84 mg scales. No soluble esterase activity was detectable in the scale and wash lanes.

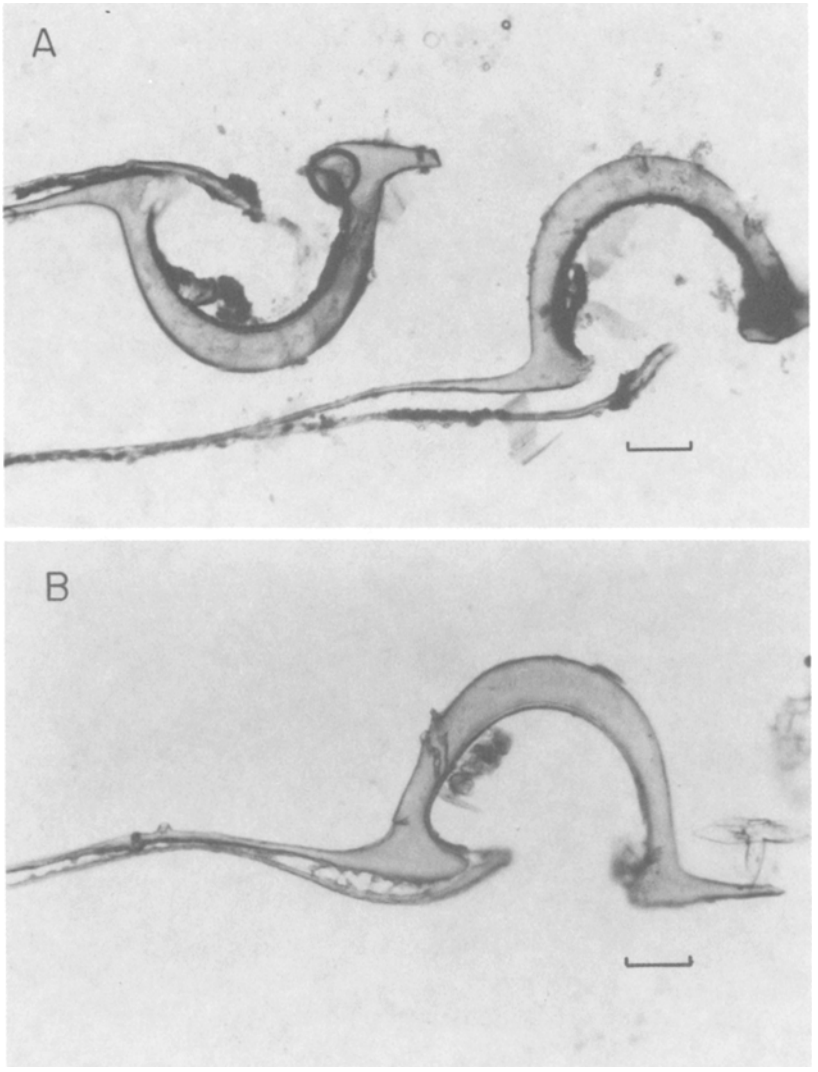

FIG. 5. Frozen sections of forewings of male *A. polyphemus*, through a wing vein. In these sections, the thin cuticle on the dorsal side of the wing vein appears broken. Figures 5A and 5B were both stained with the same esterase stain used to stain the gel in Figure 4. The section in Figure 5B was preincubated with the esterase inhibitor TFT (2×10^{-4} M, 10 min) prior to coincubation with substrate plus TFT. Esterase activity appeared as a noticeable darkening along the inside surface of the cuticle, the site of the wing epithelial cells. The size bars indicate 50 μm.

esterase stain used to stain gels (Figure 4). The stain reaction was carried out in the presence and absence of the esterase inhibitor TFT. These studies showed the presence of strong esterase activity associated with the wing epidermis (Figure 5). No definitive staining could be observed in the scales, although the pigmentation of the scales made such staining difficult to observe.

DISCUSSION

The data presented above clearly demonstrate that there is an esterase associated with the scales of *A. polyphemus* that degrades the major component of the female sex pheromone. This esterase is sensitive to specific inhibitors and to boiling, and it demonstrates substrate specificity. These findings support our earlier studies which showed that pheromone applied to the unbroken wing surface was rapidly degraded (Vogt and Riddiford, 1981b).

Previous studies have shown that homogenates or sonicates of general body parts of moths could degrade pheromone (Kasang, 1971; Mayer, 1975; Ferkovich et al., 1982). Kasang (1971) suggested that this degradation was due to surface enzymes acting to degrade adsorbed pheromone, thus preventing the animal from acting as a false pheromone source. Ferkovich et al. (1982) went so far as to suggest that enzymatic activity associated with the legs of the cabbage looper moth, *Trichoplusia ni*, might act as an antennal cleaning mechanism, where leg-associated esterases would degrade residual pheromone combed from the antenna by the legs. None of these studies, however, demonstrated the site of observed enzymatic activity.

In order for enzyme to have access to adsorbed pheromone, the enzyme must be located near the adsorptive surface of the animal. The major adsorptive surface is undoubtedly the scales, which cover the entire animal similar to shingles on a house. Considering the tremendous surface area provided by the scales, any pheromone drifting near the body surface is far more likely to adsorb onto a scale than onto the cuticle of the integument (Figure 6) (Futrelle, 1984). Our finding of a pheromone-degrading enzyme associated with the body scales of *A. polyphemus* satisfies this requirement.

This scale esterase appears to be cross-linked to the scale cuticle, presumably having been secreted with the other cuticle proteins at the time of scale formation (Greenstein, 1971). There are apparently no cellular structures present in adult scales (Greenstein, 1971). The esterase was quite stable in detergent buffer (Figures 1–3), with no activity being liberated after homogenization in Triton X-100 (Figure 4). Although activity was severely reduced by prolonged boiling (Figure 2), much of the activity returned after 24 hr at 0°C (Figure 3), suggesting that the enzyme's association with other cuticle elements may have helped to stabilize and protect it.

This insoluble scale esterase is clearly different from the soluble integumental esterase that we previously obtained from various *A. polyphemus* tissues (Vogt and Riddiford, 1981a). The histochemical studies show that the soluble esterase is restricted to the wing epidermis. This latter esterase is likely a more general esterase associated with cytoplasmic functions, and only happens to degrade the pheromone. Of course, it could also degrade any pheromone which happened to penetrate through the cuticle.

Does this scale esterase truly have a role in pheromone processing prior to mating? Certainly the esterase shows a strong substrate preference in favor of pheromone over alpha-NA. Wing scales degraded greater than 30% of 10^{-8} M pheromone in 5 min (Figure 3), while requiring in excess of 5×10^{-5} M alpha-NA to generate any observable degradation. Technically, alpha-NA degradation is easily monitored at concentrations well below 10^{-5} M (Vogt et al., 1985). The species specificity of the esterase also argues in favor of its role in phero-

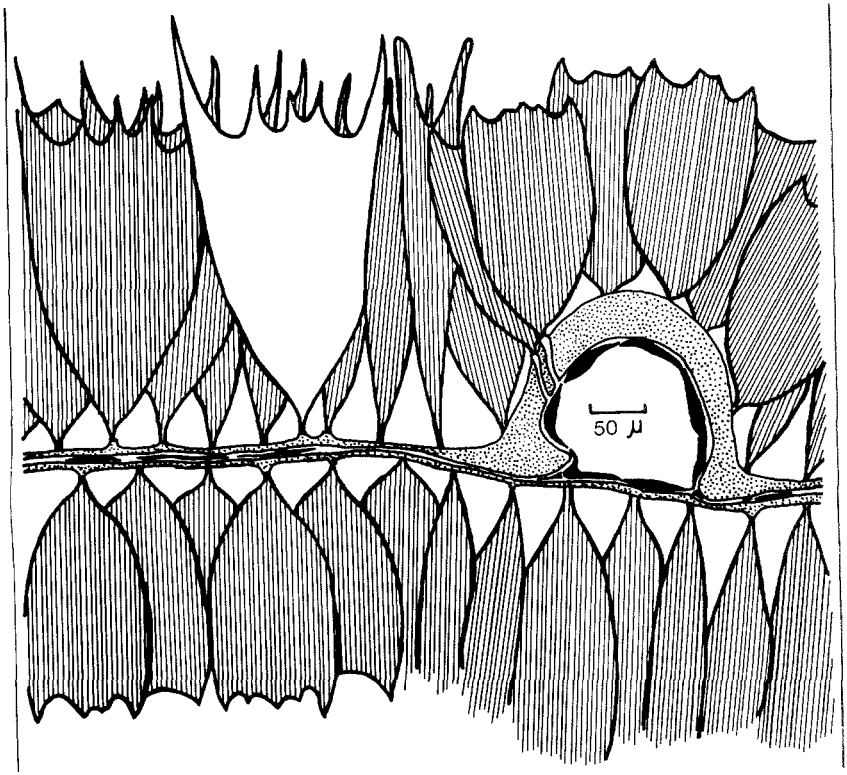

FIG. 6. Schematic diagram of a wing of *A. polyphemus*, showing the density and size relationships of scales to wing cuticle. Scales normally lie flat, overlapping each other such that there are two layers of scale covering the integumental cuticle.

mone processing. Esterase activity was either extremely low or completely absent in scales of *A. luna*, *H. cecropia*, and *S. cynthia*, all closely related species of the same subfamily as *A. polyphemus*, as well as *A. io* and *M. sexta*, somewhat more distantly related species. This pattern of species specificity suggests that the esterase does not have some general role in scale cuticle formation or pigmentation.

However, even if the esterase does have a function other than degrading pheromone, we have shown that if pheromone adsorbs to esterase-containing scales, it is degraded. Clearly then, the esterase can function in reducing background noise. How significant is this contribution in noise reduction to the precopulatory behaviors of these animals? If one considers the importance of the precopulatory sex-attractant behaviors of both males and females in terms of the selection of gene form, even the slightest contribution by the scale esterase could be quite significant. Certainly, in the present study the scale esterase was examined at a pheromone concentration (10^{-8} M) that was well within the physiological range of pheromone-sensitive sensory hair function, and nearly four orders of magnitude above the physiological threshold of these same sensory hairs (Vogt et al., 1985).

Furthermore, realizing the differences between the male and female precopulatory behaviors, the esterase may serve the sexes differently. For the male, the enzyme could function behaviorally to prevent an individual moth from becoming his own pheromone source. To other males, the enzyme could function to ensure that the local attractant source is indeed a female. And to the female, the enzyme could function to ensure control of pheromone release, allowing only pheromone from the secreting gland to leave the female undegraded. It should be noted, however, that for different moth species, the degree of control over such parameters may differ as a function of the selective pressures placed on the evolution of the relevant behavioral strategies.

It seems possible that, in the evolutionary design of the behaviors associated with sex-pheromone attraction, a single biochemical reaction may have assumed a role in more than one aspect of this sexually selected behavior. Recalling the noise-reducing role of the sensillar esterase of the pheromone-sensitive sensory hairs (Vogt et al., 1985), our findings suggest that, in the evolutionary design of the sex-pheromone attraction/reception process, multiple biochemical reactions have become optimized to maintain an overall low signal-noise level in order to maximize the efficiency of response during pheromone-elicited flight orientation.

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METABOLISM OF PHEROMONE COMPONENTS AND ANALOGS BY CUTICULAR ENZYMES OF *Choristoneura fumiferana*

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Abstract—In vivo incubation of *Choristoneura fumiferana* with (*E*)-11-tetradecenal, the major pheromone component, resulted in adsorption followed by transformation to (*E*)-11-tetradecenoic acid, the only observed metabolite. Antennae, legs, and wings from both sexes were investigated for enzyme activity. Specificity studies with the aldehyde dehydrogenase system showed that the enzyme prefers aldehydes of 14-carbon chain length or shorter.

Key Words—*Choristoneura fumiferana*, Lepidoptera, Tortricidae, pheromone components, pheromone metabolites, aldehyde dehydrogenase.

INTRODUCTION

There have been several studies undertaken in recent years aimed at elucidating the mechanisms involved in insect pheromone perception. However, this has proven not to be a simple and straightforward task. Although substantial progress has been achieved towards this goal and a framework has been established on which future research will undoubtedly be based, there are still many aspects of these processes that must be clarified.

The sequence begins with the biosynthesis and release of volatile components from within the pheromone-producing gland (usually female). These components are normally transported by air currents to the vicinity of a recipient moth, then are adsorbed onto antennae and body tissue. The sequence culminates in the transduction process and finally degradation and deactivation of the active components occurs to complete the cycle. Some of these processes have been studied in several insect species, particularly *Antheraea polyphemus* (Vogt

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and Riddiford, 1981; Kanaujia and Kaissling, 1985), *Trichoplusia ni* (Ferkovich *et al.*, 1982) and *Choristoneura fumiferana* (Percy, 1974; Silk *et al.*, 1980; Morse *et al.*, 1982), yet our understanding of them still falls short, particularly in the area of transduction (Villet, 1978).

The eastern spruce budworm moth (*Choristoneura fumiferana*) (Lepidoptera: Tortricidae) inhabits most forests of Canada and the northeastern United States; it is one of the most economically devastating insect pests, feeding on and eventually destroying spruce and fir in these areas. The primary pheromone components were initially identified (Weatherston et al., 1971; Sanders and Weatherston, 1976) and shown to consist of a mixture of 96:4 *E/Z* 11-tetradecenal. This pheromone system was later reevaluated (Silk et al., 1980; Alford et al., 1983) and shown to contain the additional minor component, tetradecanal as 2% in the blend. As for the biosynthesis and release of these pheromone components, some aspects have been investigated by Morse and Meighen. They report the identification of biosynthetic and degradative enzymes in the gland, and the evidence suggests the presence of an acetate ester precursor to the aldehyde pheromone components (Morse and Meighen, 1984a,b).

The findings reported in this paper result from an examination of the interaction of pheromone components with the cuticular surfaces after intial contact with the recipient moth. The first event is the adsorption of pheromone molecules onto all the surface tissues. Some of the molecules adsorbed on the antennal sensilla are transported through pore openings to the interior of the hairs. A percentage of the molecules will reach the vicinity of the receptor proteins associated with the dendritic portions of the sensory neurons which innervate these sensilla hairs (Kaissling, 1974). Presumably after inducing transduction, the stimulant molecule is inactivated by complexation with binding proteins, by enzymatic degradation, or both.

The first problem to be addressed concerns the fate of the pheromone molecules that do not participate in the transduction process; that is, those molecules that do not enter the pores. If the pheromone components bound to the cuticular surfaces readily desorb, this would provide false sensory information, whereas if they accumulate and remain bound indefinitely, this could lead to system inefficiency and overload in high-density populations. The most reasonable consequence would be enzymatic degradation of the components to inactive compounds.

This study identifies the primary degradation product of the major pheromone component (*E*)-11-tetradecenal, as (*E*)-11-tetradecenoic acid. The metabolic transformation is consistent with the presence of a dehydrogenase system on all the cuticular tissues examined—antennae, legs, and wings (Morse and Meighen, 1984a). The study also indicates that the enzyme system is active in female tissues as well as in the male, but to a lesser degree. An estimate of the rate and turnover capabilities to the various tissues are examined and compared.

Experiments have also been designed to determine the metabolic selectivity of the enzyme system towards the aldehyde pheromone components and several other aldehyde analogs. In addition to the aldehyde metabolism studies, bioassays have been carried out with (*E*)-11-tetradecenol and (*E*)-11-tetradecenyl acetate (previously shown to be behaviorally and electrophysiologically active) (Sanders et al., 1972; Sanders and Weatherston, 1976; Priesner, 1979), to determine if oxidase and esterase enzyme systems are present on the cuticular surface.

METHODS AND MATERIALS

Insects. Second-instar spruce budworm larvae from Great Lakes Forestry Research Laboratory, Sault Ste. Marie, Ontario, were reared on a synthetic diet (Harvey and Gaudet, 1977). The pupae were sexed and placed in separate rearing rooms. The adults were maintained on a 17:7-hr light–dark cycle (5.1–8.4 lux at 23–25°C, relative humidity 60%) and were collected daily.

Tissue. Moths 2–3 days old were used for both the in vitro and in vivo experiments. For in vitro studies, the moths were anesthetized by freezing at −15°C for 10 min. The leg and antenna tissues were removed with forceps, while the wings were excised with a razor blade and were used for bioassay immediately after their removal. For the in vivo tests, on the other hand, upon completion of the bioassay, the moths were anesthetized by freezing, and the tissues were removed and analyzed.

Pheromone Components. (*E*)- and (*Z*)-11-Tetradecenal were prepared by the following procedure. 1,10-Decane diol was treated with HBr (50%) at 95°C, and the monobrominated product was continuously extracted from the reaction medium with heptane (Pattison et al., 1956). The 10-bromodecanol was protected with dihydropyran in the presence of *p*-toluenesulfonic acid catalyst and subsequently alkylated with butyne in THF/*n*-butyllithium at 0°C (Hendry et al., 1975). The tetrahydropyranyl protecting group was removed in aqueous acidic methanol, and the resulting 11-tetradecyne-1-ol was hydrogenated under 40 lb/in^2 of hydrogen with the catalyst palladium on barium sulfate (10%) poisoned with pyridine in methanol (Su et al., 1973). These hydrogenating conditions produced the 11-tetradecen-1-ols with a *E*/*Z* ratio of 60:40, respectively, as indicated by gas chromatography. These *Z* and *E* isomers were separated on a silver nitrate-impregnated acidic resin column by elution with methanol (Houx et al., 1974). Compounds with greater than 99.5% isomeric purity were obtained as determined by GC analysis on a SP1000 capillary column. (*E*)- and (*Z*)-tetradecenols were oxidized to the corresponding aldehydes with pyridinium chlorochromate (PCC) in methylene chloride (Corey and Suggs, 1975). Tetradecanal was prepared by the oxidation of tetradecanol (Aldrich) with PCC in methylene chloride buffered with sodium acetate.

Analogs. 13-Tetradecenal was obtained as follows. Bromododecanol was prepared from 1,12-dodecane diol using the described HBr procedure, and the alcohol moiety was protected as the pyranyl ether. The bromide was then alkylated using a lithium acetylide–ethylene diamine complex in DMSO to give 13-tetradecynol (Dear and Pattison, 1963). Then deprotection of the alcohol, as above, followed by hydrogenation with 1 atm H_2 catalyzed by palladium on carbon (10%) in methanol produced 13-tetradecenol. Finally oxidation with PCC yielded the 13-tetradecenal. Hexadecanal, pentadecanal, tridecanal, and dodecanal were all prepared from the respective alcohol precursors (Aldrich) by the PCC oxidation procedure. (*E*)-11-Hexadecanal was obtained from 1,12-dodecane diol by a procedure similar to that described for the preparation of 11-tetradecenal. (*Z*)-11-Hexadecenal was obtained from Aldrich. (*E*)-11-Tetradecenol was obtained as an intermediate in the synthesis of 11-tetradecenal as described above. (*E*)-11-Tetradecenyl acetate was prepared by acetylation of (*E*)-11-tetradecenol with acetic anhydride–pyridine.

The intermediates and final products were purified by flash column chromatography (Kieselgel 60, 230–400 mesh) (hexane–ethyl acetate gradient solvent system) (Still et al., 1978). Nuclear magnetic resonance analysis was performed on either a Varian T60 or Varian XL200 instrument. The infrared spectra were obtained from a Perkin-Elmer 598 spectrophotometer. GC analyses were carried out using a Varian 6000 gas chromatography/capillary system and the GC-MS analyses were performed with a Finnigan 4021 EI-CI system coupled with an INCOS data system.

Pheromone Component and Analog Assay Solutions. Organic stock solutions of pheromone components and analogs were prepared by dissolving 10 mg of the compound in 1000 μl of hexane. The aqueous solutions to be used for in vitro bioassays were obtained by placing 10 μl of the organic stock solution in a 3-ml glass vial, evaporating the hexane under dry nitrogen flow, adding 1000 μl of distilled water and, finally, vortexing for 5 min at room temperature. The aqueous samples were immediately utilized in the assay. The components used for the in vivo bioassay were applied in neat form to filter paper.

Buffer–Cofactor Solutions. The phosphate buffer pH 7.4 was prepared by mixing 2.9 ml of 0.5 M NaH_2PO_4 and 12.4 ml of 0.5 M Na_2HPO_4 and diluting to 100 ml. A solution of 3 mM NAD^+ was prepared by dissolving 100 mg NAD^+ in 50 ml of phosphate buffer solution. The buffer solutions were stored at 2°C.

In Vivo Assays. The incubations were performed in crystallizing dishes 150 × 75 mm. A circle of filter paper (Whatman quality No. 1) was placed at the bottom of the container, and the assay component was spotted on the paper in neat form. A wire screen was positioned 2 cm above the filter paper (to prevent direct moth contact with the applied test component), and the dish was topped

with a large watch glass. After 5 min of equilibration, the moths were quickly placed in the assay chamber and the glass lid replaced. The experiments were run from 0.25 to 8 hr, after which time the moths were anesthetized and the tissue removed and washed several times with ether. The extracting solvent was concentrated under N_2 flow to 200 μl in the cases of both the antenna and leg extracts and to 1000 μl in the case of the wing extracts. Then 50 μl of diazomethane solution (Fieser and Fieser, 1967) was added to each of the concentrated extracts to convert the free acids to GC-detectable methyl esters. Two control assays were run: one in which the assay chamber contained moths and no substrate, the other in which the chamber contained substrate and no moths. The extracts were analyzed by the GC techniques described in the analysis section.

In Vitro Assay. In the bioassays utilizing intact tissue, the antennae, prothoracic legs, and metathoracic legs from 10 moths were placed in 10 × 75-mm culture tubes. To these tubes were added solutions of NAD^+ phosphate buffer (150 μl) and the aqueous substrate (150 μl, 10 μg). A fourth tube in which the tissue was omitted was used as a control. These assays provided a substrate concentration of $\sim 6 \times 10^{-5}$ M. The culture tubes were vortexed at 10-min intervals during the 1- and 2-hr incubation periods. At the end of these periods, the assay mixture was extracted with 3 × 500 μl portions of ether, then the solvent was concentrated to 200 μl under a slow stream of dry N_2. A diazomethane solution (150 μl) was added, and the extract was analyzed by GC-MS and GC.

Bioassays of homogenized tissue were employed to compare the activity of homogenized versus intact tissue. These bioassays differed from the intact tissue bioassays only in that the prothoracic legs were not assayed. The tissue was homogenized with a ground-glass hand homogenizer (3 ml), and the incubation was performed in the homogenizing tube.

Selectivity studies were carried out to determine if the dehydrogenase enzyme system displayed substrate specificity. The pheromone components and the analogs were assayed in a 1 : 1 concentration ratio with (*E*)-11-tetradecenal. The bioassays were performed with homogenized and intact tissues from 10 pairs each of antennae and metathoracic legs from 10 moths. The duration of the assays was 1 hr in the case of intact tissue and 15 min for the homogenized tissue. The shorter bioassay period for the homogenized tissue was necessary because of its higher rate of metabolism, and it was desired that only 50% of total substrate be metabolized. At 3×10^{-5} M concentration, 85% of the (*E*)-11-tetradecenal substrate was converted to the corresponding (*E*)-11-tetradecenoic acid during the incubation periods. Therefore a 2×10^{-5} M concentration each of both the analog and (*E*)-11-tetradecenal was employed.

In vitro bioassays with (*E*)-11-tetradecenol and (*E*)-11-tetradecenyl acetate were employed to determine if oxidase or esterase enzyme systems, respec-

tively, were operating on or within the antennae and legs. Antennae and legs from 20 moths were excised for these bioassays. Both homogenized and intact tissues were incubated for 1 hr at a substrate concentration of 6×10^{-5} M.

Bioassay Analysis. All bioassays were performed in triplicate and controls were run simultaneously for all experiments. The crude ether extracts were treated with a diazomethane solution to methylate the free acids prior to GC analysis. As a check for solvent efficiency, the tissue was additionally extracted in several runs with hexane and methylene chloride; however, these solvents were not superior to the ether normally used for the extractions.

The GC analyses were performed on a Varian 6000 gas chromatograph equipped with a flame ionization detector; helium was used as carrier gas at an inlet pressure of 0.9 kg/cm^2 (3 ml/min). An SP1000 (50 m $\times$ 0.5 mm) capillary column was used in conjunction with a split injection mode (1 : 5). Temperature 120°/2 min/Rate 8°/150°. Detection limits: 1 ng. The GC-MS analyses were carried out on a Finnigan 4021 EI-CI-INCOS system using a DB5 (30 m $\times$ 0.32 mm) capillary column. Helium was used as the carrier gas with injections via a Grob injector. Temperature 50°/2 min/Rate 10°/150°/Rate 20/220. Detection limits: 1 ng.

The GC peaks were identified and quantitated by a comparison of peak heights with those from internal (pentadecanoic acid methyl ester) and external [(*E*)-11-tetradecenoic acid methyl ester] standards. GC injections of each assay were replicated at least twice. The GC-MS analyses were not necessarily replicated.

RESULTS

In Vivo Metabolism. In order to investigate the metabolism of the primary pheromone components (95 : 5, *E*/*Z* 11-tetradecenal), an in vivo bioassay was designed in which the air surrounding the moths was permeated with these components. It was desirable to look for the formation of degradation products under natural conditions. Although the quantity of applied pheromone was unnaturally high, the moths were unrestricted in their movement within the assay chamber. These conditions avoided the use of an aqueous environment which could alter the adsorption and metabolism of the pheromone components.

Initially the bioassay was performed with ten 2-day-old male moths and with a filter paper source concentration of 100 mg of pheromone. The bioassay was run for 2 hr after which time the moths were anesthetized and the tissues removed and analyzed for degradation products. Under these conditions, only one metabolized product was detected by GC-MS analysis, (*E*) 11-tetradecenoic acid (Figure 1).

Experiments were then carried out to quantitate the activity of the enzyme responsible for the observed degradation. To accomplish this, bioassays were

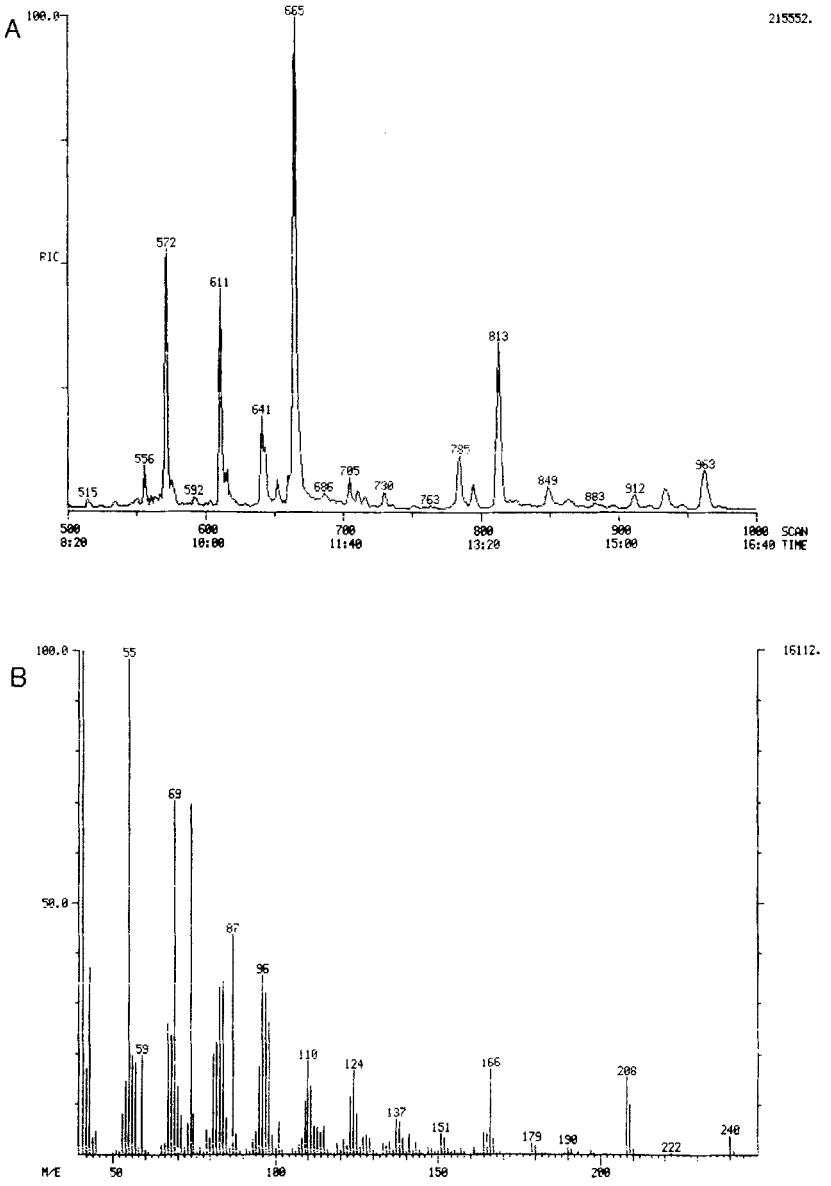

FIG. 1. (A) GC spectrum obtained with mass ion detector of antenna extract. Scan 572: diazomethane biproduct; scan 611: 11-tetradecenal; scan 665: 11-tetradecenoic acid methyl ester; scan 813: hexadecanoic acid methyl ester; scan 963: octadecanoic acid methyl ester. Note that hexadecanoic acid and octadecanoic acid are naturally present on the tissue in free acid form. (B) Mass ion spectrum of scan 665. EI spectrum at 70 eV.

run for 0.25, 0.5, 1, 2, 3, and 8 hr duration, with a saturating source concentration of pheromone. [Saturating concentration of pheromone = the maximum amount (mg) that could be employed without anesthetizing the moths; thus, a source concentration of ca. 400 mg was maintained during the assay period.] At higher pheromone concentrations the moths were anesthetized by the pheromone. The quantity of 11-tetradecenoic acid metabolite recovered from each incubation period was found to increase exponentially with time for both the male and female tissues (Figures 2 and 3). In addition, it appears that the acid

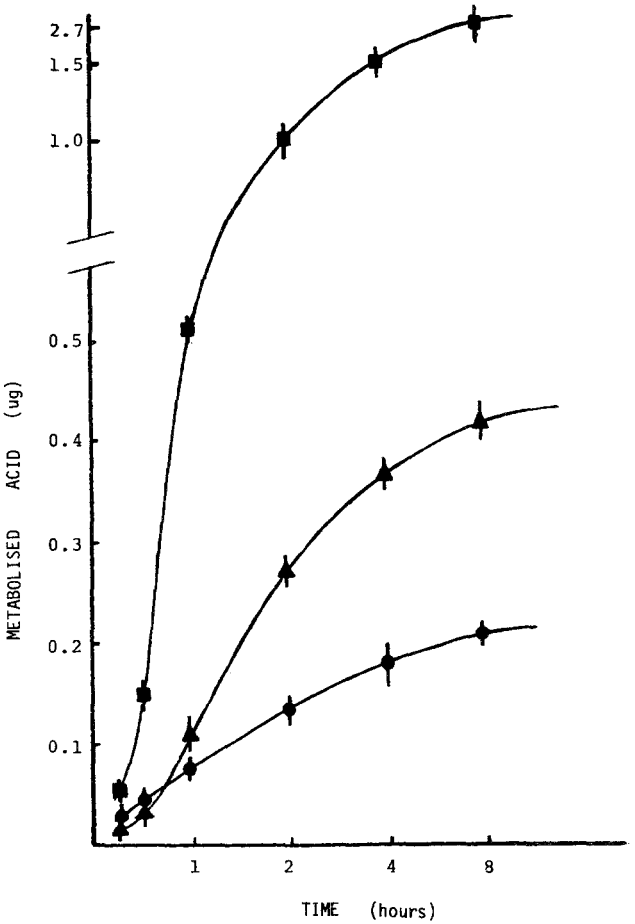

FIG. 2. In vivo formation of 11-tetradecenoic acid metabolite by male tissue. The data represent the mean value (μg) of acid obtained from 20 antennae ●, 20 metathoracic legs ▲, and 20 forewings ■. The curves fit a true exponential as determined by linear regression calculations. Bars represent standard deviation.

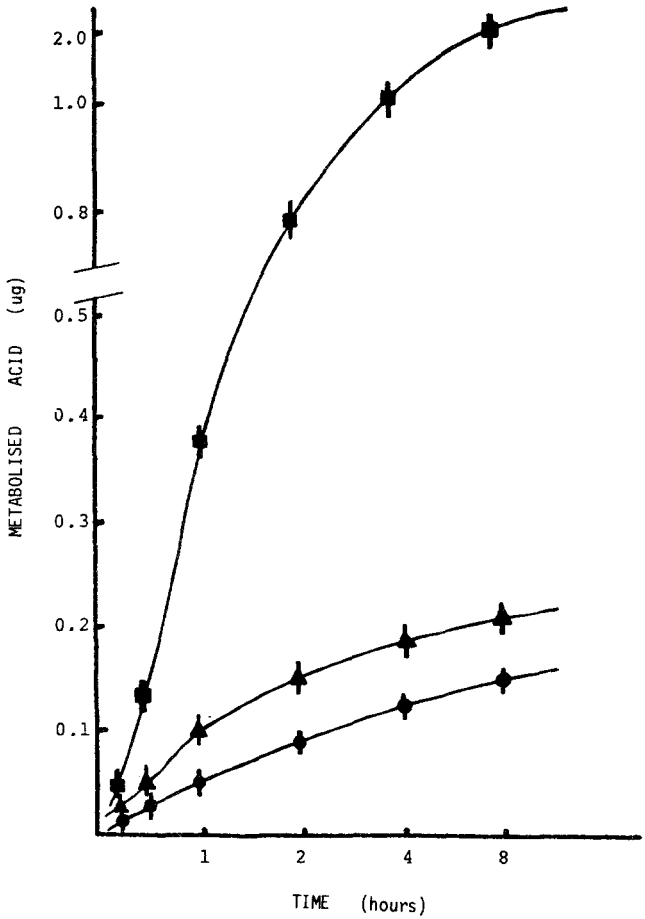

FIG. 3. In vivo formation of 11-tetradecenoic acid metabolite by female tissue. The data represent the mean value (μg) of acid obtained from 20 antennae ●, 20 metathoracic legs ▲, and 20 forewings ■. Bars represent standard deviation.

is either desorbed or converted to as yet unknown metabolite(s) (Figure 4). A study to determine whether this acid metabolite is biologically or behaviorally active or if it is further metabolized is now underway.

From the curves in Figures 3 and 4, it is evident that the decrease in amount of the acid does not entirely account for the exponential degradation of the aldehyde. It is possible that the enzyme responsible for aldehyde metabolism is restricted by the large quantities of aldehyde and acid present on the tissues.

The metabolic rates for the enzyme of the various tissues examined were calculated for the first 30-min incubation period. In the bioassays of these du-

rations, it appears (Figure 4) that the acid catabolism is not a significant factor. The following rate values are estimates made from the curves in Figures 2 and 3: (1) 1.0×10^{-13} M/sec/male metathoracic leg (350 μg), (2) 1.2×10^{-13} M/sec/male antenna (25 μg), (3) 4×10^{-13} M/sec/male forewing (490 μg), (4) 9.5×10^{-14} M/sec/female metathoracic leg (340 μg), (5) 8×10^{-14} M/sec/female antenna (20 μg), and (6) 3.4×10^{-13} M/sec/female forewing (500 μg).

Despite the fact that the metathoracic legs are 15× heavier and have a surface area ~10× greater than the antennae, the rate of metabolism of the aldehyde is very similar for both these tissues. This result may be accounted for if the antenna either has a greater number of dehydrogenase enzymes per unit area or if the antennal enzyme is more active. Whichever the case, it is

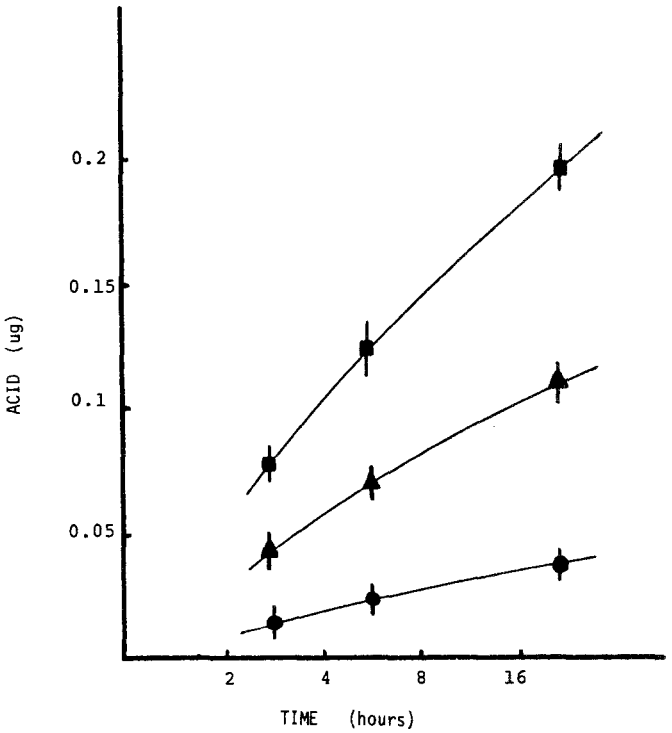

FIG. 4. 11-tetradecenoic acid removal from male tissue. The values represent the mean value (μg) of acid lost from 20 antennae ●, 20 metathoracic legs ▲, and 20 forewings ■. Standard deviations represented by bars. 40 male moths were incubated with pheromone (400 mg) for 2 hr at which time 10 moths were removed and the acid metabolite quantitated (initial acid concentration). Also at 2 hr, the remaining 30 moths were transferred to a clean chamber. After 3 hr, 10 moths were removed and the acid metabolite quantitated. This was repeated at 6 and 20 hr.

probably a reflection of the need for a quicker and more efficient removal of excess aldehyde from the antenna.

The above consideration may explain the slightly slower rate of metabolism by the female tissues as compared to the male. The female *Choristoneura fumiferana* perceive their own pheromone as determined by electrophysiological and behavioral studies (Palaniswamy and Seabrook, 1978). However, the removal of excess pheromone may not be as crucial to females versus males, since the females do no fly upwind in pheromone plumes. Perception of their own pheromone may be useful in monitoring the density of calling females in the area and may affect their calling behavior and emigration.

In Vitro Metabolism—Intact versus Homogenized Tissues. Aqueous incubations of antennae and pro- and metathoracic legs were carried out with pheromone concentrations of 6×10^{-5} M. The activities of intact and homogenized tissues were compared (Table 1). It was found, as in the case of the in vivo studies, that the intact antennae and intact metathoracic legs displayed similar enzymatic activity under aqueous conditions. The prothoracic legs produced slightly less metabolite than the metathoracic legs: perhaps a reflection of their smaller size.

When homogenized tissue was bioassayed, the quantity of recovered metabolite increased substantially, particularly in the case of homogenized antennae (from 28% to 75% conversion for a 1-hr incubation period). This increase in enzymatic activity is probably due to the release of enzymes from within the interior of the antenna upon homogenization. As additional supportive evidence that the degradation taking place with the intact tissue is the result of cuticular enzyme activity, the following additional experiments were conducted. First, scales (2 mg) from male wings were incubated with (*E*)-11-tetradecenal for 1 hr, and a 22% conversion of aldehyde to acid took place. Secondly, following several intact tissue bioassays (antennae and legs) and subsequent ether extraction, the tissue was homogenized and additionally extracted with ether. However, no significant increase in acid metabolite was observed.

TABLE 1. IN VITRO PHEROMONE METABOLISM[a]

Time (hr)	Antenna		Legs		
				Metathoracic	
	Intact	Homogenized	Prothoracic, intact	Intact	Homogenized
1	28 ± 5	75 ± 6	24 ± 6	30 ± 5	40 ± 7
2	53 ± 5	90 ± 8	45 ± 6	59 ± 9	70 ± 4

[a] Values expressed as percentage conversion of (*E*)-11-tetradecenal to (*E*)-11-tetradecenoic acid (mean ± SD). Three replicates of 10 moths for each bioassay bioassayed at pheromone concentration of 6×10^{-5} M.

In Vitro Metabolism—Competitive Analog Assay. To determine whether the dehydrogenase enzyme displayed selectivity for the pheromone components, a number of 12- to 16-carbon analogs were competitively assayed with the (*E*)-11-tetradecenal standard. The percentage conversions of aldehyde to acid for intact and homogenized tissues are presented in Table 2. At a concentration of 2×10^{-6} M, the 12-, 13-, and 14-carbon analogs were equally good substrates as (*E*)-11-tetradecenal. On the other hand, the enzyme showed less discrimination for the 15-carbon substrate with only a 25% conversion to acid after 1 hr with intact tissue as compared to a concurrent 70% conversion of (*E*)-11-tetradecenal. In the case of the 16-carbon analogs, the enzyme showed complete selectivity for the 14-carbon standard. It appears that the presence or position of the double bond in the substrate is not a factor influencing metabolism, whereas the addition of one carbon creates a serious disadvantage. Two extra carbons in the chain renders the analog an inactive substrate.

When the bioassays were conducted with homogenized tissue, the rate of

TABLE 2. COMPETITIVE ANALOG STUDY[a]

	Antenna		Legs	
Substrate[b]	Intact[c]	Homogenized[d]	Intact[c]	Homogenized[d]
(*E*)-11-Tetradecenal[e]	53 ± 6	49 ± 2	51 ± 10	54 ± 6
(*Z*)-11-Tetradecenal	51 ± 5	50 ± 5	48 ± 5	52 ± 5
13-Tetradecenal	50 ± 5	52 ± 3	51 ± 6	49 ± 4
Tetradecanal	46 ± 3	50 ± 3	50 ± 5	52 ± 5
Dodecanal	55 ± 5	53 ± 5	52 ± 4	54 ± 7
Tridecanal	52 ± 4	49 ± 4	47 ± 2	53 ± 8
Pentadecanal	29 ± 4	35 ± 3	30 ± 3	25 ± 5
Hexadecanal	5 ± 2	6 ± 1	8 ± 2	4 ± 2
(*Z*)-11-Hexadecenal	7 ± 2	4 ± 2	6 ± 2	7 ± 4
(*E*)-13-Hexadecenal	5 ± 3	6 ± 3	7 ± 6	8 ± 3
Control[f]			0–4%	
Heated tissue plus (*E*)-11-tetradecenal[g]	7 ± 3	10 ± 2	6 ± 4	7 ± 2

[a] Values expressed as percentage conversion to acid metabolite (mean ± SD). Three replicates of 10 moths for each bioassay.

[b] Analogs were bioassayed at 2×10^{-5} M concentration in the presence of (*E*)-11-tetradecenal, also at 2×10^{-5} M.

[c] Intact tissue bioassay duration = 1 hr at room temperature.

[d] Homogenized tissue bioassay duration = 15 min at room temperature for antennae, 30 min at room temperature for legs.

[e] At 2×10^{-5} M in presence of analog; the average of 28 bioassays with the five "good" competitive substrates. At 2×10^{-5} M, (*E*)-11-tetradecenal alone was 85% converted to the acid.

[f] Controls were simultaneously run for all the subtrates.

[g] Tissue heated at 80°C for 20 min prior to bioassay to determine at what temperature the enzyme is heat labile.

metabolism increased by a factor of three, but the selectivity trends were similar to those found for the intact tissues.

Other Enzyme Systems. Bioassays were carried out to determine if other enzyme systems were operating on the tissues studied. The existence of oxidase and esterase enzymes has been found in the pheromone-producing gland and other tissues of the female (Morse and Meighen, 1984a). Esterase, alcohol dehydrogenase, and oxidase enzymes have been found in the antenna and leg tissues of male *Heliothis virescens* whose pheromone consists in part as a 97 : 3 blend of (*Z*)-11-hexadecenal and (*Z*)-9-tetradecenal (Ding and Prestwich, 1985; Klun et al., 1980). However, in bioassays in which homogenized male antennae and legs were incubated with (*E*)-11-tetradecenol and (*E*)-11-tetradecenyl acetate, respectively, only unreacted substrate was recovered in 90% yield. It appears that the sensitivity of the methods employed was not adequate to enable the detection of small quantities of metabolites. A more extensive search for these enzyme systems using radiolabeled substrates is now under investigation.

CONCLUSIONS

The primary degradation product formed by enzymatic metabolism of 11-tetradecenal on cuticular surfaces, including antennae, legs, and wings, is the corresponding 11-tetradecenoic acid. This enzyme system undoubtedly serves a clearing function, to keep the body tissue free of excess pheromone. Whether this same enzyme is found in the interior of the antenna, where its function would be connected with the transduction process, is not clear. The bioassays utilizing homogenized antenna tissue do suggest that this enzyme may be operating inside the sensilla or antenna or both.

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REPRODUCTIVE ENDOCRINE INFLUENCES UPON OLFACTORY PERCEPTION: A Current Perspective[1,2]

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Abstract—An overview of relationships between psychophysical measures of olfactory function and reproductive neuroendocrine processes is presented. Human studies are emphasized, and the influences of gender, menstrual cycle phase, pregnancy, and injected gonadal hormones upon olfactory perception are discussed in detail.

Key Words—Pregnancy, olfactory threshold, odor preferences, hormones, estrous cycle, menstrual cycle, pheromone, UPSIT, sex differences.

INTRODUCTION

Hormones and other neuroendocrine factors influence not only the nature and deposition of biological secretions used in mammalian social communication, but also the sensory systems involved in detecting such materials and extracting information from them. Despite this fact, the anatomic, physiologic, or chemical substrates of neuroendocrine influences upon chemosensory function are largely unexplored. Such influences have been particularly difficult to establish in nonhuman mammals, since rather complex equipment and painstaking operant conditioning is required to accurately assess basic aspects of sensory function. Thus, most behavioral olfactory research involving animals has focused upon odor preferences, rather than upon olfactory sensitivity or other psychophysical measures requiring exacting stimulus control. The opposite is true with

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human olfactory research, where olfactory thresholds are the most common measure. Unfortunately, human endocrine-olfactory studies are somewhat limited in terms of the information they can provide, in that critical invasive endocrine or physiologic manipulations cannot be performed.

Despite such limitations, human studies form the backbone of our current understanding of relationships between the endocrine system and olfactory perception and provide us with important clues as to the underlying mechanisms. Furthermore, the results of such studies are inherently compelling, given our own speciocentric predilections and the important role of the sense of smell in (1) warning us of airborne hazards and (2) determining the flavor and palatability of foods and beverages. For these reasons, and because the animal literature on this topic is extremely limited, the present review focuses mainly upon human studies. Since much of this literature has been reviewed in detail previously, an attempt has been made to summarize the current state of knowledge, rather than to be comprehensive. The interested reader is referred elsewhere for more detailed accounts of this topic (cf. Doty, 1976, 1981, 1986a,b).

SEX DIFFERENCES IN OLFACTORY FUNCTION

Several human studies report women have lower thresholds (i.e. greater sensitivity) than men to a number of odorants, including camphor, the artificial musk Exaltolide, citral, and amyl acetate (Le Magnen, 1952; Schneider and Wolf, 1955; Koelega, 1970; Koelega and Köster, 1974; Toulouse and Vaschide, 1899). Such sex differences do not appear to be particularly robust, however, since they are not seen by all authors (e.g., Venstrom and Amoore, 1968; Punter, 1983) and, in some cases, appear to require relatively large sample sizes to be observed.

Similarly, women have been shown to outperform men on various odor identification tasks, such as the University of Pennsylvania Smell Identification Test (UPSIT; cf. Doty et al., 1984a,b). Again, the differences are not large over most of the age range, although greater female performance is clearly evident at the older and younger ages (Figure 1). This sex difference in odor identification ability is quite general, since it is present to about the same degree in American whites, American blacks, American Koreans, and native Japanese (Doty et al., 1985).

The origin of the sex difference in human olfactory sensitivity or odor identification ability is not known. A limited body of data suggests, however, that girls before the age of puberty perform better than boys on both types of tests, implying that adult differences in gondal steroid levels are not its basis. For example, prepubescent girls evidence greater basal sensitivity (i.e., lower thresholds) than prepubescent boys to amyl acetate (Koelega and Köster, 1974), in addition to obtaining higher scores on the UPSIT (Figure 1). Interestingly, while young girls outperform the boys on nearly all of the UPSIT test items, a

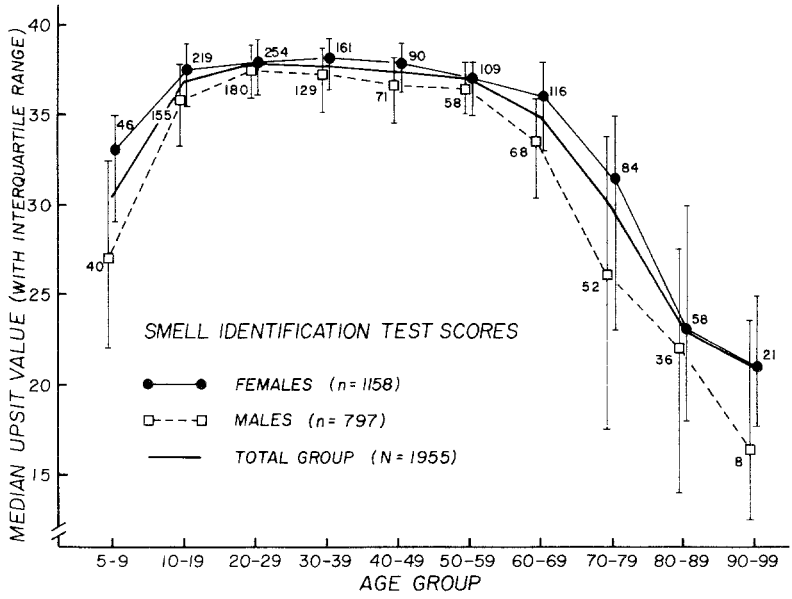

FIG. 1. University of Pennsylvania Smell Identification Test (UPSIT) scores as a function of age and gender. Numbers by data points indicate sample sizes. Reprinted with permission from Doty et al. (1984b). Copyright 1984 by the American Association for the Advancement of Science.

recent item analysis has revealed that 4- to 5-year-old boys outperform their female counterparts on the odors of motor oil and gasoline, suggesting that early cultural learning may influence the test scores. The girls outperform the boys on even these items, however, after this time (Doty, unpublished observations).

Sex differences in the rating of the pleasantness of odors have also been noted. For example, Griffiths and Patterson (1970) had 156 men and 145 women rate the pleasantness of a suprathreshold concentration of 5-androst-16-en-3-one. On the average, women rated the odor as less pleasant than did men. For both sexes, the lower a subject's threshold for the odor, the greater was his or her dislike. For subjects with the same threshold value, women rated the odor as more unpleasant than did men. Analogous sex differences have been seen for a number of odorants, including common sources of human body odor. Thus, women rate the odors of human breath, axillae, and vaginal secretions as more intense and less pleasant than do men (Doty et al., 1975, 1978, 1982b).

Although, sex differences in olfactory sensitivity have not been carefully examined in animal studies, there is some indication that the development of the olfactory system may be influenced by gondal hormones and that sex differences exist at the morphological level in at least some sectors of the chemosensory pathways (see Segovia et al., 1984). In addition, sex differences in odor

preferences are well established, and often are indicative of complex relations between experiential and physiologic factors, including endocrine state. Such relations are exemplified by the early work of Mainardi and associates (1963) which found that adult estrous female house mice (*Mus musculus domesticus*), normally reared by both parents since weaning, preferred odors from males of the same strain to those from *Mus musculas bactrianus*. Estrous *Mus musculus domesticus* females, reared only by their mothers in the absence of adult males, showed no sexual preferences and were attracted indifferently to *M. m. bactrianus* and *M. m. domesticus* male odors. Such preferences were not observed in the males. More recently, Brown (1983) noted that female rats reared with both their dam and sire strongly preferred the odor of male bedding over that of clean shavings. However, female rats reared with the dam alone did not show such a preference. The specific mechanisms underlying these preferences have yet to be elucidated.

INFLUENCE OF MENSTRUAL CYCLE ON OLFACTORY PERCEPTION

Although there have been numerous studies of olfactory function across the phases of the menstrual cycle, in many cases the sensory measures were collected too infrequently during the cycle to determine the specific nature of the fluctuations. With rare exceptions, circulating hormone levels have not served as the basis for categorizing the cycle phases. As noted elsewhere (Doty, 1979), the procedure for characterizing cycle phases can significantly alter the conclusions. Despite such problems, however, it is now clear that cyclical alterations occur in olfactory sensitivity to a number of odorants (including xylene, furfural, phenyl ethanol, and Exaltolide), with peak sensitivity occurring a day or two before the midcycle LH surge (Doty et al., 1981; Köster, 1965, 1968; Le Magnen, 1952; Vierling and Rock, 1967).

Despite the fact that circulating levels of gonadal hormones have been suggested to be the basis of the fluctuations in olfactory sensitivity across the menstrual cycle phases, only one menstrual cycle/olfactory study has measured circulating levels of the major reproductive hormones concurrently with sensory testing (Doty et al., 1981). In this work, a signal detection paradigm was used to evaluate odor detection performance to furfural across 17 menstrual cycles of normally cycling women, six menstrual cycles of women taking oral contraceptives, and six equivalent time periods of three men. During the 2- to 3-hr test periods (which occurred every other day throughout the cycles), measures of blood pressure, heart rate, body temperature, nasal airflow, and respiration rate were established, along with radioimmunoassay-determined serum or plasma levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), estrone (E_1), estradiol (E_2), progesterone (P), and testosterone (T). In general,

peaks in average sensitivity were observed midcycle, midluteally, and during the second half of menses in both women taking and not taking oral contraceptives. The serendipitous finding of systematic alterations in the detection performances of women taking oral contraceptives suggested the fluctuations are not directly dependent upon circulating levels of gonadal hormones or hypophyseal gonadotropins (Figure 2). The basis of these fluctuations is not yet known.

To determine if the fluctuations observed in sensory sensitivity across the pill cycles were present in a modality other than olfaction, we measured puretone auditory thresholds and evoked potential auditory brainstem responses every other day across two complete cycles of a 28-year-old woman taking an oral contraceptive. In addition, measures of olfactory sensitivity (d′) to phenyl ethanol, body temperature, heart rate, and circulating levels of LH, total estrogens, and progesterone were also established (Doty et al., 1982a). The results of this study (Figure 3) suggest that a number of the dependent measures fluctuated in similar fashion across the time periods examined, although the peaks in olfactory sensitivity during the second half of the cycles were relatively large compared to those observed in the average data presented in Figure 2 (possibly reflecting the use of a different odorant or an individual difference). In addition, slight differences in the times of maxima and minima were present across the variables. Since these data were collected from only one subject and because each point in Figure 3 represents a moving average with equal weights attached to three adjacent time points, some of the lack of correspondence among the variables may be due to the multiple averaging of noise, rather than to a difference in underlying rhythms. The degree to which these rhythms are related to one another requires additional study.

Cyclical changes in odor detection performance are also observed in mice and rats, with peak performance occurring during either estrus or proestrus. Such cyclicity is eliminated by ovariectomy, suggesting the hypothesis that they are due to fluctuations in circulating levels of gonadal hormones (Pietras and Moulton, 1974; Phillips and Vallowe, 1975; Schmidt, 1978). Such dependence on ovarian hormones would be in congruence with electrophysiological studies noting that estrogen augments, and progesterone depresses, evoked potential activity within olfactory-related areas of the forebrain in several species (see Kawakami and Sawyer, 1967; Oshima and Gorbman, 1969). However, it is also possible that the estrous-related sensitivity fluctuations are a primary manifestation of cyclic neuroendocrine events higher in the system than the ovary, and that removal of the gonads simply eliminates "permissive" levels of gonadal hormones necessary for their expression.

Several examples of the likely "permissive" role of ovarian estrogens in the expression of nonovarian, but estrous-related, rhythms are available from the rat literature. For example, recurrent vaginal estrus occurs in gonadectom-

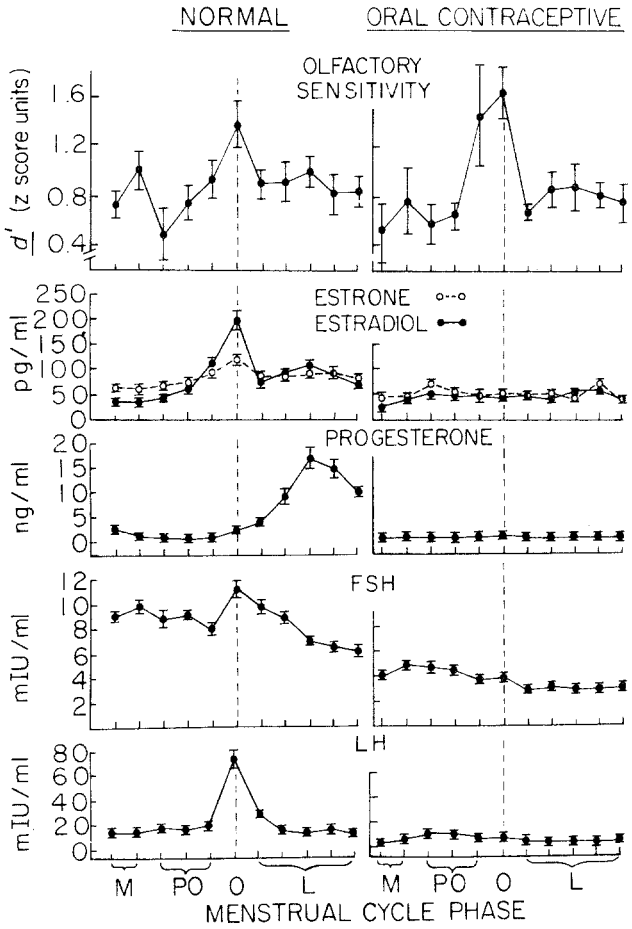

FIG. 2. Patterns of changes in signal detection measures of olfactory sensitivity and plasma or serum levels of five reproductive hormones across cycle phases of women taking and not taking oral contraceptives (data modified from Doty et al., 1981). Data are normalized and assigned to cycle phases using the Doty (1979) procedure, where M = menstrual phases 1 and 2, PO = preovulatory phases 1–3, O = "ovulatory phase" (day of LH surge or day before LH surge in normally cycling group, day 13 or 14 in oral contraceptive group, where day 1 = 1st day of menses), and L = luteal phases 1–5. Note clear fluctuation in olfactory sensitivity in both groups, and the lack of correlation between these changes and circulating levels of pituitary and gonadal hormones in the oral contraceptive group. From Doty et al. (1982a), with permission.

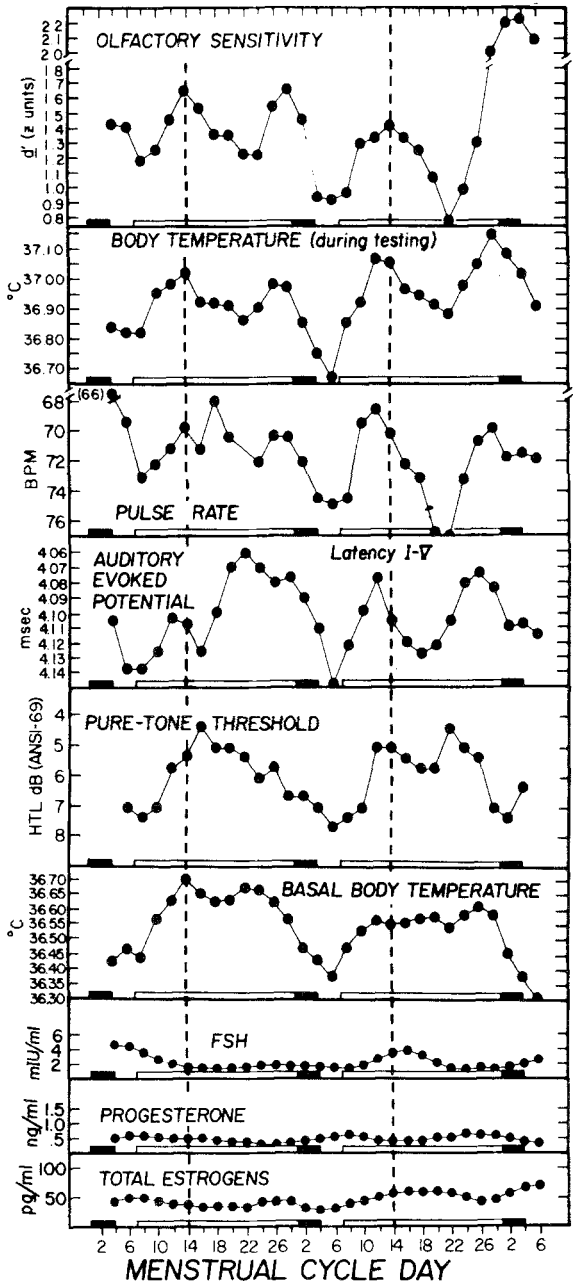

FIG. 3. Changes in nine variables across two consecutive menstrual cycles of a woman taking oral contraceptives. To diminish noise, a moving average with equal weights attached to three adjacent time points was applied to each series. Dark rectangles on the abscissae signify periods of menstrual bleeding, open rectangles days during which the oral contraceptive medication was taken. Testing took place from 9:30 AM to 12:00 PM on each test day. Pure-tone thresholds are averaged across a wide range of frequencies. From Doty et al. (1982a), with permission. Copyright 1982 by IRL Press Limited.

ized rats injected daily with a constant low dose of estrone (Bourne and Zuckerman, 1941a). The periodicity of this phenomenon is indistinguishable from that of nonovariectomized females. This cyclicity is not markedly altered by hypophysectomy, although it is eliminated or greatly affected by adrenalectomy. Interestingly, this cyclicity is related to a 4- to 5-day rhythm in adrenal weight which is present in castrated females given a constant daily injection of a low dose of estrone (Bourne and Zuckerman, 1941b). The rhythm in adrenal weight and volume is due mainly to hypertrophy of cells within the zona fasciculata of the cortex, although adrenal medullary cells were noted to increase in size as a result of estrogenic stimulation. Since adrenal insufficiency is related to enhanced odor detection performance, it is not inconceivable that rhythmic activity of the adrenal may relate to the cyclic changes in olfactory sensitivity (see Henkin and Bartter, 1966; Sakellaris, 1972).

Another example of the possible "permissive" role of estrogens in estrous-related rhythms comes from the work of Terasawa and Timiras (1968). These authors noted that estrous-related cyclic changes in electrically induced seizure thresholds in the hippocampus and amygdala, although eliminated by ovariectomy, were briefly restored, in dampened fashion, following a single injection of estradiol. That this phenomenon may be influenced by some type of early hormonal "organization" process is suggested by the finding that such restoration was most marked in rats ovariectomized in early adulthood and was not present in rats ovariectomized in infancy.

Currently, research in our laboratory is being performed to ascertain whether or not the cyclical fluctuations in olfactory sensitivity of rats can be established in the absence of the gonads when low levels of estrogen are provided on a tonic basis. If so, caution must be exercised in assuming that the rhythmic fluctuations in olfactory sensitivity of these animals is due solely to alterating levels of gonadal hormones from the ovaries.

CHANGES IN OLFACTORY FUNCTION DURING PREGNANCY

Despite the widespread incidence of cravings and aversions in many women during the time of pregnancy (cf. Dickens and Trethowan, 1971; Hook, 1978), a relationship between episodes of such phenomena and alterations in chemosensory function has yet to be experimentally established. Although the literature is inconsistent regarding alterations in smell perception during pregnancy, the few studies in which longitudinal measures were taken suggest that olfactory sensitivity is decreased during late pregnancy (Doty, 1986a). Anecdotal reports of hypersensitivity have received no strong support, although there is some suggestion that such hypersensitivity, if present, occurs during the earlier stages of pregnancy. In mice, a slight increase in olfactory sensitivity has been reported a few days prepartum, with a marked increase occurring during the postpartum week (Schmidt et al., 1982).

INFLUENCES OF CASTRATION AND/OR ADMINISTRATION OF GONADAL STEROIDS UPON OLFACTORY SENSITIVITY

To date, only a few human studies have examined the influences of oophorectomy, orchidectomy, or hormonal replacement therapy upon olfactory function. Unfortunately, double-blind procedures and rigorous statistical and methodological controls (e.g., controls for the effects of repeated testing) have been lacking in a number of these studies, so it is difficult to establish whether such manipulations influence olfactory function.

Several investigators suggest that decreases in circulating estrogen titer are associated with decreased olfactory sensitivity, and increases with increased olfactory sensitivity. In 1952, Le Magnen obtained odor detection thresholds for the musk Exaltolide in seven ovariectomized women and found them to be approximately two log units higher than normal. Five of these women were subsequently administered estradiol. Thresholds were found to drop by over two log units in two of these subjects, and by less than a log unit of the others. No change was seen in the fifth subject. In subsequent studies, Le Magnen administered estradiol to himself and noted an increase in sensitivity to trimethylamine and pyridine, and a decrease in sensitivity to safrol.

Six years after Le Magnen's study, Schneider et al. (1958) measured citral thresholds in two hypogonadal women, aged 30 and 84 years, once a week for periods extending over six months. The subjects received daily injections of either placebo or Equilin SO_4, Premarin, or estradiol interspersed in 1- to 2-week-long treatment intervals within the test period. The average thresholds were reported to be lower during the estrogen treatment periods than during the times of placebo administration, although the differences were not marked and the ranges of the daily thresholds overlapped considerably.

More recently, Good et al. (1976) evaluated olfactory sensitivity for nine days via a signal detection procedure in a woman who was reportedly anosmic to Exaltolide. The percentage of hits (reports of the presence of the odor on odor trials) and false alarms (reports of the presence of odor on blank trials) were both zero on the two pretreatment days, implying that detection was not reported on any trial. During the initial treatment days the percentage of hits and false alarms rose to about the same level, whereas during the later ones the percentage of hits rose even higher and the false alarm rate dropped somewhat.

While the aforementioned studies are cited in support of the positive influences of estrogen on olfactory function, other studies claim that testosterone decreases olfactory sensitivity. Le Magnen (1952) self-injected large doses of testosterone and found decreased sensitivity to several odors tested, including Exaltolide. Schneider et al. (1958) injected a 69-year-old woman periodically with androgen and found that during the periods of injection the thresholds for citral were higher than during the periods of noninjection. In contrast, rat studies suggest that exogenous testosterone increases, rather than decreases, odor

detection performance (Pietras and Moulton, 1974). Since supraphysiological doses of testosterone were used in the rat work, it is not clear whether this phenomenon is due to the conversion of the hormone to estrogenic metabolites.

The few animal studies available suggest that orchidectomy, per se, has little or no influence upon odor detection performance. Thus, Carr and Caul (1962) found no alteration in the ability of prepubertally castrated rats to learn a water-reinforced discrimination between the odors of receptive vs nonreceptive female conspecifics. Carr et al. (1962) found no effect of prepubertal castration on detection thresholds for estrous urine in male rats. Recently, we established odor detection performances of six postpubertally castrated rats and six sham-operated controls to ethyl acetate using a computer-controlled olfactometer and a go/no-go operant response procedure [Doty, unpublished data; see Marshall et al. (1981) for a description of the general test paradigm]. Despite the extreme sensitivity of our procedure to subtle alterations in odor detection ability, we similarly found no short-term influence of adult castration

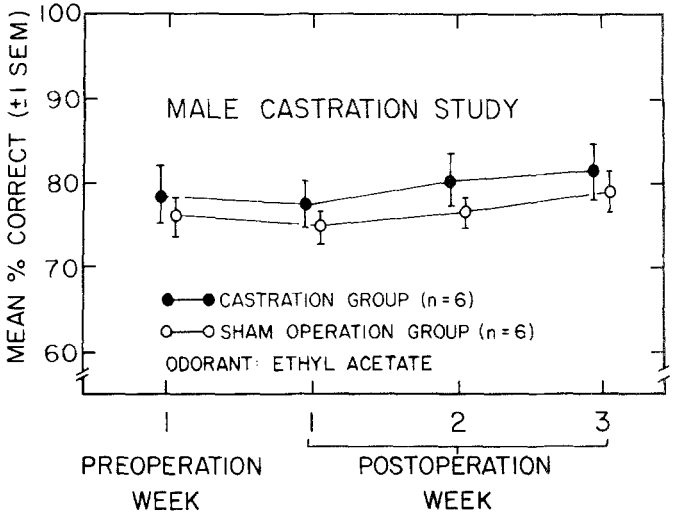

FIG. 4. Percent correct detection of ethyl acetate in a go/no-go operant task for six castrated and six sham-operated Long-Evans male rats (Doty, unpublished data). Each data point represents an average of 10,920 trials (260 trials/day/rat). Within each subject's daily test session, 60 trials were run at $10^{-4.5}$ log concentration (percent saturation) and 50 trials at each of the following log concentrations: $10^{-5.0}$, $10^{-5.5}$, $10^{-6.0}$, and $10^{-6.5}$. The concentrations were presented in a series of descending and ascending 10-trial same-concentration blocks in which half of the trials were blank. Two adjacent blocks of 10 trials were run at the beginning of the test session for the $10^{-4.5}$ concentration and at the second ($10^{-6.5}$), third ($10^{-4.5}$), fourth ($10^{-6.5}$), and fifth ($10^{-4.5}$) reversal points of the staircases. The order of presentation of the odorant and blank trials were from a quasirandom series within each of the 10 trial blocks.

on odor detection performance (Figure 4). Whether long-term castrates evidence alterations in odor detection performance requires futher study.

In contrast to the minor or nonexistent effects of orchidectomy on smell function, ovariectomy reportedly eliminates fluctuations in olfactory detection performance associated with the rat's estrous cycle (Pietras and Moulton, 1974; Phillips and Vallowe, 1975). Specifically, the peak noted in odor detection performance during estrus is not present. Interestingly, baseline performance appears to be slightly elevated in the ovariectomized animals (Pietras and Moulton, 1974). Injection of estradiol reportedly increases odor detection performance, whereas injection of progesterone decreases such performance, with latencies for the peak effects being 12 and 18 hr, respectively (Phillips and Vallowe, 1975).

CONCLUSIONS

It is becoming increasingly apparent that complex relations exist between olfactory function and neuroendocrine factors in mammals. Previous notions of a simple relationship between measures of olfactory function and circulating concurrent levels of gonadal hormones appear no longer tenable, at least in the human, and a concerted effort to understand the mechanisms underlying endocrine/olfactory interactions is sorely needed. However, even basic questions regarding human olfactory/endocrine relationships have not been answered at the psychophysical level, as pointed out in a recent review (Doty, 1985a). Questions yet to be answered include: Does olfactory sensitivity (and/or other measures of olfactory function) change systematically during pregnancy and, if so, are such changes related to factors such as nutritional needs, food intake, cravings, or aversions? Are the fluctuations in olfactory sensitivity noted across the reproductive cycle closely coupled with fluctuations in other sensory systems? If so, which ones? Are fluctuations in olfactory sensitivity present in prepubscent girls whose brains have not yet experienced cyclic changes in blood levels of reproductive hormones from the ovary? Are sex differences or endocrine-related changes in olfactory sensitivity specific to only some types of odors? If so, what are the physiochemical parameters most closely related to these changes? In a double-blind situation, what influences, if any, do exogenously administered hormones have on olfactory function? If such hormones influence olfactory sensitivity, by what means do they exert their influence?

As indicated in the present paper, most human studies have focused upon sex differences or upon naturally occurring time periods associated with hormonal alterations, such as pregnancy, menopause, or stages of the menstrual cycle. While such studies shed considerable light on possible endocrine/sensory interactions, observations from such studies need to be followed up by incisive animal studies in which the mechanisms responsible for the phenomena can be more fully elucidated. For example, a sex difference in human olfactory func-

tion could be due to factors as diverse as differences between the sexes in (1) the nasal architecture (which conceivably influences the number of odorant molecules reaching the olfactory neuroepithelium), (2) sniffing strategies or patterns, (3) the anatomy or physiology of the olfactory system proper (including subtle hormone-dependent alterations in vascular and secretory processes within the neuroepithelium), (4) the frequencies of certain habits among male and female populations (e.g., dieting); (5) attention or arousal; (6) familiarity and/or experience with odorants; and (7) acquired alterations in the olfactory system, such as neuroepithelial damage from industrial pollutants or viruses. While the relative influence of some of these variables can be assessed statistically or determined experimentally in the human, others are not amenable to such measurement and require the use of suitable animal models for their estimation.

Le Magnen, in his pioneering and classic studies of endocrine/olfactory interactions, suggested the hypothesis that the perception of only certain odorants, such as Exaltolide, is influenced by alterations in endocrine factors. More recent studies suggest, however, that alterations in sensitivity across estrous or menstrual cycles are rather general, being observed for a number of compounds (see Doty et al., 1981; Pietras and Moulton, 1974). Although the degree of such changes may vary across odorants, it is difficult at the present time to ascribe any functional significance to them, as they could simply reflect such factors as the number of receptor sites, receptor site types, or fibers recruited for each of the odorants. Furthermore, such differences might have an experiential basis, in that exposure to an odorant may alter subsequent responsiveness to it. For example, Coopersmith and Leon (1984) have demonstrated that rats, preexposed to the odor of peppermint, evidence a higher level of radiolabeled 2-deoxy-D-glucose uptake in the olfactory bulb (a measure of functional neural activity) than rats not similarly preexposed. It is also noteworthy that olfactory imprinting can occur for substances of no apparent biological relevance (e.g., cinnamon and cumin; cf. Porter and Etscorn, 1974, 1975) and that the olfactory system, per se, is exquisitely sensitive to substances never before encountered in either ontogeny or phylogeny, such as perfluorocarbons (Marshall et al., 1981). Such complexity and plasticity serves as a never-ending challenge to investigators interested in understanding endocrine/olfactory interactions in mammalian forms.

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ANALYSIS OF CHEMICAL SIGNALS IN A PRIMATE SPECIES (*Saguinus fuscicollis*): USE OF BEHAVIORAL, CHEMICAL, AND PATTERN RECOGNITION METHODS

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Abstract—Scent marking with specialized skin glands is a common behavior in the tamarin, *Saguinus fuscicollis*. The scent marks identify species, subspecies, gender, and individual, and they also contain information on the social position and hormonal condition of an animal. The marks are chemically complex, containing a large number of compounds. Analysis by means of gas chromatography–mass spectrometry has identified 16 major components (squalene and 15 esters of butyric acid). These compounds are present in the marks of males and females of two subspecies, *Saguinus f. fuscicollis* and *Saguinus f. illigeri*. Application of computerized pattern recognition techniques has shown that concentration patterns of some of the butyrates are diagnostic of the two subspecies while concentration patterns of other butyrates are diagnostic of males and females regardless of subspecies. Behavioral studies have shown that the concentration patterns of butyrates and squalene alone do not encode information on subspecies and gender. It is, however, likely that this information is partially encoded by these specific butyrate–squalene concentration patterns but that yet unidentified compounds in the scent marks serve as necessary synergists.

Key Words—Chemical communication, scent marking, pattern recognition, *Saguinus fuscicollis*, GC-MS, primate, tamarin, skin secretions, *n*-butyrate esters, concentration profiles.

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INTRODUCTION

In comparison with many other mammalian groups, primates (including man) have traditionally been classified as microsmatic. This classification has been based on a progressive reduction from prosimians to monkeys, apes, and man in the complexity of the nasal chamber, the extent of the olfactory epithelium, the development of the vomeronasal organ, and the relative sizes of the main and the accessory olfactory bulbs (Epple and Moulton, 1978; Wysocki, 1979; Epple, 1986; Maier, 1981). These findings have often been taken as indication of a poorly developed chemosensory system in infrahuman primates and humans. Questioning the validity of this judgment, Keverne (1983) has pointed out that neural projections from the olfactory system have access to the neocortex via the thalamus and that primates, with their well developed neocortex, have a more sophisticated neural backup for recognition and evaluation of olfactory sensory input patterns. "Primates do not therefore have a poorly developed sense of olfactory perception, and the ways in which this is employed may well be the most evolved of all species" (Keverne, 1983). Indeed, during recent years, evidence has accumulated suggesting that the olfactory system serves as an important sensory mediator of signals in sociosexual communication and that many primates make extensive use of chemical signals in these areas of behavior (Epple, 1974a, 1976, 1986; Schilling, 1979). Moreover, the vomeronasal system may also mediate chemical signals important in communication among conspecifics (Epple, 1986).

Much of the evidence for the use and function of chemical signals in primates is based on the fact that many species possess specialized scent-producing skin glands in various body regions, show scent marking behavior, and investigate conspecifics and their scent marks by sniffing and licking. The literature on the occurrence of specialized scent glands and scent marking behaviors and on other observational and experimental evidence for the behavioral functions of chemical signals within the order of primates has been reviewed in detail by Epple (1974a, 1976, 1986) and by Schilling (1979). According to the studies reviewed by these authors, one widespread function of chemical signals appears to be the communication of information on the identity of the species, subspecies, gender, and individual, and on the reproductive condition and social and emotional state of the individual. In addition, chemical signals play a role in a variety of behavioral contexts. Functions associated with spacing behavior and territoriality, with aggressive behavior, with reproduction, and with mother–infant interactions have either been suspected or have been demonstrated experimentally in a number of prosimians, in some New World monkeys, and in a few Old World monkeys (reviews: Epple, 1974a, 1976, 1986; Schilling, 1979).

The chemical composition of scent signals has been studied in several spe-

cies of primates (see reviews by Epple, 1986, Albone, 1984). However, characterizing the biological activity of chemical compounds of suspected communicatory function by means of bioassay experiments has proven to be very difficult. In many species the sources of chemical communicatory signals are highly complex. In most mammals, including some primates, scent gland secretions contain numerous compounds (Albone, 1984). Moreover, the scent marking behavior of some primates involves elaborate mixing of scent gland secretions with other body products such as urine or vaginal discharge (Epple, 1986). In these complex mixtures, communicatory messages may be encoded by quantitative as well as qualitative patterns (Albone, 1984; Johnston, 1983; Preti et al., 1977), which increase the difficulty of identifying the specific compounds or patterns of compounds with which particular behavioral responses are associated. Moreover, most species of primates live in societies in which not only the physiological condition of the individual but also its social status and numerous experiential factors have a profound influence on its responses to communicatory signals. Therefore, the way in which chemical and other social signals influence behavior depends on the context in which they are perceived. This fact greatly complicates the design of experiments for the evaluation of biological activity of specific chemical constituents.

Our own studies on the behavioral functions and chemical composition of scent signals in South American tamarin monkeys have certainly been confounded by the complexities described above. We review here some of the difficulties we have encountered and the progress we have made in the course of this work.

Of all monkeys and apes, the marmosets and tamarins (Callitrichidae) may rely most heavily on chemical communication. Callitrichids are the only family of monkeys in which specialized epidermal scent glands are developed to as great a degree as in some of the prosimians, a group which makes extensive use of such glands in chemical communication (Schilling, 1979). The saddleback tamarin (*Saguinus fuscicollis*), the species on which our studies have concentrated, possesses an apocrine chest gland above the sternum and a large pad of sebaceous and apocrine glands in the circumgenital–suprapubic area (Perkins, 1966) (Figure 1). Males and females routinely scent mark with the circumgenital–suprapubic gland, depositing glandular secretions which are mixed with urine and possibly genital discharge. The sternal gland is used less frequently, sternal marking occurring predominantly in situations of high arousal. Investigation of the scent deposits by all members of a group includes sniffing and occasionally licking, both of which result in contact between the chemical stimulus and the muzzle.

The high frequency of circumgenital–suprapubic marking during daily routine activities of a group and the large amount of investigatory behavior shown by all group members indicate that the chemical signals deposited in the marks

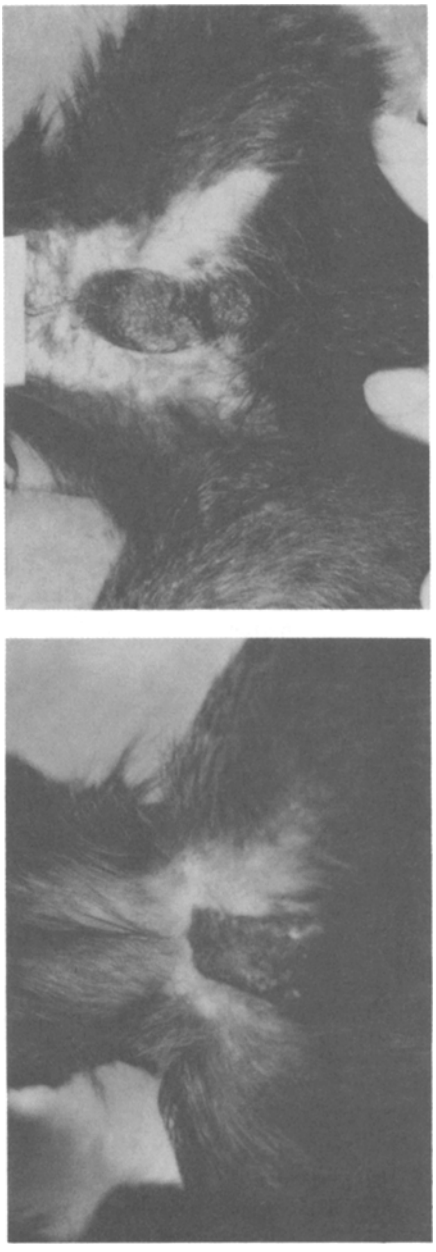


FIG. 1. Scent glands of *Saguinus fuscicollis* male (left), female (right).

are of considerable importance in social and sexual behaviors of saddle-back tamarins. During recent years, therefore, our work has been concerned with an analysis of the informational content of the circumgenital–suprapubic scent marks, their chemical composition, and the way in which the chemical constituents encode the various messages.

BEHAVIORAL STUDIES

Methods

Adult *Saguinus fuscicollis* males and females served as subjects and as donors of scent marks. The spontaneous responses of subject monkeys to scent marks produced by donor monkeys or to samples of voided urine were recorded quantitatively, measuring their investigation of novel stimulus objects of standard size which were scented with the material to be tested. Under laboratory conditions, the tamarins readily investigate novel objects placed into their cages. Therefore, it was important to evaluate the monkeys' response to "odorless" novel objects and to objects carrying control odors vis-à-vis their response to identical objects carrying the odor of conspecifics. Aluminum plates or perches of white pine (61 × 5 × 0.6 cm) were used as stimulus objects, and the investigatory responses measured were contacting, sniffing, and scent marking of the stimulus object. Wooden perches were discarded after one use but aluminum plates were reused after thorough cleaning. The results of the experiments reviewed below have been published, and detailed descriptions of all methods can be found in these reports (Epple, 1971, 1974b, 1978; Epple et al., 1980). We therefore only summarize the testing procedures here.

Single Plate Tests. These tests were used to assess the responses of the tamarins to differently scented, single-stimulus objects. For each test, the subject was isolated from its group mates in one compartment of their double-unit home cage. A stimulus plate was placed in the center of the cage floor for a period of 5 min. The 5-min test was divided into 60 intervals of 5 sec. Contacting, sniffing, and scent marking on the stimulus plate were scored in the following way: For each behavior a score of 1 was given for every interval during which it was shown, regardless of its actual frequency of occurrence. After a series of tests, the mean contacting, sniffing, and marking scores of each subject were computed, and differences in responses to the different stimuli were analyzed statistically (Siegel, 1956). Significant differences in response intensity were interpreted as evidence that the subjects discriminated between the stimuli.

Choice Tests. Most of our behavioral testing employed choice tests. This method allows the animal to investigate simultaneously two stimulus plates, each carrying a different type of scent. Some of our earlier tests lasted for 15

min (Epple, 1971, 1973, 1974b). More recently, 5-min tests have been used since the response of the animals in 5-min tests did not differ from that in 15-min tests. In all tests, the left/right positions of the stimuli are counterbalanced to avoid a reflection of side preferences. All test periods were divided into 5-sec intervals, and scoring was done in the same manner as in single-plate tests. The occurrence of a statistically significant difference in the intensities of contacting, sniffing, and marking the stimulus plates was interpreted as evidence that the animals are able to discriminate between them. Discrimination among the following scent types was tested.

1. Genus, species, subspecies
   (a) *Saguinus* versus *Callithrix*
   (b) *Saguinus fuscicollis* versus other *Saguinus* species
   (c) *S. fuscicollis fuscicollis* versus *S. fuscicollis illigeri*
2. Gender
   (a) Males versus females
   (b) Intact males versus castrated males
   (c) Castrated males versus intact females
3. Individuals
   (a) Male A versus male B
   (b) Female A versus female B
4. Rank
   (a) Dominant males versus subdominant males

Habituation Tests. These tests (Halpin, 1974) were used when animals did not show a spontaneous preference for one sample over another. A subject was presented with a single plate carrying one type of scent for 5 min. The plate was then removed, and a 5-min test, presenting a choice between a second sample of the previous type and a novel scent, was given. It was expected that animals who can discriminate between the samples will investigate the novel scent more frequently than the sample to which they have been habituated.

Collection and Preparation of Scent Material

Natural scent marks were collected by placing a clean stimulus plate into the home cage of a donor monkey. If several donors were required, the plate was placed with each one in succession. The tamarins scent marked the stimulus plates readily. The marked plates then were transferred to the cage of the subject to be tested without manipulation of the marks. In some experiments, scent marks from several donors were collected on glass plates and processed into methanol as described in Chemical Methods (see below). In these cases, the methanol solution of three scent mark equivalents was applied to aluminum plates, and the plates were placed into the cage of the subject immediately after all solvent had evaporated. When urine samples were tested, uncontaminated

urine was aspirated from plastic sheeting placed under the home cage of a donor and samples of 1 ml or 0.4 ml were placed on a stimulus plate.

Behavioral Results

Control Experiments. Because of the interest of the tamarins in all novel objects which are introduced into their environment, a number of control tests were performed. These tests compared their responses to novel objects carrying conspecific scent material with their responses to either unscented novel objects or to objects scented with control odors.

In choice tests offering two clean aluminum plates identical to those used as substrates for scent presentation, the animals did not discriminate between the plates. Their responses to these plates were much lower than to plates carrying scent marks (Epple et al., 1980). In tests offering a choice between a plate scented with exaltolide (a synthetic musk), and a clean plate, no preference was shown (Epple, unpublished). In tests offering a single plate at a time, sniffing and scent marking did not differ in response to clean plates, plates scented with exaltolide, and plates scented with synthetic mixtures of squalene and those butyrates which are among the components of natural scent marks (see below). Plates scented with natural scent marks, on the other hand, elicited significantly more sniffing and marking than any of the controls (Epple et al., 1980). These results show that, although novel objects and arbitrary novel odors elicit sniffing and scent marking, conspecific odors do so at a significantly higher level.

Signal Content of Natural Scent Marks and Urine. The responses of the tamarins to natural scent marks and urine suggest that the scent marks and, to a much lesser degree, voided urine, contain a number of communicatory messages. When *S. fuscicollis* were presented with choices between scent marks or urine samples of their own species and equivalent material from other species of callitrichids (*Callithrix jacchus*, the common marmoset, and *Saguinus labiatus*, the red-bellied tamarin), they sniffed and scent marked the material from conspecifics significantly more frequently (Epple et al., 1979). Furthermore, when given a choice between the scent marks of two subspecies maintained in our colony (*S. f. fuscicollis* and *S. f. illigeri*), the former subspecies preferred its own scent over that of *S. f. illigeri* (Epple et al., 1979).

When test animals were presented with choices between marks of males and marks of females, they sniffed and scent marked samples from males more frequently than those from females under a variety of conditions. The response appears to be largely independent of the relative amounts of the presented material. This response was shown when each stimulus plate was scent marked by a single donor male or female (Epple, 1971), and when two donors marked each plate (Epple, 1974b, and unpublished). When one plate carried the marks of two females and the other plate carried the marks of only one male, male scent was preferred. This response was also shown when a plate marked by two

males was offered as an alternative to a plate marked by only one female (Epple, 1974b). Preference for material from males was also shown when a choice between scent marks of eight males and eight females, pooled in solvent, was presented (Epple, 1974b). When given a choice between a mixture of male and female marks, and female marks alone, the test animals sniffed both with equal frequency. However, the scent marked the plate carrying the mixture more frequently than the plate carrying female marks, suggesting that a signal for maleness was contained in the mixture (Epple, 1978).

When marks from males and females were presented under a screen which made it impossible for the monkeys to contact the samples directly, the same behavioral responses were observed. This indicates that the male–female discrimination can be made on the basis of volatile constituents of the mark alone (Epple, 1978). When voided urine alone was presented, female subjects preferred urine from males over urine from females, but male subjects did not discriminate between the samples (Epple, 1978). Moreover, the complex scent marks were highly preferred over voided urine from the same individual when a choice between marks and urine was given (Epple, 1978). In another experiment, marks from intact males were preferred over those from castrates, implying a discrimination of hormonal state (Epple, 1979). Marks of castrated males, on the other hand, were not preferred over those of intact females (Epple, unpublished).

The ability of the tamarins to discriminate between scent marks of unknown male individuals was demonstrated by means of habituation tests (Epple et al., 1979). An additional experiment showed that the animals actually utilize the scent to identify known individuals. The tamarins were allowed to interact with either a strange male or female for 10 min, during which they exchanged aggressive displays with the stranger. It was expected that this interaction would motivate the animals at a later time to be particularly interested in their opponent's marks. Each encounter was followed by a choice test, offering marks from the recent opponent versus those of a familiar donor of the same sex. The subjects preferentially investigated the recent opponent's marks. This response was shown even when the tamarins were tested several days after they had encountered the opponent (Epple, 1974b). However, subjects did not discriminate between urine samples of two individuals presented under the same conditions (Epple, 1978).

Social status also appears to be encoded in scent marks, either by the amount of scent deposited or perhaps by a difference in scent quality between dominant and subdominant animals. When the monkeys received a choice between marks of unfamiliar dominant and subdominant males, the scent marks of the dominant males were preferred (Epple, 1973).

The stability of the chemical signal over time has been investigated. In series of single-plate tests and choice tests, freshly marked plates were sniffed and marked more frequently than unscented plates, plates carrying control odors

(butyrate–squalene mixtures or exaltolide), and plates carrying scent marks which had been aged at room temperature for various periods of time. Depending on the conditions of testing, aging scent marks remained attractive to the monkeys for 1–3 days. Signals identifying gender also appeared to persist for this period of time. The animals discriminated between scent marks from males and scent marks from females on the basis of fresh material, and 1- and 2-day-old material, but not on the basis of 3- and 4-day-old marks (Epple et al., 1980).

In summary, the results of the behavioral studies reviewed above demonstrate that the complex scent mark material of *S. fuscicollis* is an important vehicle for communicating the identity of species, subspecies, gender, and individual; its social status and hormonal condition; as well as the age of the material. Urine alone contains information on species and gender, but not on individuality, and is behaviorally much less attractive than scent marks.

CHEMICAL STUDIES

Methods

Our findings that the scent mark material, and not urine, is the important vehicle for communication via chemical signals led us to an investigation of the chemical nature of the scent marks. Initially, gas chromatography (GC) together with mass spectrometry (GC-MS) was used as the analytical method of choice. Most of these results, together with experimental details, have been published. We present here a brief description of the procedures employed in the chemical analysis of the scent material. The reader is referred to the original publications for further descriptions (Smith et al., 1976; Yarger et al., 1977; Golob et al., 1979).

Preparation of Scent Material

Scent marks from one or more donors were obtained by allowing the animals to mark frosted glass plates (61 × 5 × 0.6 cm) attached to aluminum plates of the same size. The deposited material was removed by rinsing with 30 ml nanograde methylene chloride–methanol (3 : 1, v/v), and the resulting solutions stored at −76°C. Prior to analysis, the solvent was removed by careful rotary evaporation at reduced pressure. For analysis, the residue was dissolved in nanograde hexanes and further reduced in volume (15–20 μl) under nitrogen. When used for bioassay, the residue was taken up in nanograde methanol or hexanes (0.5 ml per three scent marks).

Analytical Procedures

The identification of the major volatile components of the hexane-soluble material was accomplished using GC and GC-MS as the primary tools. Both

packed columns and high-resolution glass capillary columns were employed. Additionally IR, NMR, and total synthesis of each component established the structures (Smith et al., 1976; Yarger et al., 1977; Golob et al., 1979).

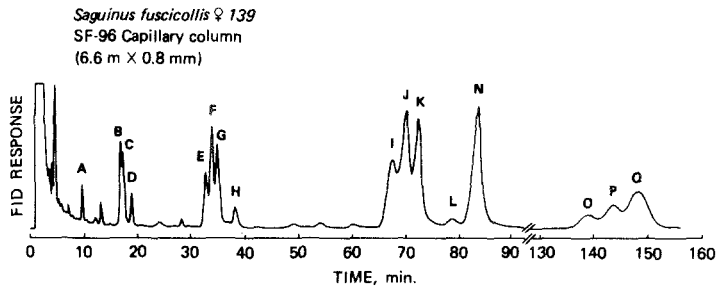
Quantitative analysis of the volatile constituents of scent material for individuals of different donor types (i.e., males or females of different subspecies) was performed on a Perkin-Elmer gas chromatograph interfaced to a Perkin-Elmer PEP-II computer. High-resolution SF-96 coated open tubular glass capillary columns were employed for fractionation. Quantification of the major components was accomplished by integration of their GC peak areas. These values were expressed as an area percentage of the total eluate of all these components.

Chemical Results

Analytical studies employing gas chromatography and combined gas chromatography–mass spectrometry demonstrated that the major volatile constituents of hexane-soluble material extracted from natural scent marks are squalene and a number of saturated and unsaturated alcohols esterified with butyric acid (Figure 2). Under our conditions of analysis, these components constituted approximately 96–98% of the volatile material of the scent mark (Smith et al., 1976; Yarger et al., 1977). The structures of these naturally occurring butyrates were confirmed by comparison of their IR, NMR, and mass spectra with the respective spectra of the butyrates obtained by total synthesis (Golob et al., 1979). Approximately 0.5 g of each butyrate was prepared. The availability of synthetic samples of each butyrate (99.9% pure) permitted the formulation of synthetic butyrate–squalene scent marks.

All of the butyrates and squalene were found to be present in the scent marks of males and females of all subspecies of *S. fuscicollis* investigated and of their hybrids. No qualitative differences between the marks of males and females or between different subspecies and hybrids could be detected in the retention time area of these compounds.

The remaining 4% of the volatile scent mark components are generally of higher volatility, eluting by GC before the first butyrate ester. The composition of the highly volatile region is quite complex, containing numerous compounds, among them aldehydes, esters, ketones, and acids. Analytical work on the chemical constituents of higher volatility is presently in progress. A few compounds other than the butyrates and squalene, among them some additional acids, are also present in the butyrate–squalene region and may be of some importance to the communication system. The scent marks also contain a number of constituents of high molecular weight, the presence of which can be demonstrated by other techniques such as UV absorption, gel electrophoresis, and thin-layer chromatography (Belcher, unpublished). Little is known currently about the composition of the low volatility compounds.



Peak	Molecular Formula	Identification
		SATURATED
A	$C_{20}H_{40}O_2$	
D	$C_{22}H_{44}O_2$	
H	$C_{24}H_{48}O_2$	
L	$C_{26}H_{52}O_2$	
		MONOENES–TYPE A
B	$C_{22}H_{42}O_2$	
F	$C_{24}H_{46}O_2$	
J	$C_{26}H_{50}O_2$	
P	$C_{28}H_{54}O_2$	
		MONOENES–TYPE B
C	$C_{22}H_{42}O_2$	
G	$C_{24}H_{46}O_2$	
K	$C_{26}H_{50}O_2$	
Q	$C_{28}H_{54}O_2$	
		DIENES
E	$C_{24}H_{44}O_2$	
I	$C_{26}H_{48}O_2$	
O	$C_{28}H_{52}O_2$	
		SQUALENE
N	$C_{30}H_{50}$	

FIG. 2. Major volatile components of *Saguinus fuscicollis* scent marks. Reprinted with permission from Smith, A.B. III et. al. (1985). *Science* 228:175–177. Copyright 1985 by the AAAS.

As mentioned above, no qualitative differences in the butyrate–squalene area (the area of the major volatile constituents) were detectable in the scent marks from different subspecies and from males and females. Therefore, we tested the hypothesis that differences in the concentrations of these compounds are responsible for encoding some of the communicatory signals which our behavioral studies have indicated to be present. The relative concentrations of all butyrates and of squalene in a series of scent marks of individual donors were determined as described in Chemical Methods. The concentration profiles

of these compounds in the scent marks of a number of males and females of *S. f. fuscicollis*, *S. f. illigeri*, and of some hybrids were monitored in this way over extended periods of time.

The concentration profiles in the scent marks of these males and females remained remarkably constant. Figure 3, showing the "scent print" of a hybrid male (♂100) over a period of three years, documents this finding. The "scent prints" of the two subspecies appeared to differ from each other, and those of hybrids were different from those of each purebred subspecies (Epple et al.,

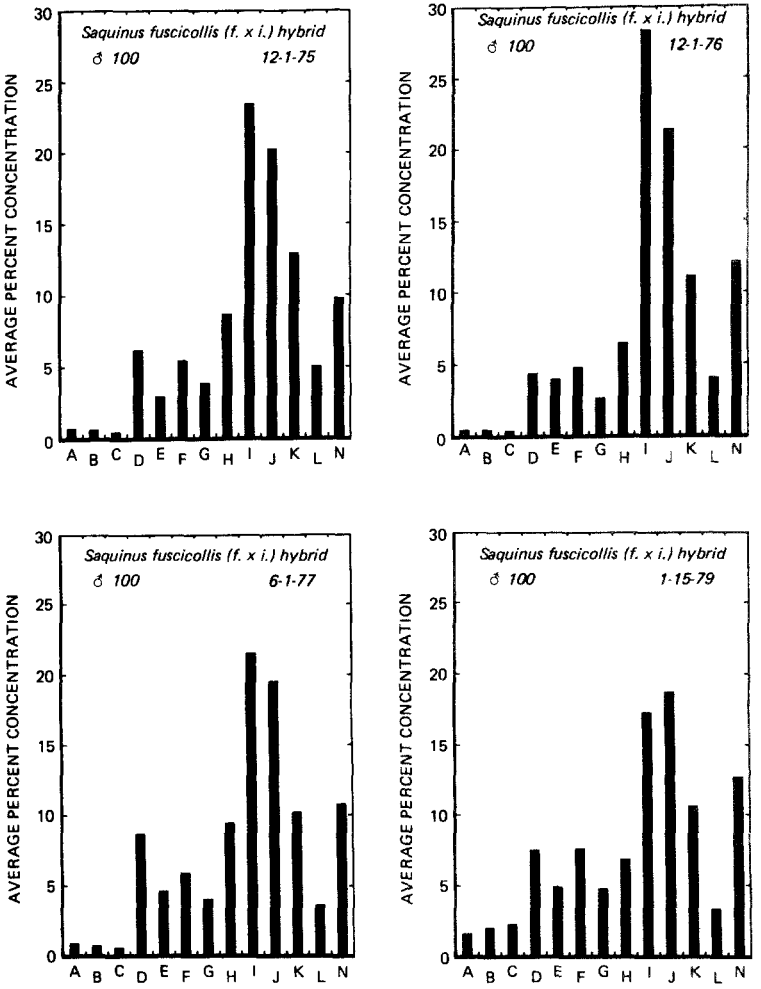


FIG. 3. Scent prints of *Saguinus fuscicollis* male over time.

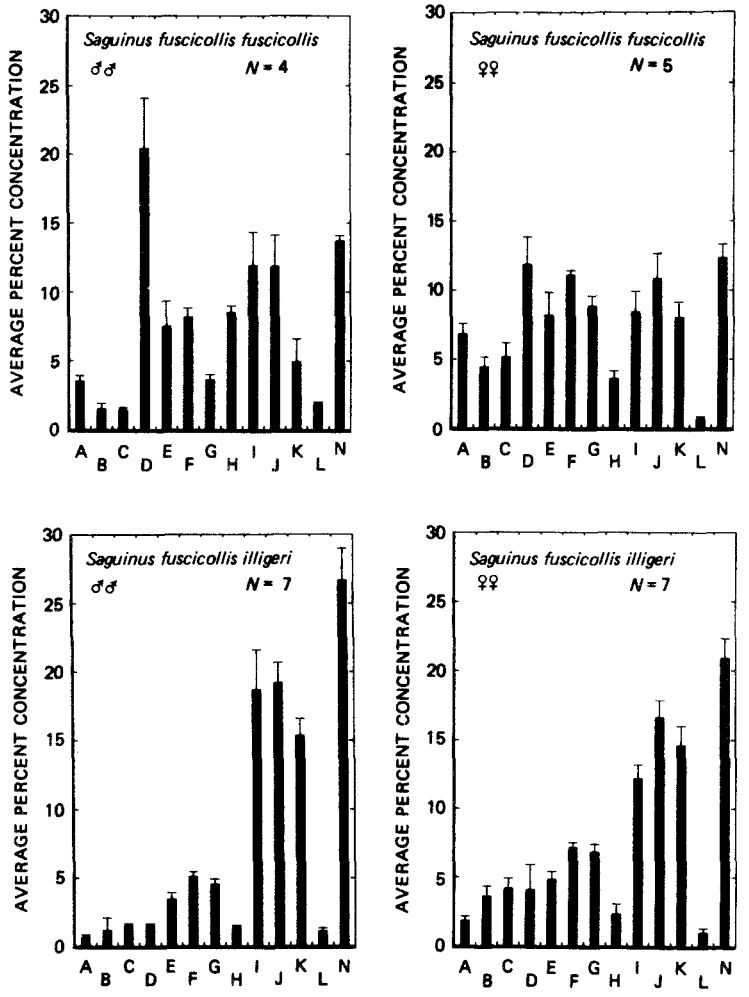


FIG. 4. Average concentration profiles of *Saguinus fuscicollis* males and females of two subspecies. Reprinted with permission from Smith, A.B. III et. al. (1985). *Science* 228:175–177. Copyright 1985 by the AAAS.

1979). Moreover, there also appeared to be differences in the concentration of some of the butyrates between males and females. Figure 4 shows average concentration profiles for males and females of both subspecies. Differences in profiles are apparent. In an attempt to examine more rigorously these differences, computerized pattern-recognition analyses were used to classify the concentration profiles of the scent marks as to gender and subspecies (Smith et al., 1985).

PATTERN RECOGNITION STUDIES

In general, pattern recognition methods are employed to classify samples or patterns according to a particular set of measurements or descriptors. The sample or pattern is represented as a point in *n*-dimensional space where the axes of this space are defined by the set of measurements used to describe the pattern. Ideally, for a binary classification problem, the patterns will cluster in two different regions of space and meaningful differences between the two groups will be reflected by this clustering.

In our study, pattern recognition techniques were employed to classify the individual scent prints according to a particular set of descriptors, i.e., the relative concentrations of 13 major volatile components of the scent marks. (The three latest eluting components were not included, as integration of their relatively broad peaks gave inconsistent results.) Means and standard errors for the relative concentrations of 12 butyrate esters (A–L) and squalene (N) (see Figure 2) were calculated for the scent print of each individual, and these data were submitted to pattern recognition analysis. Classification was done in two different studies, one on the basis of gender (male and female), and the other on the basis of subspecies (*Saguinus fuscicollis fuscicollis* and *Saguinus fuscicollis illigeri*).

For the gender study, 48 donors (25 females and 23 males) of both subspecies, including hybrids, were used. Forty of these individuals were used as a training set, and their descriptors used to generate classification schemes (discriminants). The remaining eight individuals comprised a prediction set, whose descriptors were used to test the validity of the discriminants. Members of these sets were determined by random lot. The data were standardized by autoscaling and examined by standard pattern-recognition techniques. Using linear discriminant analysis (Jurs and Isenhour, 1975), a classification scheme was developed from descriptors B and C. The SIMCA pattern recognition method (Wold, 1976), which develops an individual mathematical model for each class to which patterns are to be assigned, developed a principal component model for each class based on compounds B, C, and H.

The subspecies study used 23 individuals (9 *Saguinus fuscicollis fuscicollis* and 14 *Saguinus fuscicollis illigeri*). Due to the small number of donors available, a prediction set was not used. Principal component analysis (Jackson, 1983) was performed on all 13 descriptors to develop a classification scheme from the first principal component. Additionally, using the SIMCA method, a one-principal-component model was developed for each class from seven compounds (A, D, F, G, H, K, and N).

Pattern Recognition Results

The pattern recognition studies statistically document the differences in the concentration profiles between males and females and between the two subspe-

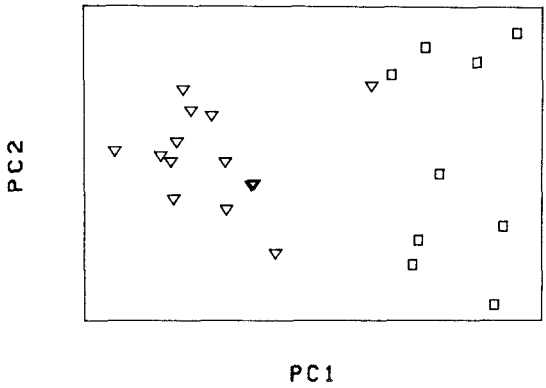


FIG. 5. A principal component representation of the pattern space defined by A, D, F, G, H, K, and N. Squares represent *S. f. fuscicollis*, the triangles represent *S. f. illigeri* individuals. Reprinted with permission from Smith, A.B. III et. al. (1985). *Science* 228:175–177. Copyright 1985 by the AAAS.

cies. In the gender identification study, the classification scheme based on descriptors B and C (butyrates B and C, Fig. 2) correctly classified 38 of the 40 members of the training set. When tested on the prediction set, it was 100% successful. Additionally, the SIMCA method correctly classified 36 members of the training set and all members of the prediction set. The subspecies study also produced good results. Both principal component analyses and the SIMCA method produced discriminants that correctly classified 22 of 23 animals. Figure 5 displays a two-dimensional representation of the clustering pattern for the subspecies study.

These results demonstrated that information derived solely from the concentration patterns of the major volatile components of scent material can be used mathematically to classify an animal as to gender and subspecies. Furthermore, it appears from the gender study that some components are more important than others in providing this information. However, the limited number of animals available in the subspecies study did not allow this point to be examined.

DISCUSSION

Pattern recognition has provided us with a new method for evaluation of complex chemical signal mixtures. The demonstration of mathematical classification of the butyrates and squalene concentrations into subspecies and gender categories does not necessarily imply that this information is utilized by the animals for the same purpose. The existence of such categories, however, suggests that they may be involved in structuring communicatory messages.

We have attempted to evaluate further the behavioral significance of the butyrates and squalene (Belcher et al., unpublished). A number of studies were carried out in which we altered the scent mark secretion by adding synthetic butyrates in concentrations sufficient to change the total butyrate–squalene profiles. In general, the scent prints of *S. f. illigeri* males differed from those of females of the same subspecies in the relatively lower concentrations of the first eight eluting butyrates. Those butyrates implicated by pattern recognition to be important for gender classification [B, C, and G (an earlier pattern recognition analysis included descriptor G as well)] were tested in the following manner.

Scent mark material was collected from male *S. f. illigeri* donors and to half of it was added synthetic butyrates B, C, and G sufficient to mimic the concentrations found in scent material from female *S. f. illigeri* donors. The spiked and unspiked samples were then presented to test animals in two-choice preference tests. Those butyrates implicated in subspecies discriminations (A, D, and H) had higher concentrations in material from *S. f. fuscicollis* than from *S. f. illigeri*. Therefore, in like manner, another pool of scent-mark material from *S. f. illigeri* male donors was divided, and to one half was added synthetic butyrates, A, D, and H sufficient to mimic the concentrations found in material from *S. f. fuscicollis* male donors. Again, the spiked and unspiked samples were subjected to standard two-choice preference tests. In neither bioassay did the subjects show a significant preference for one sample over the other. One explanation for these results is that other components in the scent mark which are essential for complete biological activity informed the subjects about the true identity of the donor type.

In another study, synthetic mixtures of squalene and all butyrates were prepared from compounds synthesized in our laboratory. These mixtures were formulated so that their gas chromatographic profiles mimicked the profiles of four individual males, two *S. f. fuscicollis* and two *S. f. illigeri*. Behavioral tests were then conducted, offering the monkeys a choice between synthetic formulations mimicking the squalene–butyrate areas of both subspecies. In these tests, the subjects showed significantly more scent marking in response to synthetic *S. f. fuscicollis* mixtures, while sniffing and contacting both formulations with equal intensity (Epple et al., 1979). The differences in scent marking behavior, although significant, were small, and a replication of this experiment did not yield significant differences in response to the two formulations.

Additional studies have evaluated the behavioral significance of fractions of scent mark material (Epple, unpublished). Scent marks collected in methylene chloride–methanol were evaporated and the residue dissolved in either hexane or methanol. Bioassay choice tests were conducted both with scent material dissolved in hexane and with material dissolved in methanol. In both cases, fractions of material from males were preferred over fractions of material from females, a response identical to that given to natural marks. However, when

fractions of the marks of individual donors were tested against the chemically untreated marks placed on the plates by the same donors, untreated marks were preferred over the hexane fractions from the same individual. Methanol fractions, on the other hand, appear to retain full activity. No discriminatory response was shown when plates carrying such fractions were tested against plates carrying natural marks from the same individual. The difference in activity between methanol and hexane fractions is not due to the presence of solvent since scent marks to which these solvents had been added and then allowed to evaporate were not discriminated from untreated scent marks.

Behavioral analysis of gas chromatographic fractions of the hexane-soluble scent material was also performed. [For preparative work, the scent material was chromatographed through packed columns (SE-30, Carbowax, or Dexsil on solid supports) and trapped via a Brownlee-Silverstein thermal gradient collector (Brownlee et al., 1969).] Earlier in our work, we encountered technical problems created by the use of methanol in gas chromatography and therefore used the hexane fraction in our further analytical and fractionation procedures. (Chemically-bonded fused silica columns now make possible the use of methanol as a solvent.) This was done with the understanding that, although biological activity was somewhat diminished, hexane fractions still contained the information concerning gender. This information was retained after preparative GC when the total eluate was collected as one fraction. However, GC fractionation of scent marks from males and from females into fractions containing the highly volatile compounds fixed in squalene, and into fractions containing only the butyrates and squalene, resulted in a loss of biological activity in both fractions (Epple, unpublished). These results suggest that neither the highly volatile compounds by themselves nor the butyrates and squalene alone contain all of the information. It is therefore likely that compounds from both regions work in concert to encode gender.

SUMMARY

The results of our behavioral studies are consistent in suggesting that compounds from both areas of volatility are necessary to encode messages on subspecies and on gender. It may well be that what is required for transmission of these messages is an elaborate pattern of chemicals which presents a total "chemical image" (Albone, 1984). Beauchamp et al. (1976) has compared the search for "the pheromone" to the search for "the note" of a song. However, we may find that certain "phrases" in the chemical profile identify the message and its donor as certainly as we identify a musical composition and its composer from a fragment or excerpt.

Defining these key phrases will require a multidisciplinary attack, and new avenues of analysis doubtless will be required. Our novel approach, using the

statistical methods of pattern recognition, has proven of immense value in targeting potentially significant chemical components. We expect that further use of this technique will be important in reconstructing significant phrases in complex chemical communication codes.

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ENZYMATIC ADAPTATIONS OF HERBIVOROUS INSECTS AND MITES TO PHYTOCHEMICALS[1]

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Abstract—A variety of oxidases, reductases, esterases, epoxide hydrolases, and group transferases in herbivorous insects and mites detoxify and facilitate the excretion of toxic phytochemicals (allelochemicals). Current theory indicates that the cytochrome P-450-dependent mixed-function oxidases (MFOs) are by far the most important enzymes because they have many attributes that are essential for an effective detoxification system. Data presented here on the midgut microsomal MFO activity of larvae of the gypsy moth, *Lymantria dispar*, are discussed in the light of previous work and support the theory. In the gypsy moth, the MFO levels exhibit a parallel trend with changes in specific feeding rates, and changes in the specific activity of the enzyme appear to be regulated ontogenetically and by inductive effect of chemicals in the diet. The specific activity of the MFOs rises more sharply on leaves of a highly preferred type-1 plant, the pin oak, than on an artificial wheat germ diet; the increase from mid-second instar to mid-fifth is 4.5- and 1.8-fold, respectively. The relationship of food consumption rate to increase in body mass (W) was slightly in excess of a 1 : 1 ratio for both pin oak and the artificial diet, indicating that the feeding rate surpasses the increase in W (a rare phenomenon in insects). Moreover, the surface-to-volume ratios are fairly constant for combined data of gut lumen and epithelium in second to fifth instars, because the volume occupied by the epithelial cells is much larger than in older ones. Thus, it is concluded that greater specific activity of the MFO is necessary with larval advancement to higher instars in order that they may process dietary allelochemicals with an efficiency comparable to younger larvae. Additional data suggest that MFO level increases reflect further adaptation to: (1) normal, seasonal changes in plants' allelochemical composition and concentration; (2) increase in allelochemical concentration in response to leaf damage; and (3) the risk faced by dispersing larvae of

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encountering a greater amount and variety of allelochemicals on suboptimal/less suitable plants. Evidence also has emerged recently for MFO-catalyzed metabolism/deactivation of numerous plant allelochemicals, including compounds that induce the enzyme. MFOs are further adapted for participation in the biogenesis of substances physiologically important to insects. Moreover, the catalytic center of the MFO system, cytochrome P-450, occurs in multiple forms; the significance of this important feature is discussed.

Key Words—AK-reductases, arginase, cytochrome P-450, enzyme induction, esterases, epoxide hydrolases, *Lymantria dispar*, Lepidoptera, Lymantriidae, mixed-function oxidases, rhodanese, transferases, urease.

INTRODUCTION

Herbivores have been subject to an astonishing array of toxins and other slow-acting but nevertheless fitness-reducing secondary chemicals produced by plants; these phytochemicals are commonly called allelochemicals. Consequently, herbivores have adapted to deal with chemical defenses, and the present day relationships reflect an ongoing evolutionary process termed "coevolution" (Ehrlich and Raven, 1964). Recent studies have revealed great complexity and diversity in insect–plant relationships, particularly in mechanisms of host recognition and nonhost rejection (reviews in Ahmad, 1983a; Bell and Cardé, 1984). After the initial search has brought an insect in close proximity with potentially favorable plants, it must then make a critical assessment of host suitability for feeding and/or oviposition on the basis of its behavioral and biochemical adaptations to avoid or neutralize the deleterious effects of allelochemicals present in the food plants (Brattsten and Ahmad, 1986).

Biochemical adaptations to plant allelochemicals include penetration barriers, special excretion, temporary binding with carrier proteins, storage in adipose tissues, sequestration of allelochemicals in specialized organs or body spaces for defensive purposes, modification of target sites, and enzymatic detoxifications (reviews in Rosenthal and Janzen, 1979). Five major enzyme systems particularly suited for the deactivation and elimination of plant allelochemicals are: cytochrome P-450-dependent monooxygenases, commonly called mixed-function oxidases (MFOs); reductases; esterases; epoxide hydrolases; and glutathione-*S*-transferases. Several other group transferases also are important.

These enzymes can be grouped conveniently into primary (phase I) and secondary (phase II) metabolic processes. Generally, the MFOs, reductases, esterases, and glutathion-*S*-transferases are considered phase-1 enzymes because they can attack the lipophilic compound directly. Glutathione-*S*-transferases are also phase-II enzymes, e.g., they attack epoxide intermediates of phase-I reactions. The resulting product of a phase-I reaction is usually innocuous or less toxic and more hydrophilic than the parent compound. Metabolites of phase-

I reactions often are rendered more polar for excretion by conjugation with amino acids, glutathione, sugars, phosphate, and sulfate by specific group transferases (reviewed by Brattsten, 1979a; Ahmad et al., 1986).

In insects, carbohydrate conjugates of alcohols, phenols, aromatic carboxylic acids, amines, and sulfhydryls are formed by UDP-hexosyltransferase. Phosphotransferase (a kinase) and sulfotransferase reactions are known in insects and other arthropods but to a lesser extent. Acyl (*N*-methyl and *N*-acetyl) conjugations and amino acid conjugations have not been studied in insects. The epoxide hydrolases (previously called epoxide hydratases or hydrases) are important inactivating enzymes of arene oxide (epoxide) intermediates of phase-I reaction catalyzed by the MFOs. The enzymatic action is that of a hydrolase except that the catalysis does not dissociate the parent compound, but involves conversion of epoxides to diols by stereospecific addition of a molecule of water (Brattsten, 1979a). Epoxide hydrolases occur in many phytophagous insect species, and in the two-spotted spider mite, *Tetranychus urticae*, and also are inducible by plant allelochemicals (Mullin et al., 1982; Mullin and Croft, 1984; Yu, 1986). Further, these studies suggest enzyme multiplicity, the *trans*-epoxide hydrolase being more commonly associated with anthropod herbivory than the *cis*-epoxide hydrolase. Glutathione-*S*-transferases are of wide occurrence in insects. In the fall armyworm, *spodoptera frugiperda*, they are highly inducible by many allelochemicals and by plants that are widely different in their secondary chemistry (Yu, 1986, and refs. therein). There is no direct evidence for allelochemical metabolism by these enzymes, but the induction suggests transferase–allelochemical interactions and hence, by inference, metabolism. As discussed later, a glutathione-*S*-transferase may be involved in detoxification of the epoxide of precocenes in insensitive insects.

Regarding phase-1 enzymes, of the many specific reductases, the cytosolic aldehyde ketone (AK) reductases are of special interest as they are capable of attacking a broad range of aldehydes and ketones, two structures which are common to many toxic or repellent allelochemicals (reviewed by Brattsten, 1979a). They have been shown to reduce naturally occurring benzaldehyde, daunorubicin, and many synthetic substrates.

The esterases, arylesterase (Mn^{2+} and Co^{2+}) and carboxylesterase, differ in their rate of substrate hydrolysis and in their sensitivity to organophosphates (arylesterases being insensitive). Their endogenous roles are poorly understood, except for JH-specific esterase (Sparks and Hammock, 1979), and a pheromone-deactivating enzyme in receptors of the cabbage looper moth *Trichoplusia ni* (Ferkovich et al., 1982). These esterases occur in both the cytosol and microsomes and are of wide occurrence in mammals, insects, and mites. Together, the two esterases can hydrolyze a variety of aliphatic, phenolic, polycyclic, and heterocyclic esters and lactones. Esters of benzilic acid, chlorobenzilate, chloropropylate, and bromopropylate were found to be hydrolyzed by microsomal carboxylesterase of the rat liver (Knowles and Ahmad, 1971). Syn-

thetic pyrethroid insecticides were also metabolized by microsomal esterase, but the MFO-catalyzed oxidation of naturally occurring pyrethrins was more predominant in insects (Abernathy and Casida, 1973; Jao and Casida, 1974). The pyrrolizidine alkaloid esters can be hydrolyzed by the carboxylesterase and thus detoxified. MFO attack produces an active toxin, a pyrrole derivative, in mammals. In danaid butterflies, the speculation is that the MFOs' action on these compounds produces male courtship pheromones (see Brattsten, 1979a, and refs. therein).

The esterases are induced by various plant allelochemicals. Feeding on celery, potato, and parsley induced the enzyme in larvae of the fall armyworm (Yu, 1986). In two-spotted spider mites, esterase activity is elevated more when fed umbellifers such as celery and carrot, compared with snap bean (Mullin and Croft, 1983). In polyphagous Lepidoptera, the activity of midgut carboxylesterase is correlated with a wide host range (Turunen and Chippendale, 1977). In larvae of gypsy moth, *Lymantria dispar*, the postmitochondrial fraction of the midgut exhibited 91.5% of the total carboxylesterase activity of all tissues (Kapin and Ahmad, 1980). In herbivorous insects, a lipase is known to be involved in the utilization of triglycerides during embryogenesis, but after hatching the "true lipase" is not detected (Kapin and Ahmad, 1980, and refs. therein). Perhaps the function of carboxylesterase is in the utilization of lipids by hydrolysis to substrates suitable for energy-generating metabolism, but an additional outcome of this process may be that toxic lipids are deactivated. Since plants produce many toxic esters and lactones, including JH-like compounds, the role of esterases in the deactivation of plant allelochemicals deserves thorough investigation.

The phase-I enzymes have the potential of working in concert with phase-II enzymes to bring about deactivation of lipophilic molecules. This renders them highly water-soluble to facilitate their excretion. Recent work has also unravelled specific detoxification enzymes. In the bruchid beetle, *Carydes brasiliensis*, the arginase level is high, accompanied by an exceedingly active urease (rare in insects). Thus appreciable amounts of L-canavanine, a nonprotein amino acid, are converted to canaline and urea by arginase action, and the urease further metabolizes urea to CO_2 and NH_3; the latter is recycled as a source of primary nitrogen (Rosenthal et al., 1982). Long and Brattsten (1982) and Beesley et al. (1985) demonstrated that the enzyme rhodanese, is present in small amounts in insects that feed with impunity on plants containing cyanogenic compounds. This enzyme can convert cyanide to less toxic thiocyanate. However, Long and Brattsten (1982) concluded that rhodanese is not important in cyanide detoxification. A better possibility would be its incorporation into β-cyanoalanine by a specific synthetase known from plants only. This enzyme is under investigation for its presence in insects (Beesley et al., 1985).

The cytochrome P-450-dependent MFOs are by far the most important enzyme system because they detoxify a wide range of foreign chemicals (e.g.,

drugs and pesticides), and also because phase-II reactions depend on the primary oxidative attack of this phase-I enzyme system. Tissue/cell distribution, enzyme components, catalytic events, and reactions catalyzed by the MFO system were thoroughly reviewed (Ahmad, 1979; Brattsten, 1979a). The catalytic center, cytochrome P-450, requires for oxidative activity reducing equivalents from NADPH (supplied via a reductase, NADPH cytochrome P-450 reductase), and molecular oxygen. The basic reaction that results in the formation of alcohols and phenols is:

$$RH + O_2 + NADPH + H^+ = ROH + NADP^+ + H_2O$$

where RH is the substrate and ROH the hydroxylated metabolite. The monooxygenation reaction, however, gives rise to several other biotransformations, depending on substrate structure and stability of the intermediate metabolite. Well-known reactions are: aliphatic and aromatic hydroxylations, arene oxide or epoxide formation, *O*-, *S*-, and *N*-dealkylations, and thiophosphate oxidation. The oxidation of sulfides (thiothers) to sulfoxides and sulfones previously considered to be a cytochrome P-450 reaction, is apparently catalyzed by a FAD monooxygenase (Brattsten and Ahmad, 1986).

In 1971, the current MFO theory came into focus, indicating for the first time deactivational interactions of plant allelochemicals with MFOs (Krieger et al., 1971). Gut enzyme level was found to be significantly higher in polyphagous Lepidoptera than in oligophagous and monophagous species. The need for higher MFO level was in agreement with the greater risk for generalists in contacting plants potentially richer in allelochemical diversity and concentration compared with specialists which usually are well-adapted to the host's specific defensive chemicals (Feeny, 1976; Rhoades and Cates, 1976). The theory received impetus with the discovery, six years later, that the gut MFOs of larvae of the southern armyworm, *Spodoptera eridania*, a polyphagous lepidopteran, were induced rapidly by a variety of allelochemicals and that, following induction, the larvae became more tolerant of the alkaloid, nicotine (Brattsten et al., 1977). Brattsten (1979b) found significantly greater induction of southern armyworm's gut MFOs when larvae fed on umbellifers such as carrot, parsley, coriander, and *Spananthe*, than when they fed on their favored food plant, the legume, lima bean.

In another lepidopteran, the variegated cutworm *Peridroma saucia*, feeding on peppermint induced the larval gut MFOs more than on another plant, snap bean (Yu et al., 1979). In the gypsy moth, *Lymantria dispar*, the MFO titer was found to increase both ontogenetically and by induction, indicating an increase in biochemical defense congruent with larval development and concomitant increase in food consumption (Ahmad, 1982). In the polyphagous Japanese beetle, *Popillia japonica*, the gut MFO was induced rapidly and attained maximal levels within 24 hr of feeding on sassafras, phlox, and broccoli (Ahmad, 1983b). The induction was highest in field polyphagous beetles, moderate

in laboratory-simulated polyphagy, and lowest on single-plant feeding. As in gypsy moths, MFO activity was reduced throughout all nonfeeding developmental stages, but was maintained at high levels in active feeding stadia. Mullin et al. (1982) reported that the MFO level was higher in the phytophagous two-spotted spider mite than in the predatory mite, *Amblyseius fallacis*.

The MFO theory has been recently criticized, however. Dowd et al. (1983) provided many examples of MFO-catalyzed metabolism of allelochemicals by insects, but as discussed later, they questioned the broader implications of the "MFO–plant–toxin" relationship. Gould (1984) claimed that "evidence presently available does not offer strong support of the theory, because key pieces of information are lacking."

Presented in this paper are new data on the gypsy moth's MFOs which, along with previous published work and new findings by other researchers, provide a better assessment of the overall role of MFOs in herbivory in insects and answer most, if not all, of the recent criticisms. Areas requiring future investigation are discussed. Also briefly discussed is the role of MFOs in the biogenesis of substances physiologically important to insects.

METHODS AND MATERIALS

Insects. Gypsy moth larvae used to monitor growth, feeding rates, and MFO activity were hatched in the laboratory from field-collected egg masses and reared at 23° ± 1°C, 55% relative humidity, and 14:10 light–dark photocycle, on a wheat-germ based artificial diet or on leaves of pin oak, *Quercus palustris*, a preferred (type I) food plant of the larvae (Doane and McManus, 1981).

Larval Development and Feeding Rates. Control diet blocks, 1.5 × 1.5 cm, and pin oak leaves were first weighed to obtain fresh (wet) mass and then dried at 90°C for 6 hr and reweighed to obtain dry mass. The water content of the diets was thus determined. The uneaten portion of the diet block or pin oak leaf was weighed after drying at 90°C for 6 hr to determine dry mass. Dry mass of food consumed was then calculated using the ratio of wet to dry mass as mentioned.

Measurement of Gut Surface Area and Volume. The mid-instars of first to fifth stadia were dissected and the guts removed and fixed in Bouin's fluid. Fixed tissues were embedded in paraffin, and 15-μm serial sections were prepared and mounted on slides. Although the lepidopteran gut is a long undifferentiated tube (Berridge, 1970), the midgut could easily be distinguished from the much thinner and more transparent foregut and the constriction at the junction with the hindgut (Figure 1). The sections and a piece of 1-cm^2 paper were placed in a photographic enlarger and their outlines were traced onto sheets of white paper. Areas of these enlarged sections were measured using a Houston

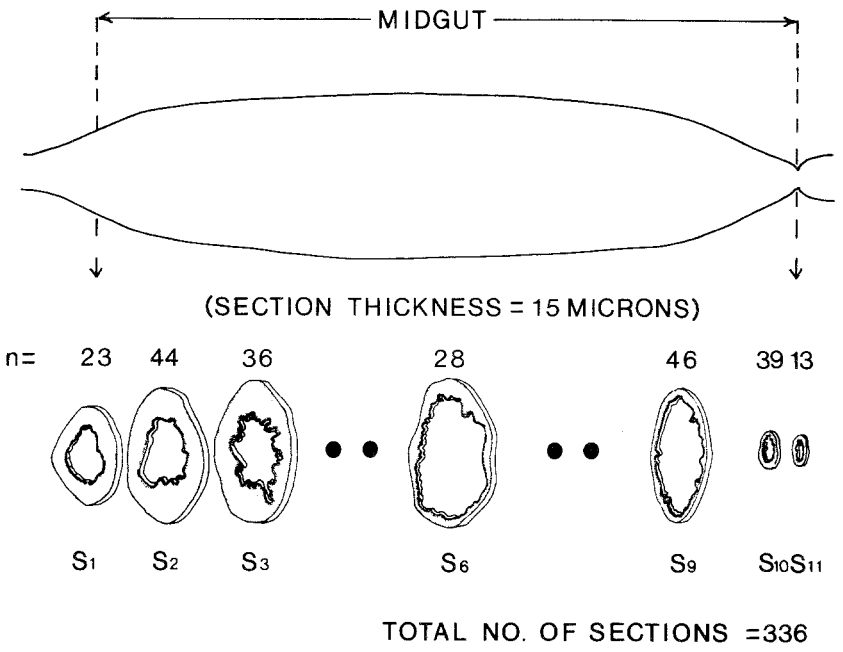

FIG. 1. Simple graphic presentation of gypsy moth's 3rd-instar gut indicating the position the midgut was excised from between fore- and hindgut. Midguts of other larval stages were similarly dissected and 15-μm thick sections were prepared. S and n denote slide numbers and number of serial sections on slides. Area occupied by epithelial cells is depicted in gray.

Instruments Highpad digitizer attached to a MINC laboratory computer (Digital Equipment Corp., Maynard, Massachusetts).

The digitizer was programmed to transform the circumference of an irregular circle (due to villi or folding of the epithelial layer) to an equivalent completely round circle. This permitted the calculation of the radius from circumference of each section from the measured flat surface area. The section thickness (h) being a known constant (15 μm), the surface areas and volumes were computed using a program utilizing the three related equations: (1) circumference = $2\pi r$ (mm), (2) surface area = $2\pi rh$ (mm^2), and (3) volume = $\pi r^2 h$ (mm^3). Summation of measurements of all sections from a midgut provided its total surface area and the volume enclosed. These two parameters were computed for gut lumen, lumen plus gut epithelium, and gut epithelium alone. The total surface area in each case was divided by total volume to obtain the surface-to-volume ratios. Number of sections (n) measured in this manner for a single midgut of each instar was: first, 159; second, 292; third, 336; fourth, 459; and for fifth instar, 1023.

Assessment of Food Intake Increases with Body Mass. The food intake is correlated to body mass, setting a limit to the size of the gut and to the rate at which food can be processed. This is an exponential function represented by the general form:

$$y = b \cdot a^x \tag{1}$$

The logarithmic form of this equation is:

$$\log y = \log b + x \log a \tag{2}$$

Using equation (2), feeding rate and body mass relationship was plotted as a linear function between $\log_e$ of y (feeding rate) plotted againt $\log_e$ of x (body mass). The scaling factor between body mass (W) and feeding rate (exponent of W = slope of the regressed line) was established according to Islam (1981; in Crawley, 1983).

Leaf Moisture. The moisture content of pin oak leaves (from a tree that had been free of any infestation over the past 10 years) of various ages from newly flushed to the time larvae reached mid-fifth instar was determined by subtracting dry weight from wet weight; dry weight was obtained by drying leaves at 90°C for 6 hr, cooling in a desiccator overnight, and then reweighing.

In another experiment designed to determine changes in water content upon herbivory, leaves from another pin oak tree that was partially infested in 1983 with gypsy moth larvae were used. Water content in undamaged leaves was determined as described above. Larvae from infested leaves were prevented from moving onto undamaged leaves as described by Schultz and Baldwin (1982).

Assay of MFO activity. Optimal assay conditions for gypsy moth microsomal gut MFO activity had previously been investigated using the substrate *N,N*-dimethyl-*p*-nitrophenyl carbamate (DPNC) (Forgash and Ahmad, 1974). In this investigation, I used *p*-chloro-*N*-methylaniline (PCNMA), which produced higher turnover than did DPNC and provided essentially the same flat bell-shaped enzyme activity–pH profile, as reported for DPNC. Maximal activity occurred at 33–34°C (instead of 35–36°C for DPNC), with only ca. 3–4% less activity at 32°C. Addition of BSA, which was found to enhance DPNC activity by 24.5%, had no effect on *N*-demethylation of PCNMA. The reaction rate was linear up to 30 min. All conditions of the MFO assay were the same as in similar work on the southern armyworm (Brattsten et al., 1977), and the Japanese beetle (Ahmad, 1983b).

Clean midguts obtained by dissecting the larvae were homogenized and centrifuged to obtain microsomal pellets (cf. Ahmad, 1983b); 500 midguts of second- and third-instar larvae, 350 midguts of fourth-instar larvae, and 250 midguts of fifth instars were used. After suspension of microsomes in isotonic KCl (Ahmad, 1983b), the protein concentration was determined by the method

of Miller (1959). Immediately after protein determination, the MFO activity was assayed. Upon termination of the reaction, chromogenic reagent was added and the clear supernatant was read for absorbancy at 450 nm against an appropriate blank in the double-beam mode in a Beckman spectrophotometer (model 35). The specific activity of the MFO-catalyzed *N*-demethylation was obtained and the activity expressed as picomoles per milligram protein per minute (at 32°C).

RESULTS

Larval Body Mass and Feeding Rate. Studies of gypsy moth development on the two diets were completed up to the eclosion of adults. However, in Table 1, the data are provided only up to mid-fifth instar, commensurate with MFO activity measurements to this developmental stage. The following trends are evident: (1) increase in mass on leaves of pin oak and the artificial diet was the same until the third instar ($P > 0.05$ by t test), (2) a dramatic increase in mass occurred by the mid-fourth instar, (3) mass of fourth and fifth instars was greater ($P < 0.05$ by t test) when larvae were reared on artificial diet than when reared on pin oak, and (4) food consumption rates (dry mass/48 hr) followed closely the increase in larval mass. Fifth instars that developed into male pupae (data not included in Table 1) continued feeding for additional 4–5 days and greatly increased their mass by 2.5-fold on pin oak leaves and by 2.0-fold on the artificial diet. Gut clearance and pupation followed. Fifth instars that developed into sixth-instar larvae (females) exhibited a great surge in food consumption and nearly doubled their mass relative to the terminal fifth-instar males. Despite differences in mass gain and increase in food intake between the two diet groups, mass of male and female pupae, sex ratio, and days for adult eclosion were the same for larvae reared on either food. Therefore, gypsy moths fared equally on both diets, and the developmental synchrony must be due to intrinsic regulation (most likely hormonal), especially at the time of pupation.

In both diet groups, relative feeding rates dropped as larvae approached the next molt (Table 1). Larvae from 12 to 24 hr of apolysis had less food in their gut than larvae in mid instars; all late-instar larvae were characterized by retraction of the head capsules. Moreover, relative feeding rates (dry mass/1.0 g body mass/48 hr) increased from first-mid instar to the fifth-mid instar, the trend being more pronounced in pin-oak fed larvae than in the artificial-diet group. The rate of feeding as larvae advanced in age was increased more than the increase in body mass. This finding has important implications vis-à-vis increase in the specific activity of gut MFO enzyme, as discussed later.

Gut Surface Area and Volume Relationship. Surface area-to-volume ratio of the gut is an important factor when studying the amount of food that can be taken up into the gut lumen, subsequent passage of food materials through the

TABLE 1. BODY MASS AND FEEDING RATES OF GYPSY MOTH LARVAE IN THEIR MID AND LATE 1ST TO 5TH INSTARS[a]

Larval instar	Days from hatch	Larval mass (mg) ($\bar{X} \pm$ SE)	Food consumption (dry mass, mg/48 hr, $\bar{X} \pm$ SE)[b]	Relative feeding (mg/1.0 g larval mass/48 hr)
Pin oak				
Mid-1	7	7.5 ± 0.21	2.6 ± 0.30	347
Late-1	10	12.3 ± 0.39	2.9 ± 0.49	236
Mid-2	13	17.0 ± 0.45	6.1 ± 0.67	359
Late-2	16	26.1 ± 0.46	7.3 ± 0.84	280
Mid-3	20	29.1 ± 0.52	11.6 ± 1.42	399
Late-3	23	36.2 ± 0.73	12.0 ± 0.94	331
Mid-4	28	54.4 ± 1.31	25.9 ± 1.49	476
Late-4	33	122.4 ± 8.90	23.2 ± 1.42	190
Mid-5	37	181.8 ± 11.60	80.0 ± 3.66	440
Wheat-germ diet				
Mid-1	7	7.6 ± 0.11	2.7 ± 0.10	355
Late-1	9	12.8 ± 0.34	2.7 ± 0.09	211
Mid-2	12	20.1 ± 0.61	6.1 ± 0.38	303
Late-2	14	27.3 ± 0.67	5.6 ± 0.66	205
Mid-3	17	37.3 ± 0.80	11.2 ± 0.65	300
Late-3	20	46.0 ± 0.94	10.2 ± 0.90	222
Mid-4	25	146.6 ± 1.97	81.5 ± 0.63	556
Late-4	30	199.5 ± 6.35	56.7 ± 1.83	284
Mid-5	36	248.1 ± 13.44	119.5 ± 5.52	482

[a] Means are derived from eight replicates; each replicate contained a batch of 10 developmentally synchronized larvae.
[b] Consumption per larva.

gut epithelium, and associated gut enzymes that are not secreted. The smaller midguts of early instars have more surface relative to volume than the midguts in later instars (Table 2). Surface area of midgut lumen of first to fifth instars increased greatly in larvae reared on pin oak; volume increases were even greater, giving rise to a decrease in the surface-to-volume ratio from 185.8 in mid-first instar to only 19.8 in the mid-fifth instar (Table 2). However, when surface area and volume were considered for gut lumen in combination with gut epithelium, this trend was not obvious; surface-to-volume ratios were fairly constant in second to fifth instars. This pattern is evident from the data on gut epithelium only, which indicates that the volume occupied by epithelial cells, on a relative scale, is much greater in younger larval stages than in older stages (Table 2). Thus, the gut epithelium of later instars is much thinner (by volume; relative to lumen volume) than that of younger larvae. This result has implications in better understanding of the need for greater specific activity of midgut MFOs in later instars than in younger larvae, in order to process dietary allelochemicals with comparable efficiency.

TABLE 2. COMPUTER-GENERATED MEASUREMENTS OF SURFACE AREAS, VOLUMES, AND SURFACE-TO-VOLUME RATIOS OF GUT LUMEN, GUT LUMEN + GUT EPITHELIUM, AND GUT EPITHELIUM ONLY IN MID-1ST TO MID-5TH INSTARS OF GYPSY MOTH REARED ON PIN OAK

Larval instar	Surface area (mm^2)	Volume (mm^3)	Surface-to-volume ratio
Gut lumen			
1	20.44	0.11	185.8
2	188.74	2.02	93.4
3	200.84	5.00	40.2
4	258.93	8.04	32.2
5	1046.07	52.80	19.8
Gut lumen + gut epithelium			
1	39.34	9.52	4.1
2	238.96	12.80	18.7
3	307.17	18.05	17.0
4	470.50	26.09	18.0
5	1912.56	175.63	10.9
Gut epithelium only			
1	18.90	9.41	2.0
2	50.22	10.78	4.7
3	106.33	13.05	8.2
4	211.57	18.05	11.7
5	866.49	122.83	7.1

Food Intake and Body Mass. In insects, food intake generally increases with $W^{0.8}$ where W is body mass; by comparison, mammalian food intake scales to about $W^{0.7-0.75}$ (Crawley, 1983, and refs. therein). Food intake in the gypsy moth increased by about the same scaling factor on both diets, $W^{1.09}$ for pin oak and $W^{1.15}$ for the artificial diet (Figure 2), indicating an ability to process much more food than hitherto reported, except for cinnabar moth, *Tyria jacobaeae*, where the scaling factor is 0.88 (Crawley, 1983). This relationship, established by regression analysis, was highly significant ($r^2 = 0.99$ for pin oak and 0.98 for artificial diet). Despite greater mass for fourth and fifth instars in the artificial-diet batch, the scaling of W to rate of food intake is nearly the same ($P > 0.05$; comparisons of slopes) as in the larvae reared on pin oak. Although the gut volume of the bigger and heavier artificial-diet-reared larvae was not measured, the data suggest that the gut volume may be increasing proportionately with W. Clearly this finding deserves further study as it has implications in understanding the overall energetics of the gypsy moth.

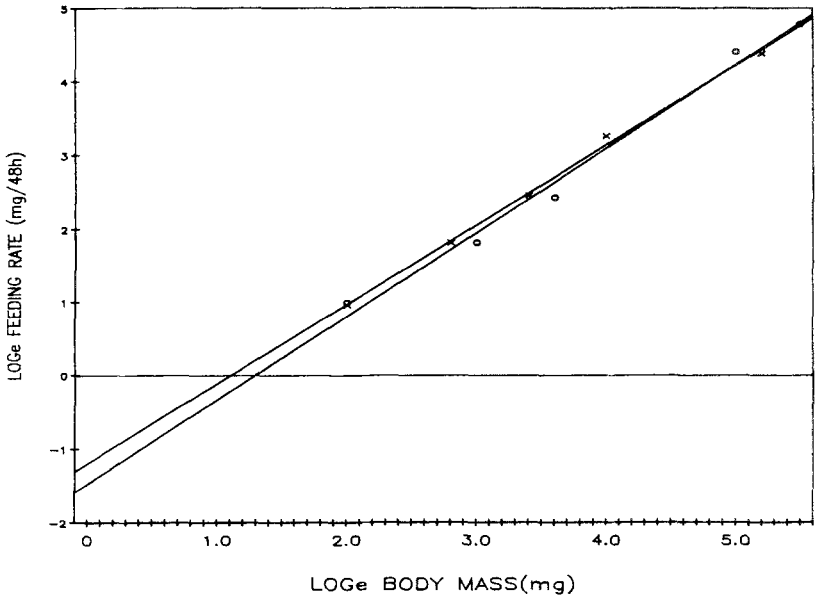

FIG. 2. The relationship of specific feeding rates to body mass (W) of first to fifth instars of the gypsy moth. Open circles and crosses denote data from artificial-diet- and pin-oak-reared larvae, respectively. The data were transformed to natural logs ($\log_e$) for regression to obtain a linear relationship. The regression revealed scaling factors of $W^{1.09}$ and $W^{1.15}$ for pin oak and artificial diet groups of larvae, respectively. Both regressions are highly significant ($r^2 = 0.99$ for pin oak and 0.98 for artificial diet). However, comparison of slopes indicated that the difference in the two scaling factors was not significant.

TABLE 3. CHANGES IN LEAF MOISTURE OF GYPSY MOTH'S HOST PLANTS[a]

Plant species	Leaf moisture (%, $\bar{X} \pm$ SE)[b] Newly flushed leaves	Mature leaves	References
Free of insect feeding/defoliation			
Sugar maple	74	60	Hough and Pimentel (1978)
White oak	75	60	Hough and Pimentel (1978)
Red oak	74	59	Hough and Pimentel (1978)
	66.8 ± 4.9	57.3 ± 0.9	Schultz and Baldwin (1982)
Pin oak[c]	73.1 ± 2.6	53.0 ± 1.6	Ahmad, unpubl. data
Following feeding/defoliation			
Red oak	66.3 ± 1.9	55.1 ± 5.9	Schultz and Baldwin (1982)
Pin oak[c]	73.0 ± 1.9	49.9 ± 1.9	Ahmad, unpubl. data

[a] Data are condensed to reflect overall changes from newly flushed leaves (concurrent with appearance of hatchlings) to mature leaves (when larval development is completed). The time of bud break and appearance of hatchlings is sychronized; however, the date when ths event occurs in the northeast differs from state to state because of differences in climatic conditions.
[b] Hough and Pimentel data did not provide standard deviations or errors of the means.
[c] Means are derived from 10 replicates. The difference between newly flushed and mature leaves is significant ($P < 0.05$ by t test).

Leaf Moisture. The drop in moisture content in pin oak was greater (20%) than in other hosts (10–15%) (Table 3). Moreover, the reduction was slightly higher (23%, but not significant) in pin oak leaves as a result of larval feeding on the plant. The relationship of changes in water content to allelochemical concentration and its implication to MFO activity is discussed below.

MFO Activity. The following trends are evident (Figure 3): (1) the enzyme level in all late instars (when feeding decreases, then ceases) is always lower than in the corresponding mid instars; (2) a progressive increase occurs in late-instar enzyme level, the rise is gradual and not different in the larvae of both diet groups, either in titer or in the slope ($P > 0.05$ by t test); and (3) in the actively feeding mid instars, MFO activity rises sharply from second to fifth stage: the rise was significantly higher ($P < 0.05$ for differences in the slopes) in larvae reared on pin oak (slope = 36.04) than those reared on the artificial diet (slope = 24.88), and the difference between mid-fifth and mid-second instar was much greater (4.5-fold) on pin oak than on the artificial diet (1.8-fold). Figure 4 clearly shows a parallel trend in the MFO specific activity and specific feeding rates of larvae fed leaves of pin oak.

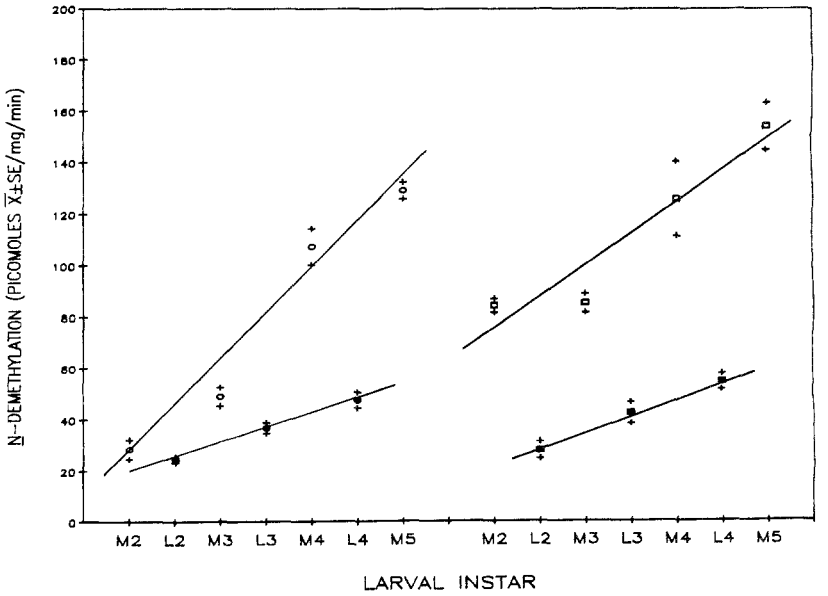

FIG. 3. MFO-catalyzed *N*-demethylation specific rates for the substrate PCNMA of midgut microsomal preparations of second to fifth instars of the gypsy moth. Data to the left are for larvae reared on pin oak; open and closed circles denote data for actively feeding (mid instars) and in reduced feeding stages (late instars), respectively. Data to the right are for larvae reared on wheat germ artificial diet; open and closed squares denote data for active and reduced feeding stages, respectively. All data are means derived from four replicates; plus symbols denote ±1 SE.

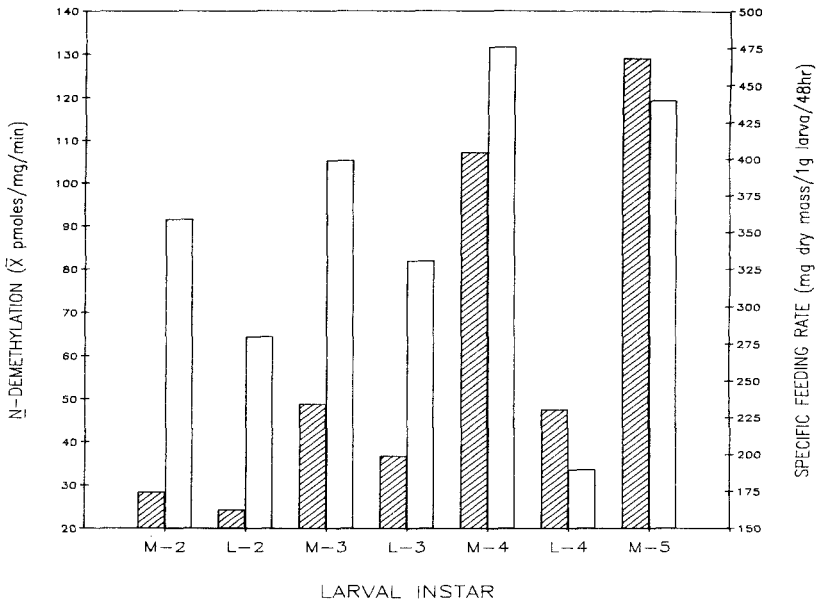

FIG. 4. Patterns of MFO activity and specific feeding rates of mid (M) and late (L) second- to fifth-instar larvae of the gypsy moth. MFO activity is high in all mid instars, rising with larval advancement and concomitantly with increases in specific feeding rates. Preecdysial depression in MFO activity coincides with the preecdysial suppression of specific feeding rates in late instars. Striped bars represent MFO activities, and open bars the specific feeding rates.

DISCUSSION

Results presented in this paper are discussed in light of the criticisms of the MFO theory by Dowd et al. (1983), and Gould (1984). The criticisms found in the above papers can be categorized in six specific statements.

1. Variation in MFO Specific Activity with Insect Development. Patterns of variation in MFO activity exist among developing stages in insects, e.g., southern armyworms (Brattsten, 1979b), gypsy moths (Ahmad, 1982), and Japanese beetles (Ahmad, 1983b), indicating that the activity is highest in feeding stages, is lowered during the molting process, and kept low during nonfeeding quiescent developmental stages. Gould (1984) stated that although the fifth instars of the gypsy moth consume ca. 10 times more food (artificial diet) than third instars, they are ca. 10 times heavier than the thirds; therefore the relative consumption rate for the two instars is equal. However, no data were provided on larval mass. Data in the present paper (Table 1) show that body mass difference between the mid-fifth and mid-third instars is only 6.65-fold. The difference in consumption rate per larva is 10.67-fold, however. Gould further

stated that changes in MFO specific activity were related to diet consumption on a per larva basis rather than on per unit larval mass, which is a common measure of relative feeding rate (Scriber and Slansky, 1981). Here I provide a clear relationship between relative consumption rates (mg dry mass of food eaten/1.0 g larva/48 hr) and the specific activity of larval gut MFO (Figure 4).

2. *Problem of Scaling Gut Volume/Surface Area to Enzyme Level.* The finding (Ahmad, 1982) that larger gypsy moth larvae (i.e., fifth instars) had higher specific activity than smaller stages (i.e., third instars), was contradicted by Gould (1984), because he speculated that higher specific activity existed in smaller larvae, since their volume-to-surface ratio is smaller (or conversely, surface area-to-volume ratio is higher, as per this report) than in larger older instars. Assuming that plant toxin molar concentration remained unchanged in the diet, the dose of plant toxin(s) expected to contact the gut wall of smaller larvae would be relatively higher. Gould is correct in pointing out this "geometric" aspect; however, he overlooked the fact that MFOs are not associated with membranes of epithelial cells but are distributed throughout the cell's network of endoplasmic reticulum, being membrane-bound to that structure. Thus, any consideration of gut volume/surface area (and the amount of food it can hold and process) must be viewed in the context of cellular volume.

In agreement with Gould, I found that larvae reared on pin oak leaves show a 480-fold increase in gut lumen volume from mid-first to mid-fifth instar; consequently, the surface-to-volume ratio dropped from 185.8 in the first instar to 19.8 in the fifths (Table 2). However, because the volume of the gut epithelium increased only 13.05-fold between the first and fifth instars, the surface-to-volume ratio showed a reverse trend as larvae developed, increasing from first- to fourth-instar larvae (Table 2). Schmidt-Nielsen (1979) had pointed out that organs may not scale in the same proportion as the animal size.

In the gypsy moths reared on pin oak, body mass and relative feeding rate scales to $W^{1.09}$ (Figure 2). This is a reflection of great increases in gut lumen volume that can hold greater amounts of food in older larvae and, also, to enhanced feeding rates. However, the large amount of food in the guts of larger larvae must pass through a volume-wise thinner layer of epithelial cells than in smaller larvae. Therefore, it is logical to predict that larger larvae cope with plant toxins with an efficiency at least comparable to that in smaller larvae because the specific activity of MFOs in their midgut epithelium is higher.

In support of his criticism, Gould (1984) further stated that "there have been no successful demonstrations of any MFO activity in first and second instars of Lepidoptera." In the present study MFO activity was quantified in second-instar larvae of the gypsy moth, and the data support earlier predictions (Ahmad, 1982; Ahmad, 1983b) of an increase in MFO specific activity with feeding-stage development (Figure 3). Although enzyme levels of first-instar larva remain to be investigated, the data for first instars most likely will fit the profile presented in this report.

3. Induction and Subsequent Metabolism of Inducers. One of the most significant features of the gut microsomal MFO system is its ability to respond rapidly via induction by allelochemicals (Brattsten et al., 1977). Dowd et al. (1983) and Gould (1984) claimed that, following induction, subsequent metabolism of an inducer has never been shown. However, as documented in the following examples, recent work in large measure alleviates their concern: (1) (+)-Limonene is hydroxylated as well as epoxidized in herbivorous mammals (Brattsten, 1983, and refs. therein). (2) The mint monoterpene, pulegone, is transformed in vitro to 9- and 10-hydroxypulegone by southern armyworm midgut microsomes (Trammell, 1982). (3) α-Pinene and other cyclic terpenes have been shown to be metabolized in rats and bark beetles; in the latter, some oxidized products are used as pheromones (Brattsten, 1983). (4) Xanthotoxin, a linear furocoumarin, is metabolized by MFOs of the fall armyworm and larvae of the black swallowtail butterfly, *Papilio polyxenes*; metabolic rates are different in the two species, however (Ivie et al., 1983). (5) In a thorough study discussed in more detail below, Yu (1986) demonstrated MFO-calalyzed turnover of many other structurally diverse allelochemicals in the fall armyworm.

One of several inducers which is not metabolized is the organochlorine compound, mirex (Barker *et al.*, 1972). Natural products tend to be more biodegradable than synthetic compounds, e.g., pyrethroids (Abernathy and Casida, 1973; Jao and Casida, 1974). Plant enzymes must be able to detoxify allelochemicals (therefore allelochemicals must be biodegradable) when injury occurs, in order to escape autointoxication (Rosenthal and Janzen, 1979; Harborne, 1982). Moreover, plants translocate allelochemicals during early growth and at the time of leaf senescence when they are broken down and recycled. The synthetic insecticides, especially the chlorinated hydrocarbons, on the other hand, often are less biodegradable, a desirable attribute for persistent insecticidal activity. High acute toxicity of such compounds makes it extremely difficult to demonstrate induction of the insect cytochrochrome P-450. Recent work on the mechanism of dioxin induction and gene expression shows that induction is of a specific form of cytochrome P-450 (Jones et al., 1985; Jaiswal et al., 1985). Dioxins are polychlorinated compounds (in studies cited here, the compound is TCDD, 3, 4, 7, 8, tetrachlorodibenzo-*p*-dioxin), and like mirex, they induce cytochrome P-450 but are not metabolized. The compounds are extremely toxic; the LD_{50} is in the microgram per kilogram range. As we learn more about the nature of the binding site on the cytosolic receptors of other substrates in relation to that of cytochrome P-450s, we will be able to better understand why mirex and dioxin have such a high affinity for the cytosolic receptor but not for the enzyme they induce. Other acutely toxic substrates (e.g., pyrethroids) that are metabolized by the MFOs apparently do not induce the enzyme activity at nonlethal dose levels.

4. Ineffectiveness of MFOs against Specialized Allelochemicals. It has been emphasized by many researchers (Feeny, 1976; Rhoades and Cates, 1976;

Brattsten 1979a) that enzymatic detoxification represents only one of many other biochemical adaptations against plant allelochemicals. It is not surprising then, that plant toxins such as cardiac glycosides (of milkweeds; family Asclepiadiceae) are tolerated by nonenzymatic means (e.g., insensitivity of target site) by larvae of the monarch butterfly *Danaus plexippus*, which sequesters cardenolides for defense against vertebrate predation (Blum, 1978). Other insects which rarely or never eat cardenolide-containing food (e.g., *Periplaneta americana*) may also not be harmed as cardenolides are not absorbed by the gut (Scudder and Meredith, 1982).

Nicotine is tolerated by both metabolic deactivation and by specialized excretion (Brattsten, 1979a, and refs. therein). The extent to which one insect species uses one mode of tolerance more than the other probably represents selection of genetic variables for the two attributes that differed in populations of different insect species. Nicotine tolerance like that of cardenolides represents a highly specialized historically established relationship between chemically well-defended plants and their equally well-adapted feeders. These examples are improper in nullifying the MFO theory which had never claimed that all allelochemicals are subject to attack by the MFOs. On the contrary, the theory assigned a greater role for MFOs in generalists that need to detoxify a broad range of lipophilic allelochemicals than in specialists that encounter specific toxin(s).

Gould (1984), using the two cases discussed above, reached the conclusion that (in reference to enzymatic detoxification of allelochemicals) "metabolism seems unimportant." Discussion so far has provided a firm basis for a major role for enzymatic deactivation of allelochemicals in herbivores. Even in cases such as the antimetabolite L-canavanine, previously thought to be tolerated only because of a modified tRNA synthetase, Rosenthal et al. (1982) demonstrated enzymatic detoxification. Marty and Krieger (1984) have provided evidence that in the larvae of the monarch butterfly, uscharidin, a cardiac glycoside, undergoes enzymatic metabolism in the fat body and midgut by enzymes other than MFOs. Thus enzymatic involvement is indicated even where we thought other defense mechanisms were solely operative in imparting insect tolerance to plant allelochemicals.

5. Lethal Biotransformations by MFOs and MFO Inhibitors. Some plant compounds, instead of being detoxified, are activated to more potent toxins. In the argument that MFOs do not necessarily deactivate allelochemicals, Gould (1984) cited two examples of potentiation of pyrrolizidine alkaloids (Mattocks, 1972) and aflatoxins (Tilak et al., 1975). Recently, lethal bioactivation of plant and synthetic precocenes to reactive epoxides by MFOs in the corpora allata (CA) of sensitive insects (e.g., *Locusta migratoria*) also has been shown (Ellis-Pratt, 1983). The CA is the organ of synthesis of hormonal epoxides (juvenile hormones). The epoxide of precocenes spontaneously rearranges to a carbonium

ion, and in this form it is a potent alkylating agent reacting with CA's macromolecules, culminating in complete necrosis of the organ. In nonsensitive species, precocenes are ineffective due to metabolic deactivation and excretion. Precocenes can undergo MFO-catalyzed *O*-demethylation or epoxidation. The epoxide is toxic, but not if attacked either by an epoxide hydrolase and then conjugated or by a glutathione-*S*-transferase; in both cases (and also *O*-demethylation), the result is detoxification, presumably occurring in the gut, fat body, or other tissues with a full and sufficiently active enzyme complement, but apparently not (sufficiently) in the CA. (Ellis-Pratt, 1983; Bowers, 1983; Dowd et al., 1983). Clearly this requires work on MFOs of the CA, gut, and fat body to better understand why precocenes are activated and persist in toxic form in sensitive insects but not in insensitive ones.

Vertebrate herbivores generally avoid eating toxic plants, although mistakes occasionally occur. Insects avoid such plants altogether. Exceptions are several species of danaid butterflies, where the males are strongly attracted to certain pyrrolizidine-containing plants. For example, feeding on nectar of *Crotalaria* sp. (Fabaceae) provides monocrotaline, a pyrrolizidine alkaloid that is converted to male courtship pheromone(s) (Boppré, 1978). Interestingly, the biotransformation of monocrotaline to the pheromones may be MFO-catalyzed (Brattsten, 1979b). It is not known whether this occurs in the gut, integument, or a specialized organ. The nature of this MFO requires study. In vertebrates, oxidation produces a highly toxic and carcinogenic metabolite in the liver, and the hepatotoxicity is fatal (Brattsten, 1979a).

Aside from toxic substrates (to which at least some insects are refractory), plants also are known to produce methylenedioxyphenyl compounds called lignans that inhibit MFO activity. Some examples are sesamin and sesamolin from sesame oil, myristicin in parsnip, and apiol from parsley leaves (Brattsten, 1979a; Bowers, 1983). A synthetic lignan, piperonylbutoxide has been commercially used as an insecticide synergist. Studies with piperonylbutoxide show that it is essentially an inhibitor, ultimately undergoing degradation by the MFOs (Brattsten, 1979a, and ref. therein).

The above examples of toxin sythesis or enzyme inhibition are predictable by the coevolutionary scenario (Ehrlich and Raven, 1964). Accordingly, insects and plants are engaged in an evolutionary arms race. As insects have developed enzymatic adaptations to neutralize toxic allelochemicals, plants in turn have developed countermeasures to offset the insects' responses. We have seen that some of the plant's countermeasures have been breached by some insects, a further indication of ongoing coevolution. In my opinion, therefore, the relatively few examples of lethal biotransformations or MFO inhibitors do not negate the generalization that the MFOs act, in large measure, as a detoxification system for a great majority of plant toxins.

6. Enzyme Multiplicity and Method of Assay. Until 1979, cytochrome mul-

tiplicity had been shown for the mammalian system and in insecticide-susceptible and -resistant house flies. Ahmad (1979) stated that in the immediate future research will be directed to resolve the question "whether the insect gut-MFO system is dependent on a single form or on multiple forms of cytochrome P-450." Recent evidence involving differential induction and metabolism showed that the order of induction of MFO-catalyzed epoxidation in the fall armyworm was indole 3-carbinol > flavone > indole 3-acetonitrile; the order for biphenyl hydroxylation was flavone > indole 3-carbinole > indole 3-acetonitrile, and for parathion desulfuration the order was indole 3-acetonitrile > flavone > indole 3-carbinole (reviewed by Yu, 1986). Ivie et al. (1983) showed that xanthotoxin is substantially metabolized by the gut MFO of larvae of the black swallowtail butterfly, but the enzyme in the fall armyworm larva metabolized only a very small amount of xanthotoxin. Gunderson et al. (1986) have shown the existence of cytochrome P-449 and P-450 (CO-complex peaks) in larvae of the southern armyworm. Moreover, the spectral form P-449 was found to hydroxylate pulegone at the C-10 position three times faster than the P-450 form in vitro.

Gould (1984) suggests that despite multiplicity, MFO activity has been measured mainly by epoxidation of dieldrin to aldrin as per Krieger et al. (1971). The following are some examples of different substrates and biotransformations used to assess MFO activity: (1) hydroxylation of aniline in gypsy moth larvae (Forgash and Ahmad, 1974) and in southern armyworm larvae (Brattsten et al., 1976); (2) hydroxylation of dihydroisodrin in six species of saturniid larvae (Krieger et al., 1976), in black cutworm (*Agrotis ypsilon*), and in cabbage looper larvae (Thongsinthusak and Krieger, 1976); (3) overall NADPH oxidation (inhibited by CO and SKF-525A) in gypsy moth larvae (Ahmad and Forgash, 1973, 1978); (4) allelochemical-dependent NADPH oxidation (inhibited by CO and piperonylbutoxide) in the fall armyworm (reviewed by Yu, 1986) and southern armyworm (Brattsten, 1983); (5) *O*-demethylation of *p*-nitroanisol in the housefly (Hansen and Hodgson, 1971), in gypsy moth larvae (Forgash and Ahmad, 1974), and in fall armyworm larvae (Yu, 1986, and refs. therein); (6) *O*-demethylation of methoxyresorufin in southern armyworm larvae (Brattsten et al., 1980); (7) *N*-demethylation of *N*,*N*-dimethyl-*p*-nitrophenyl carbamate in the house fly (Hansen and Hodgson, 1971) and in gypsy moth larvae (Forgash and Ahmad, 1974; Ahmad and Forgash, 1978); and (8) *N*-demethylation of *p*-chloro-*N*-methylaniline in saturniid larvae (Krieger et al., 1976), larvae of the southern armyworm and black swallowtail butterfly (Brattsten et al., 1977; Brattsten, 1979b), and the Japanese beetle (Ahmad, 1983b). These examples refute Gould's (1984) comment that MFO activity has been assessed mainly by one method.

The most powerful evidence of a major allelochemical-detoxifying role for the MFOs is an impressive list of allelochemicals that are now known to be metabolized by this enzyme system. The following allelochemicals are metab-

olized by insect gut MFOs: phytoecdysones (cf. Bowers, 1983), oxidative metabolism of precocenes (cf. Bowers, 1983; Ellis-Pratt, 1983), metabolism of the monoterpene, pulegone (Brattsten, 1983; Gunderson et al., 1986), and metabolism of three indoles, safrole, flavone, *trans*-anethole, estragole, a cardenolide (digitoxin), three coumarins, a phenylpropane (eugenol), alkaloids (atropine, strychnine, caffeine), 13 monoterpenes, two sesquiterpenes, a diterpene (phytol), stigmasterol, sitosterol, ergosterol, squalene, and β-carotene (Yu, 1986). A number of cyclic terpenes are also oxidized, some of which are used as pheromones (cf. Brattsten, 1983).

Further Interpretations of Results on Diet and Age-Related Changes in Gypsy Moth Gut MFOs. The MFO activity was higher on artificial diet than on pin oak leaves using the *N*-demethylation assay of the substrate PCNMA. This may be because pin oak (closely related to red oak and a type-1 plant) is a highly preferred host plant for the gypsy moth. In the southern armyworm (also by the same assay) larval enzyme level on artificial diet was 0.70 nmol (Brattsten et al., 1977); whereas on lima bean it was 0.29 nmol/mg protein/min (Brattsten, 1979b). In the Japanese beetle also, the induction of MFOs was lower on sassafras (2.59 nmol), a highly preferred host plant, than on less preferred broccoli (2.93 nmole/mg protein/min) (Ahmad, 1983b). Conceivably, the gypsy moth's MFO occurs at higher levels on less preferred/nonhost plants as, for example, has been shown for the southern armyworms (MFO activity was 2.73, 1.92, and 1.24 nmol on carrot, *Spananthe*, and parsley, compared to only 0.29 nmol on lima bean; Brattsten, 1979b). This interesting possibility needs experimental validation. Higher induction of MFOs by the artificial diet fed to gypsy moths could be due to the presence of many potential inducers such as components of wheat germ and agar, cholesterol, antibiotics, and antifungal compounds (ODell and Rollinson, 1966).

Nonetheless, data in Figures 3 and 4 reinforce the conclusion reached earlier (Ahmad, 1982) that MFO titers in the gut of the gypsy moth increase ontogenetically and by induction during feeding stages (Figure 3), suggesting an increase in biochemical defense commensurate with larval development and concomitant increase in food intake. Analysis of leaf water content as a function of leaf-aging and herbivory has shown a marked drop (Hough and Pimentel, 1978; Schultz and Baldwin, 1982; and data on pin oak leaves as per this report); the molar concentration of leaf allelochemicals will therefore increase. Moreover, leaves' phenolics and tannins also increase with time (Baldwin and Schultz, 1983). Other allelochemicals have not been analyzed from this perspective, but it is possible that similar increases occur in ubiquitously present "general green leaf" compounds (e.g., various hexenals, hexanols, hexanylacetate, etc.); this increase in concentration is probably the result of degradation of leaf constituents with aging and senescence (cf. May and Ahmad, 1983, and refs. therein). Some of these compounds have been shown to be inducers of MFOs and also metabolizable (Brattsten et al., 1977; Yu, 1986). An example

is the compound *trans*-2-hexenal, which is so highly reactive that it is used along with *p*-benzoquinone and methacrylic acid as a defensive substance in some insects (Blum, 1978). These compounds inactivate macromolecules such as proteins by reacting with nucleophilic groups such as NH_2 and SH. Simpler phenolics that are present in most leaves (and give rise to more complex flavonoids, e.g., rotenone; and polyphenols, e.g., tannins) also may be metabolized by the MFO system. The phenolics increase in concentration in oak leaves with time and as a result of plant response to herbivory (Baldwin and Schultz, 1983).

The leaf tannin content significantly increases as leaves mature and in response to leaf damage (Schultz and Baldwin; 1982; Baldwin and Schultz, 1983), and this lowers the availability of primary nutrients, nitrogen and sugar. At the same time, allelochemical concentration increases as a consequence of decrease in leaf water. Futher, as the larvae disperse seeking other hosts, the risk of encountering plants poor in nutritional quality and well-defended by toxic allelochemicals increases. From this I conclude that the gypsy moth is able to complete development by its ability to maintain high food consumption rates on nutritionally poor diets and to cope with allelochemically adverse plants by its remarkable ability to increase MFO levels. Similarly, high food consumption rates as compensation for poor diets is known for the polyphagous larva of the southern armyworm (Scriber, 1984). My conclusion is supported by (1) correlation of MFO activity with increase in specific rate of dietary intake with larval advancement, (2) by the nearly 1 : 1 relationship of food consumption rates to increase in body mass (Figure 2) and (3) the sharper rise in MFO activity on pin oak leaves, 4.5-fold from second to fifth instar, an indication that more allelochemicals that need to be detoxified are increasing in the plant concomitant with the insect development. The comparatively smaller increase, 1.8-fold, seen when larvae were fed artificial diet perhaps merely reflects a response of the MFO enzymes to greater total amounts of inducers as a result of an intrinsically increased specific rate of food consumption, rather than the molar concentration of inducers which remained unchanged.

Future Directions. The data suggest that MFOs in herbivorous insects have all the necessary attributes to act as a major enzymatic defense mechanism to allelochemicals. Moreover, specific multiple forms seem to have been elaborated, enabling some insects to deal more efficiently with specific allelochemicals of their particular hosts. However, a great deal remains to be investigated. Particularly interesting studies involve (1) some inducers that are not metabolized, (2) more thorough studies of cytochrome P-450 induction and multiple forms, (3) how efficient the system is in metabolizing relatively less innocuous but lipophilic allelochemicals in contrast to potent toxins in various species, (4) the full identity of MFO-metabolizable allelochemicals present in preferred and nonpreferred/nonhost plants of the gypsy moth and other insects, (5) further work on phytophagous mites which have been shown to possess higher activi-

ties of MFOs and *trans*-epoxide hydrolase than in a predacious mite, and (6) the interesting observation (C. A. Mullin, personal communication, 1984) that sucking phytophagous arthropods have lower levels of MFOs and other detoxification enzymes than chewing arthropods.

Aside from gut MFOs, sizable microsomal MFO activity is associated with the fat body and Malpighian tubules of insects. In some species, such as the gypsy moth and the Japanese beetle, the fat-body-specific microsomal MFO activity is 55 and 85%, respectively (Ahmad and Forgash, 1973; Ahmad, 1983b). In the cabbage looper and the American cockroach, *Periplaneta americana*, microsomal MFO activity in the fat body surpasses that in the gut (Brattsten, 1979, and refs. therein). Ahmad (1982) hypothesized that the fat body–MFO system may "provide insects with a secondary line of defense from toxic allelochemicals." Recent work suggests that in some insects precocenes are deactivated in the gut, fat body, and Malpighian tubules by MFO-catalyzed *O*-demethylation and/or by dihydrodiol formation and subsequently conjugated and excreted (Ellis-Pratt, 1983). The fat body microsomal MFOs are inducible enzymes, unlike the mitochondrial system, which is noninducible and whose function is to carry out hydroxylation of molting prohormone α-ecdysone to the active β-ecdysone (Bollenbacher et al., 1977). Whereas Ahmad's (1982) hypothesis requires clarification, a complicating factor requiring resolution is the evidence that steroid hydroxylation is not always mitochondrial, but is also carried out by the microsomal systems of the peripheral organs, e.g., the fat body and Malpighian tubules (Feyerseisen and Durst, 1978, and refs. therein).

Finally, enzymatic adaptations, especially the MFO system, may also enable herbivorous insects to utilize phytosterols in the biogenesis of cholesterol (Ahmad, 1979), cyclic monoterpenes, and plant olefins to produce sex attractants (Brattsten, 1979b, 1983) and to convert pyrrolizidine alkaloids to male courtship pheromones (Boppré, 1978; Brattsten, 1979b). Also, as discussed above, the MFO system in the corpora allata functions in the synthesis of juvenile hormone (JH) from farnesoate (Ellis-Pratt, 1983) and in regulating the JH titers along with expoxide hydrolase and also an esterase (cf., Brattsten, 1983).

The site of cholesterol synthesis from phytosterols is the insect gut. An intermediary reaction in the dealkylation of C_{28-29} phytosterols, e.g., sitosterol, to C_{27} cholesterol is the formation of an epoxide (fucosterol epoxide), and the entire conversion is dependent on NADPH and oxygen. This hypothesis, first advanced by Ahmad (1979), requires experimental support. This biotransformation has its root in the inability of de novo biosynthesis of the cyclic steroid nucleus in insects. In fact, insect association with plants has lead to complete dependence by insects on cyclic compounds of plant origin, as an evolutionary answer to costly ring closure reactions. Thus it is not surprising that cyclic monoterpenes through MFO oxidation also are utilized as pheromones. The same can be said with regard to cyclic oxidative derivatives of toxic pyrrolizi-

dine alkaloids in serving as male courtship pheromones in danaid butterflies. However, future investigations should resolve whether these "biogenesis" reactions of the microsomal MFOs are catalyzed by specific forms of the cytochrome P-450 and, also, the tissue and organ where pheromonal synthesis occurs, especially in the danaids which release monocrotaline-derived pheromones from their hair pencils.

Brattsten (1979b) hypothesized that the gypsy moth's sex attractant, disparlure, an epoxide of an olefin, is synthesized by an MFO system. However, it remains to be seen if this conversion is effected in the pheromone gland or the midgut. Unpublished results (L.B. Brattsten, C.F. Wilkinson, and T. Taylor, cited in Brattsten, 1979b) show that gut microsomal MFO of the armyworm can catalyze the epoxidation of the olefin. Therefore, it is possible that disparlure may also be synthesized in the midgut and then sequestered in the pheromone gland. This possibility awaits experimental investigation.

Compounds such as disparlure and many other epoxide pheromones may be inactivated by *trans*-epoxide hydrolase, known to be present in many tissues such as midgut and fat body, but so far this enzyme has not been found in pheromone glands. Earlier, it was pointed out that a pheromone-specific esterase is present in the cabbage looper's receptors, which would deactivate the esterpheromone after it has elicited a response in the male. Likewise, there is the need to study existence of AK-reductases in pheromone receptors for their role in deactivating (after elicitation of a response is over) a large number of aldehyde and ketone pheromone molecules of insects.

From the foregoing, it is clear that MFOs have been harnessed by insects in allelochemical deactivation and also in the utilization of substances physiologically important to insects. Both detoxification and biosynthetic roles of these enzymes should be considered in the future investigations of the MFOs and other allied allelochemical-interacting enzyme systems.

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THE HAUSTORIUM AND THE CHEMISTRY OF HOST RECOGNITION IN PARASITIC ANGIOSPERMS

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Abstract—Two parasitic angiosperms, *Agalinis purpurea* (Scrophulariaceae) and *Striga asiatica* (Scrophulariaceae), are compared as to the chemical recognition events involved in host selection. *Agalinis* is a hemiparasite which can mature to seed-set without a host, whereas *Striga* is a holoparasite and survives for only a very limited time without a host. Both parasites, however, attach to a host through a specialized organ known as the haustorium and regulate the development of this organ through the recognition of chemical factors from host plants. We now describe the discovery of 2,6-dimethoxy-*p*-benzoquinone (2,6-DMBQ) as an haustoria-inducing principle from *Sorghum* root extracts. Our investigation of this compound has led us to suggest that one level of host recognition in these parasitic plants is mediated through their enzymatic digestion of the host root surface. Degradation of surface components liberates quinonoid compounds, such as 2,6-DMBQ, which in turn trigger haustorial development.

Key Words—*Agalinis*, *Striga*, parasitic angiosperms, 2,6-dimethoxy-*p*-benzoquinone, haustoria, organogenesis, laccase, phenol oxidase, parasite-host recognition.

INTRODUCTION

The viability of an organism depends on its ability to recognize and respond to events occurring in its environment. An efficient and specific recognition system is particularly critical for organisms that have evolved as parasites dependent on a specific association with a host. Parasitic plants, being sessile organisms, respond to potential hosts through processes of growth and development. These responses are readily observed and quantified, and therefore

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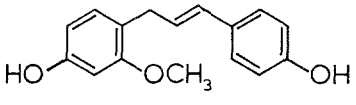
these organisms represent a simplified system for investigating the chemistry of such recognition events.

At present, there are tens of thousands of plant species which have evolved the ability to parasitize other plants. Some of these parasites have a narrow host range and must have developed methods for selecting between host and non-host. The parasitic angiosperm *Agalinis purpurea* responds to the presence of host plants by controlling the development of an organ specialized for host attachment termed the haustorium (Kuijt, 1969; Riopel, 1979; Riopel and Musselman, 1979). Preliminary experiments suggested that materials exuded from host plants induced the development of the haustorium in *Agalinis*. In fact, by screening fractions from a commercially available plant exudate, gum tragacanth, for the ability to induce haustoria in axenic cultures of *Agalinis*, the first



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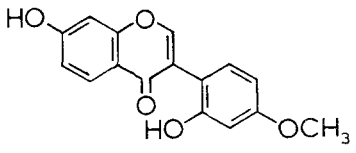

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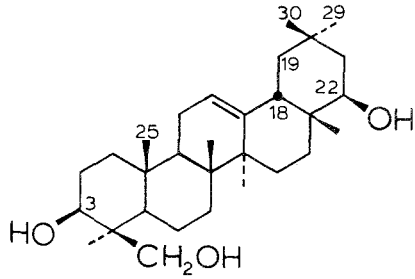

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haustorial inducers, xenognosin A(1) and B(3), were isolated and characterized (Lynn et al., 1981; Steffens et al., 1982). Several synthetically prepared analogs of the xenognosins (Kamat et al., 1982) established the *m*-methoxy phenol functionality and the propene double bond as structural features critical for biological activity (Steffens et al., 1982). Work in other laboratories (Bell, 1981; Carlson and Dolphin, 1981; Dewick, 1975) has shown the xenognosins to be phytoalexins and plant-stress metabolites. Taken together, these findings have led us to suggest that the xenognosins are stable constitutive antibiotics necessary for host-plant defenses and therefore likely recognition cues of a suitable host. The identification of the xenognosins, then, provided the first experimental support that parasitic angiosperms mediate host selection through the identification of specific molecules present in and potentially exuded by their hosts.

Isolation of the triterpene soyasapogenol B(3) exuded from the *Agalinis* host, *Lespedeza sericea* (Leguminosae), was quite unexpected in light of the strengths of the arguments in favor of the phenylpropanoid recognition cues identified in gum tragacanth (Steffens et al., 1983). However, many of the find-

ings which support the phenolics also favor 3 as a recognition cue. The haustoria-inducing activity of 3 is dependent on certain structural features. Neither the 22-keto derivative, soyasapogenol E, nor a series of other olean-12-ene triterpenes possessed any detectable activity. The soyasapogenols have been found exclusively in the Leguminosae and therefore may have restricted occurrence, but their chemotaxonomic usefulness is not as well established as that of the flavanoids. The soyasapogenols have also been attributed with defensive roles. In their glycosylated form, they are toxic and reduce herbivory in leguminous seeds (Langenheim, 1981). A group of more highly oxidized oleane triterpenes, the averacins, are found in oat roots and are potent resistance factors to "take all" disease caused by the fungus *Gaeumannomyces graminis* (Crombie et al., 1984).

Measurements of the levels of root exudation of these triterpenes and a single flavanoid, genistein, from *Lespedeza* suggested that terpenoid exudation is under active metabolic control and that phenolic exudation is extremely low. (Steffens et al., 1986; Lynn, 1985). If this low exudation level of phenolic compounds from *Lespedeza* can be taken as representative of potential hosts, then it seems unlikely that quantities of these phenolics sufficient for the induction of haustorial development would be exuded. Therefore, the structural specificity, the stable and specific metabolic production, and the metabolic control over the exudation of the soyasapogenols by *Lespedeza* suggest that the terpenoids may be more appropriate substances for these parasites to use as recognition cues.

The haustoria-inducing activity of soyasapogenol B is, however, much weaker than that of xenognosin A. In a filter paper disk assay, 20 nmol of soyasapogenol B is the lowest quantity which will induce haustoria, whereas xenognosin A will induce large numbers of haustoria at 1 nmol. Haustoria are not induced when soyasapogenol B is presented as a 10 μM solution, but xenognosin A is quite active at this concentration. Measurements of the rate of exudation of 3 from 2-week-old seedlings suggest that, without any environmental alteration or microbiological metabolism, sufficient amounts of soyasapogenol B for haustorial induction would not accumulate. Unlike the xenognosins, which constitute virtually all of the activity of gum tragacanth, the soyasapogenol B activity represents only a portion of the activity of *Lespedeza*. While there is a very real possibility of synergism occurring with the multiple components from *Lespedeza*, it is clear that the complete story of the interaction between *Agalinis* and *Lespedeza* root exudation has not been defined.

Agalinis purpurea, while a much larger and more vigorous organism when attached to a host, is a hemiparasite capable of maturing to seed-set without host attachment. Host recognition would, therefore, be expected to be less critical for *Agalinis* than for a holoparasite, such as *Striga asiatica*, which is incapable of survival for more than 10 days without a host. *Striga* has a host range that is restricted to grasses, such as wheat, corn, and *Sorghum*, and has

been commonly called "witchweed" because of its damaging effects on grain crops. It is now clear that *Striga* has at least two levels of host recognition, one at the level of germination and one associated with the development of the haustorium. *Striga* initiates its commitment to a host through germination. A sesquiterpenoid, strigol, has been found to be exuded by cotton roots as a potent *Striga* germination stimulant. However, cotton does not serve as a host for *Striga* and, in fact, is used as a trap crop to reduce *Striga* infestation. Both corn and *Sorghum* serve as hosts for *Striga* and have been shown in our laboratory to produce separate stimulation factors for both germination and haustorial development.

Haustorial development is also very tightly regulated in *Striga*, and it has been shown that the induction of the haustorium is not possible four to five days postgermination (Riopel and Baird, 1986). Perhaps of even more importance is that *Striga* forms only a terminal haustorium (Figure 1) and radicle elongation ceases with the induction of haustorial development. For successful host attachment, this induction must occur within a 50-μm distance of the host. Germination must occur within a distance where the growing radicle can reach the host's surface within five days postgermination. Therefore, not only must *Striga* carefully select a suitable host, but it must also be able to determine the distance between itself and its host. The chemical challenge with *Striga*, then, is to explain not only how it recognizes its host, but how it recognizes the distance from its host. In this paper we will attempt to lay the groundwork for the explanation of both aspects of this biological recognition.

METHODS AND MATERIALS

Seeds. Striga asiatica seeds were obtained from R.E. Eplee, U.S.D.A. Witchweed Methods Development Laboratory, Whiteville, North Carolina, and handled under USDA quarantine at Charlottesville, Virginia. *Agalinis purpurea* seeds were obtained from L.J. Musselman, Old Dominion University. *Sorghum bicolor* (Var. Sudan) seeds were grown in vermiculite under greenhouse conditions.

Bioassays. Haustorial induction was determined by first germinating *Striga* seeds in distilled water with strigol (Nickrent et al., 1979). The 24-hr-old seedlings were then transferred and exposed to selected extracts in distilled water. Haustoria were counted 24–48 hr later.

Isolation of Haustorial Inducer from Sorghum Roots. Two-month-old vermiculite-grown *Sorghum* roots (600 g fresh weight) were exhaustively extracted with CH_2Cl_2–MeOH (4:1), and the extract was filtered and dried in vacuo. This material was extracted with $CHCl_3$ and the washings were concentrated to yield 174 mg of a dark green oil. The oil was applied to a Sephadex LH-20 column (2.5 × 37.1 cm, CH_2Cl_2–MeOH, 40:60), and following 125 mL of

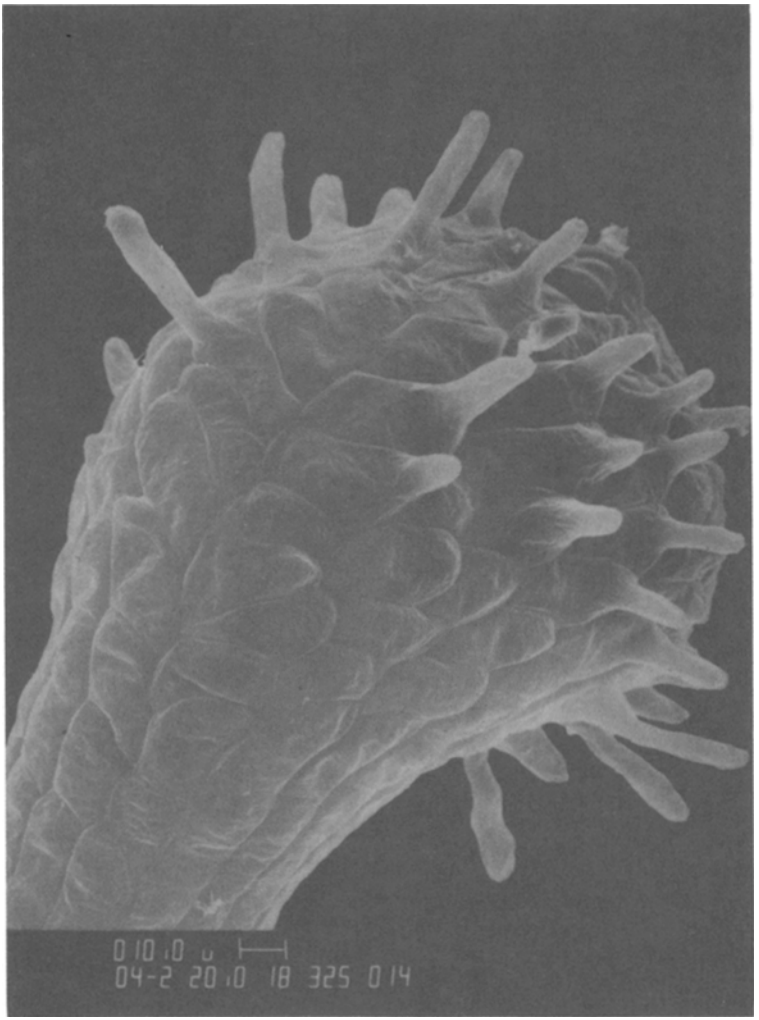


FIG. 1. Electron micrograph of the developing haustorium of *Striga asiatica*. The haustorium forms at the meristematic tip and radicle elongation is halted. (Courtesy of Dr. Vance Baird).

eluant, a biologically active red-colored band was collected and dried (38 mg). Further purification on a reverse-phase C18 flash column (1.1 × 2.1 cm, 40% MeOH–H_2O) gave an early eluting orange fraction (7.9 mg), which was subsequently chromatographed on SiO_2 (0.55 × 6.1 cm, CH_2Cl_2) to yield a yellow sample (1.1 mg). Final HPLC purification on Waters Radial Pak (SiO_2, 5 μm, 8 mm i.d. × 10 cm, 92% hexane–6% CH_2Cl_2–2% MeOH) gave 100 μg of a

crystalline solid capable of haustorial induction. This procedure was repeated four additional times throughout the process of the structural characterization and yielded a total of approximately 600 μg of material.

Chromatography. Gel filtration chromatography was performed using Sephadex LH-20-100 (25–100 μm particle size) obtained from Sigma Chemical Co. Reverse-phase chromatography was performed by using octadecylsilane-bonded silica gel (40 μm particle size) supplied by J.T. Baker Chemical Co. Silica gel 60 (230–400 mesh) obtained from EM Reagents, Inc., was used for SiO_2 column chromatography. High-pressure liquid chromatography (HPLC) was performed both analytically and preparatively on 5 μm Waters Radial Pak SiO_2 columns. Solvents were blended in a Dupont series 8800 four-solvent pump operated by a Dupont series 8800 gradient controller. Effluent was monitored at 284 nm with a Dupont series 8800 Spectro variable wavelength detector.

Syntheses of Compounds 2,6-Dimethoxy-p-*benzoquinone* (4), was synthesized by the method reported earlier (Bolker and Kung, 1969).

For *2,5-Dimethoxy*-p-*benzoquinone* (5), to a stirred solution of 2,5-dihydroxy-*p*-benzoquinone (50 mg, 0.36 mmol) in 3 mL methanol, diazomethane (0.72 mmol) was added at room temperature, and the stirring was continued for 2 hr. Solvent was evaporated in vacuo, the residue dissolved in CH_2Cl_2, and washed with 5% Na_2CO_3 (2×) and H_2O (2×). Concentration of the organic layer gave 5 (40.8 mg, 68%).

2,3-Dimethoxy-p-*benzoquinone* (6), was prepared by oxidation of 1,2,3-trimethoxybenzene with hydrogen peroxide and potassium ferricyanide (Matsumoto and Kobayashi, 1985).

2-Carboxymethoxy-6-methoxy-p-*benzoquinone* (7), was prepared by nitric acid oxidation (Bolker and Kung, 1969) of 1,2-dimethoxy-3-phenoxyacetic acid (synthesized from 2,3-dimethoxyphenol by the method of Burgstahler and Worden, 1973). [^{1}H] NMR ($CDCl_3$), δ 3.81 (s, 3H), 4.60 (s, 2H), 5.72 (d, J = 2Hz, 1H), 5.84 (d, J = 2 Hz, 1H). FT-IR (CH_2Cl_2), 1756, 1697, 1644, 1595 cm^{-1}.

2-Ethoxy-6-methoxy-p-*benzoquinone* (8). A solution of 2,3-dimethoxyphenol (15.8 mg, 0.103 mmol) in ethyl bromide (2 mL) and K_2CO_3 (19.9 mg) was refluxed for 9 hr. The solvent was evaporated in vacuo, the residue dissolved in ether and washed with saturated $NaHCO_3$ (3×), then H_2O (3×). The organic layer was dried over anhydrous $MgSO_4$, concentrated in vacuo, and purified on SiO_2 (0.55 × 6.3 cm, benzene–MeOH, 98:2) giving 1,2-dimethoxy-3-ethoxybenzene (2,3 mg, 13%). Subsequent oxidation with nitric acid as above, gave VIII. [^{1}H] NMR ($CDCl_3$), δ 1.46 (t, H = 7 Hz, 3H), 3.79 (s, 3H), 3.97 (q, J = 7 Hz, 2H), 5.27 (s, 2H). FT-IR (CH_2Cl_2), 1697, 1644, 1593 cm^{-1}.

2-Methoxy-p-*benzoquinone* (9) was prepared by the reaction of *p*-benzoquinone with methanol at room temperature for 24 hr. Purification on SiO_2 column (1.6 × 9.5 cm, benzene–ether, 90:10) gave IX. [^{1}H] NMR($CDCl_3$), δ

3.73 (s, 3H), 5.84 (s, 1H), 6.60 (s, 2H). FT-IR (CH_2Cl_2), 1679, 1658, 1622, 1589 cm^{-1}. UV (CH_2Cl_2), λ_{max} 358 nm (log ϵ 1.20), λ_{max} 254 nm (log ϵ 2.18).

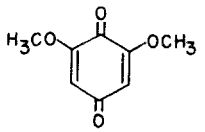
Histochemical Assay for Detection of Phenol Oxidases (Harkin and Obst, 1973). *Sorghum*, peas, and lettuce seeds were surface sterilized with 50% commercial bleach for 20 min. *Agalinis* seeds were surface sterilized as reported earlier (Riopel and Musselman, 1979). Treated seeds were then rinsed with sterile distilled water and grown on a modified Murashige and Skoog (1962) medium under a 12 hr photoperiod at 27°C. Seedlings were transferred from the agar to microscope slides, and a solution of 0.1% syringaldazine in ethanol (1–2 drops), followed by 0.3% H_2O_2 (1–2 drops) was added to the roots. The red-purple color developed immediately and tended to fade after 15 min.

Spectroscopy. [^{1}H] NMR data were recorded with a home-built console interfaced with an Oxford 500-MHz magnet, a 1280/293C data system, and NMCFT software. FT-IR spectra were performed on a Nicolet 20SXB with 1 cm^{-1} resolution. The spectrum was stored and solvent background was computer subtracted. UV data were obtained using a Perkin-Elmer Lambda 5 UV/VIS spectrophotometer. High- and low-resolution mass spectral data were obtained with a VG 70-250 spectrometer.

RESULTS

A general screen for materials which are able to induce the development of haustoria in *Striga* has been developed (Nickrent et al., 1979). The use of this screen to direct the purification of compounds from *Sorghum* roots is shown in Scheme 1. This purified haustorial inducer proved to be the only active fraction in *Sorghum*. The bright yellow compound was slightly soluble in H_2O and very soluble in organic solvents, and possessed a strong long-wavelength UV chromophore with λ_{max} 284 nm (log ϵ 4.01) and 376 nm (log ϵ 2.65). [^{1}H] NMR ($CDCl_3$, 500 MHz) indicated only two signals at δ 5.83 and 3.75, with a relative integrated ratio of 1:3, respectively. EI-MS (70 eV, 200°C) showed a molecular ion at *m/z* 168, and this assignment was supported by DCI-MS (isobutane, M + H ion at *m/z* 169). Exact mass measurements established a molecular formula of $C_8H_8O_4$ (*m/z* 168.0389, calc. 168.0422).

These data uniquely support a dimethoxy-*p*-benzoquinone structure. Orthoquinones are generally dark red in color and possess a considerably higher redox potential than the para isomers. This redox chemistry is readily observed as an intense $(M + 2)^+$ ion in the mass spectrum (Patai, 1974). The three isomeric



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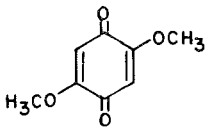

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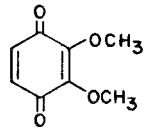

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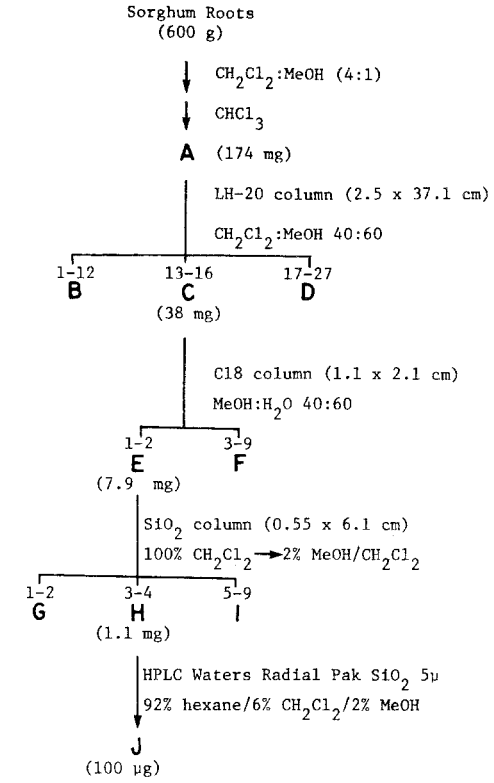


Fraction	ED_{75}(μg/mL)@
A	60*
C	4
E	1
H	0.5
J	0.05

SCHEME 1. The schematic representation of the isolation procedure followed for the purification of the haustorial inducer from *Sorghum* root extract. The activity of each fraction is represented as the concentration of the extract necessary to induce 75% of the seedlings to develop haustoria. @Minimum concentration required to induce 75% haustoria. *Swelling only, no viable haustoria observed. All other fractions were inactive.

p-benzoquinones 4–6 are not readily distinguishable by the data thus far presented.

The 2,3-substituted quinone, (6), reflects the symmetry observed in the system by NMR, but the quinonoid protons would be expected to resonate farther downfield (δ 6.3–7.3) than those observed in the haustorial inducer. The electronic spectrum of the unsubstituted *p*-benzoquinone has three absorptions:

an intense $\pi \rightarrow \pi^*$ transition at 246 (log ϵ = 4.42), a medium intensity $\pi \rightarrow \pi^*$ band at 288 (log ϵ = 2.50), and a weaker $n \rightarrow \pi^*$ absorption at 439 nm (log ϵ = 1.35) (Patai, 1974; Morton, 1965). Introduction of a methoxy substituent produces a small bathochromic shift of the first band (< 10 nm), but the second band undergoes a more noticeable red shift of the order of 69 nm (Patai, 1974; Thomson, 1971, and references therein). The third band is little affected and is obscured by the second maximum. Introduction of a second substituent has much less effect on the second band than the first, and is the greatest for the 2,3-disubstituted derivative (Patai, 1974). The observed bands in the haustorial inducer at 284 and 376 nm do not fit with the expected transitions of the 2,3-dimethoxy isomer (6). In addition, neither the favorable retro-Diels-Alder fragmentation, nor the $(M + 2)^+$ ion expected for 6 are observed in the mass spectrum (Bowie et al., 1966). The mass spectrum of the natural product shows ions at *m/z* 112 and 80 which could arise from the retro-Diels-Alder cleavage of either 4 or 5 (Figure 2).

Of the two possible isomers remaining, the 2,5-isomer would be expected to give two different electronic transitions of equal intensity around 280 nm. In addition, the carbonyl stretching frequencies in the 2,5-isomer should be of the same frequency. FT-IR (CH_2Cl_2) of the haustorial inducer showed intense bands

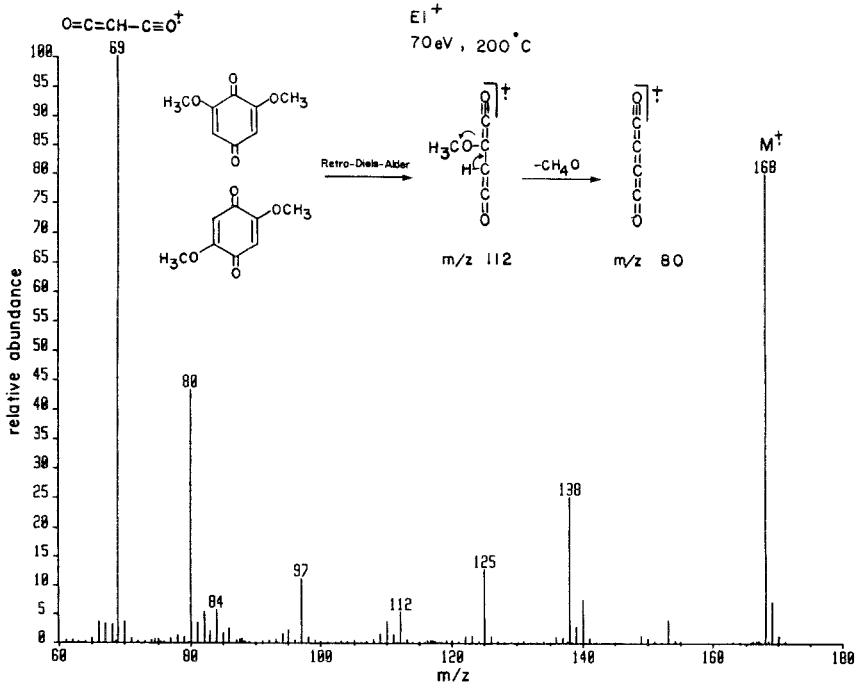


FIG. 2. Electron impact mass spectrum (70 eV, 200°C) of the haustorial inducer from *Sorghum*. The sample (ca. 3 μg) was volatilized from a solid probe on a VG 70-250.

at 1698 (νC = 0), 1646 (νC = 0), and 1597 cm^{-1} (νC = C) (Figure 3). These electronic and vibrational transitions uniquely established the haustorial inducer as 2,6-dimethoxy-*p*-benzoquinone (4 2,6-DMBQ).

Synthetic studies have finalized the assignment. Nitric acid oxidation of 1,2,3-trimethoxybenzene gives 4 in reasonable yields (Bolker and Kung, 1969), whereas oxidation with hydrogen peroxide and potassium ferricyanide gives 6 and 4 in a 3 : 1 ratio, respectively (Matsumoto and Kobayashi, 1985). The commercially available 2,5-dihydroxy-*p*-benzoquinone can be efficiently methylated with diazomethane to give 5. These synthetic samples gave the anticipated spectral data (Table 1) and confirmed the assignment of 2,6-dimethoxy-*p*-benzoquinone as the haustorial inducer.

By similar synthetic methods, several benzoquinone analogs have been prepared. These studies reveal a strict structural requirement for biological activity. Figure 4 compares the haustoria-inducing activity of the monomethoxy (9), and ethoxymethoxyquinones (8), both of which show reduced activity when compared with 4. Unexpectedly, replacement of the ethyl substituent of 8 with the corresponding carboxy methylene (7), increases the analog's activity to a level comparable to 2,6-DMBQ. More drastic structural alterations completely abolish activity (Table 2). Like the xenognosins, a methoxy functionality ap-

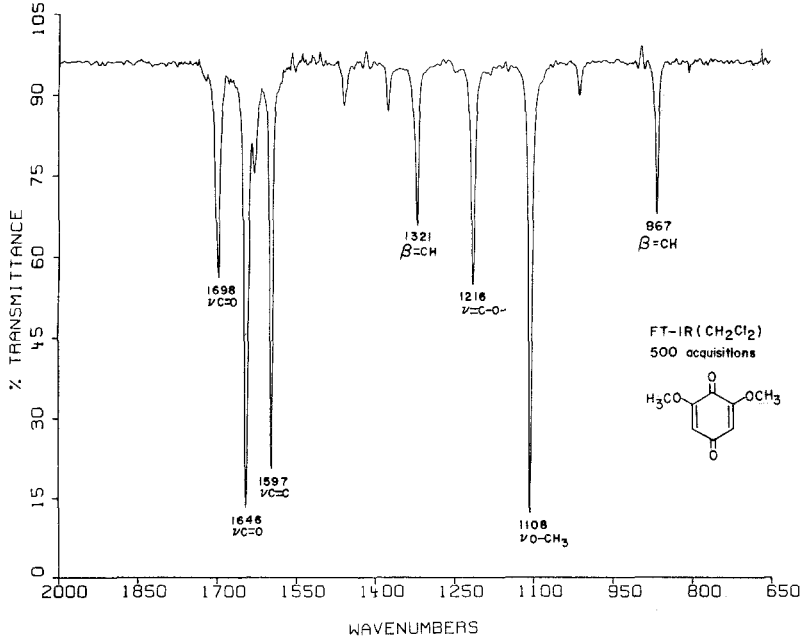
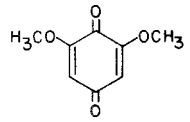
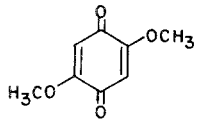
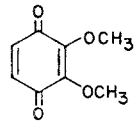


FIG. 3. Fourier transform infrared spectrum of the haustorial inducer from *Sorghum*. The spectrum was taken in methylene chloride, and the solvent background was computer subtracted.

TABLE 1. COMPARISON OF THE SPECTRAL DATA OF SYNTHETIC BENZOQUINONES WITH THE HAUSTORIAL INDUCER FROM *Sorghum*

	IR (CH_2Cl_2) cm^{-1}		UV (CH_2Cl_2)	
	$\nu C=O$	$\nu C=C$	λ_{max} nm (log ϵ)	[^{1}H]NMR ($CDCl_3$)
	1698 1646	1598	283.5 (4.28) 377 (2.78)	5.86s 3.84s
	1666	1600	278 (4.37) 284 (4.38) 370 (2.48)	5.85s 3.88s
	1662	1592	253 (3.87) 389 (2.92)	6.58s 4.01s
Haustorial Inducer	1698 1646	1597	283.9 (4.01) 375.6 (2.65)	5.83s 3.75s

pears to be critical, and the unsubstituted benzoquinone, as well as the dimethyl and 2,5-dihydroxy derivatives, are totally inactive.

The active quinones also show a distinct concentration for maximal activity, and at high concentrations they inhibit haustorial induction. This inhibition may be related to the broad spectrum of toxic effects documented for these quinones (Bundenberg de Jong et al., 1955; Hausen et al., 1972; Jones et al., 1981; Kodaira et al., 1983; Redfearn and Whittaker, 1962; Schultz et al., 1979), however, there is no necrosis or obvious growth inhibition at high concentrations. 2,3-DMBQ and 2,6-DMBQ are both inhibitors of mitochondrial respiration (Chappel and Hansford, 1972; Redfearn and Whittaker, 1962) and are capable of inducing haustoria. In contrast, the 2,5-isomer shows no such inhibition of respiration and does not induce haustoria. The connection between the inhibition of respiration, the general toxicity, and haustoria-inducing activity of these quinones is still not clear.

Recognizing that pathogenic fungi secrete phenol oxidases to catalyze the breakdown of lignins to various phenolics including 2,6-DMBQ (Umezawa et al., 1982), we investigated whether parasitic angiosperms produced the same enzymes. The addition of syringaldazine (Figure 5) and hydrogen peroxide to

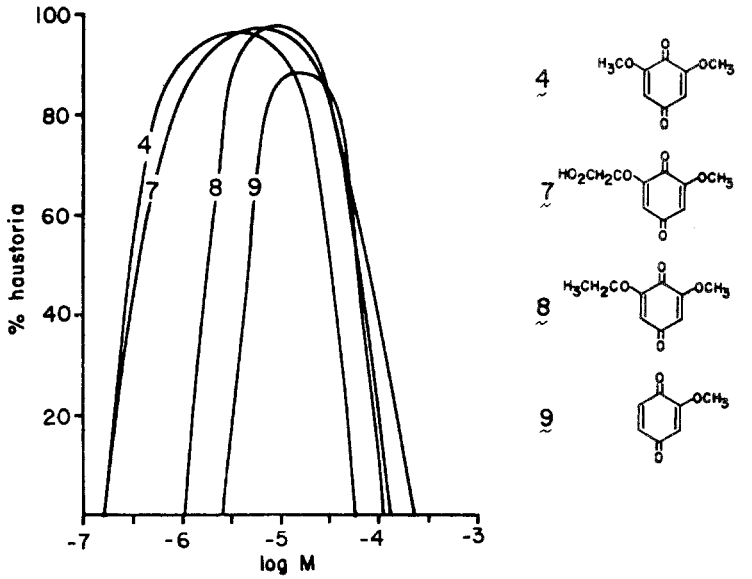


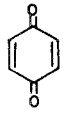
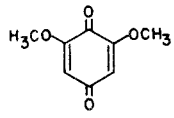
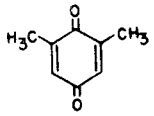
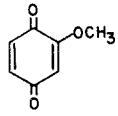
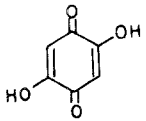
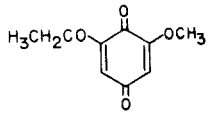
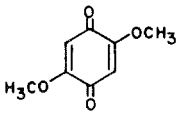
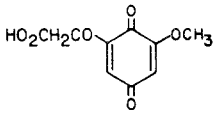
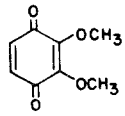
FIG. 4. Logarism of the concentration of the haustorial inducer plotted against the percentage of 2-day-old *Striga* seedlings that are induced to develop haustoria. The assay is performed on 20–50 *Striga* seedlings in 1 mL of distilled H_2O and the number of haustoria are counted after 24–48 hr exposure time. The striking inhibition at high concentrations of the analogs does not appear to be due to insolubility nor are there any visible toxic effects on the seedlings.

the roots of lettuce, pea, and *Sorghum* seedlings immediately turned the root hair zone of these plants a dark reddish purple. By contrast, this dye is oxidized at the meristematic root tip in *Agalinis* seedlings. None of the potential host plants, thus far tested, show evidence for any phenol oxidase activity at the root tip.

DISCUSSION

Striga asiatica responds through the development of the haustorium to only a single compound in *Sorghum* extract. This specific response is much simpler than the chemical responses seen between *Agalinis* and *Lespedeza*. *Striga*, being an obligate parasite, has a far more restrictive time frame in which to identify its host than does *Agalinis*. This limitation in time means that *Striga* must germinate at a distance where the growing radicle can reach the host root within five days, about 0.3–0.4 cm. Likewise, haustorial induction, which stops radicle elongation, must occur well within 50 μm of the host root, or the parasite will not attach or survive. So *Striga*, much more critically than *Agalinis*, must

TABLE 2. COMPARISON OF THE BIOLOGICAL ACTIVITY OF DIFFERENT BENZOQUINONE ANALOGS[a]

	ED_{50}		ED_{50}
	inactive		3.2×10^{-7} M
	inactive		5.0×10^{-6} M
	inactive		2.0×10^{-6} M
	inactive		4.7×10^{-7} M
	$0.6\text{-}3.0 \times 10^{-6}$ M		

[a]The ED_{50} represents the concentration of the analog necessary to induce haustorial development in 50% of the *Striga* seedlings.

utilize host chemistry to signal not only the presence of a suitable host, but also to serve as a cue that the host is within the required distance for attachment. How then can the exudation of stable phenolic compounds account for this host and distance selection given that such materials would be expected to accumulate very differently depending on the soil, weather, and microbial conditions?

Work over the last 40 years has focused on the identification of the germination factor for *Striga* from natural host plants. This work has not been successful primarily because of the reported physical instability of the germination stimulants. Strigol was identified from a nonhost, cotton (Cook et al., 1972); this stimulant is quite stable and can accumulate around the root of the cotton plant. *Striga's* ability to respond to a physically unstable compound could eliminate any buildup around the host root and may provide a mechanism for

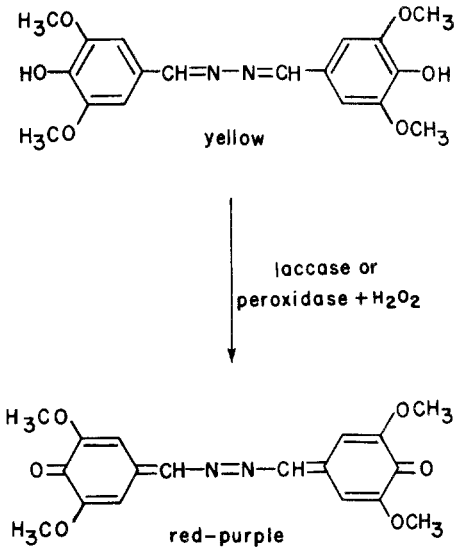


FIG. 5. Oxidation of syringaldazine to the red-purple pigment used for the histochemical detection of laccases or peroxidases.

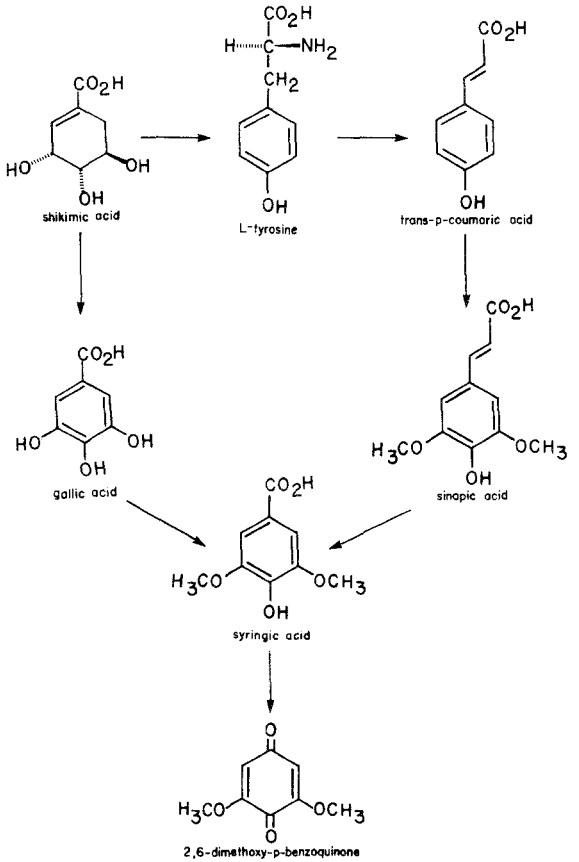
measuring distance. 2,6-DMBQ is chemically stable, so that under appropriate conditions it could accumulate at distances far from the root surface.

2,6-DMBQ is broadly distributed in the plant kingdom, occurring in 48 genera within 29 different families (Handa et al., 1983). The benzoquinones are biosynthesized through two independent pathways, both branching from shikimic acid (Scheme 2). Direct dehydrogenation gives rise to benzoic acid derivatives, such as syringic acid. Alternatively, oxidative removal of the propene side chain of phenylpropanoids, such as sinapic acid, gives the same benzoic acid which is oxidatively decarboxylated to the quinone (Harborne, 1977; Haslam, 1974). Both of these pathways are found in large numbers of plants and the widespread occurrence of 2,6-DMBQ is not surprising. However, all our efforts to identify 2,6-DMBQ in the root exudate of *Sorghum* have failed, and in fact, carefully collected exudate from healthy *Sorghum* seedlings does not induce haustorial development in *Striga*.

Xenognosin A, which induces haustoria in *Agalinis* and *Striga*, is structurally similar to 2,6-DMBQ. Both compounds contain the same metamethoxyphenol functionality, differing only in oxidation state. Structural modifications altering this functionality dramatically affect the activity of both compounds. Our report (Lynn et al., 1981) of phenolic compounds inducing haustorial development in *Agalinis* led MacQueen (1984) to study the activity of other phenolic compounds. Ferulic and sinaptic acids are active on *Striga hermonthica* at 10^{-6} M, whereas syringic acid shows half maximal activity at 10^{-7}

M. The level of haustorial induction by these phenolic acids is comparable to those observed by the similarly substituted quinones. Our studies have shown that 3,4-dihydroxycinnamic acid and 1,2,3-trimethoxybenzene, both of which either lack the methoxy or free hydroxy groups, show no activity whatsoever in *Striga asiatica*. These results suggest that the activity of all of these phenolics is mediated through a similar mechanism in *Agalinis* and *Striga* species, but it does not explain how these compounds are being detected by the parasite.

Umezawa et al. (1982) have shown that pathogenic fungi are capable of degrading lignin model compounds, such as 12, to 2,6-DMBQ by the action of phenol oxidases (Figure 6). In fact, simple syringic and sinapic acid derivatives give 2,6-DMBQ when treated with laccases or peroxidases (Caldwell and Steelink, 1969; Ishihara and Ishihara, 1976). A specific dye, syringaldazine, has been developed to test for the presence of these enzymes (Figure 5). The com-



SCHEME 2. Biosynthetic pathways for the production of 2,6-DMBQ.

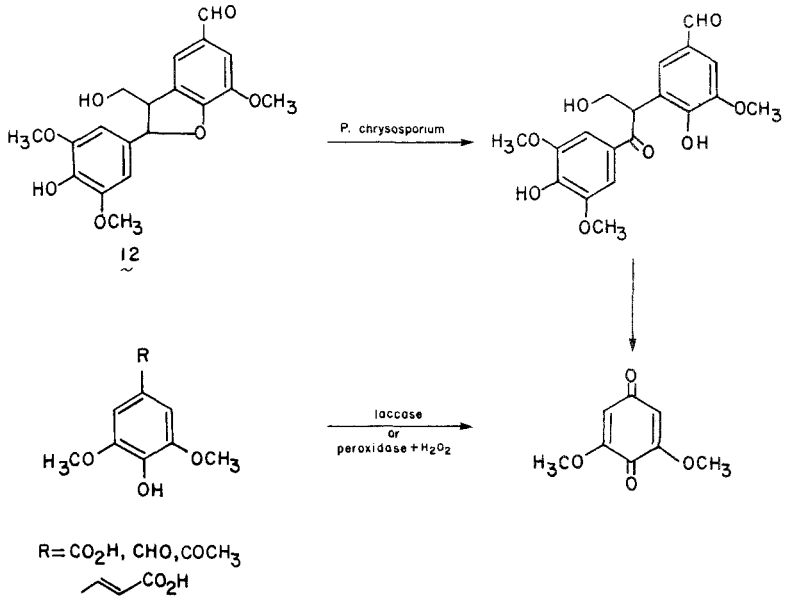


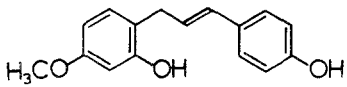
FIG. 6. Enzymatic breakdown of a lignin model compound by the pathogenic fungi *Phanerochaete chrysosporium*. Oxidation of alkyl phenols is possible with laccases or peroxidases to give compounds similar to 2,6-DMBQ.

pound, which is yellow in its reduced form, is oxidized to the red–purple quinone methide in the presence of laccases or peroxidases (Harkin and Obst, 1973).

Harkin and Obst used this dye to screen large numbers of fungi for phenol oxidases and were able to obtain a correlation between plant pathogenic fungi and excreted phenol oxidases. This correlation supported the specific use of these enzymes by the fungi to digest host-plant cell walls. Our results have shown that *Agalinis* has similar enzymatic activity apparently localized at the meristematic root tip. While the identification of this enzymatic activity says nothing about its specificity, the differential location of these enzymes in the parasite appears to be significant.

The presence of such activity would allow these parasites to oxidatively degrade phenylpropanoid surface components from proximal root surfaces. If a root surface with the proper phenylpropanoid is enzymatically degraded to the appropriately substituted quinone, such as 2,6-DMBQ, then the parasite responds by initiating the development of the host attachment organ. Most importantly, the strategic localization of these enzymes at the meristematic tip would guarantee proper distances being established between host and parasite before haustorial development is initiated. Structural modifications of xenognosin A that would be expected to effect this oxidation, such as reduction of

the propene double bond or the methoxy regioisomer (11), drastically reduce the biological activity. These other phenylpropanoids could be active as a result of their conversion into the active quinones. Such a theory of surface component degradation to the appropriate quinones would unify the mechanism of distance regulation and host selection in *Agalinis* and *Striga* and may be widespread among the Scrophulariaceae. Efforts are currently underway in our laboratory to further substantiate this mechanism of biological recognition.



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What we hope to have demonstrated is that plants, like animals, have evolved very specific recognition systems that allow them to respond to and regulate their environment. It is critical that parasitic angiosperms regulate and respond to environmental cues, and they have provided a very fruitful system for study. Such plant–plant interactions may prove to be far more prevalent and highly evolved than we ever dreamed possible. Through a better definition of the control of plant growth and development, we hope it will be possible to define more precisely the communication occurring between members of the plant kingdom.

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COMPONENTS OF MORIBUND AMERICAN ELM TREES AS ATTRACTANTS TO ELM BARK BEETLES, *Hylurgopinus rufipes* AND *Scolytus multistriatus*[1]

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Abstract—*Hylurgopinus rufipes* male and female beetles were attracted to American elms infected with Dutch elm disease, and to American elms killed by injection of cacodylic acid. *H. rufipes* was also attracted to solvent extracts of elm, or to Porapak Q-trapped volatiles from elm. The major components of attractive fractions of Porapak Q-trapped volatiles were isolated, identified, and tested in field bioassays. Several artificially compounded mixtures of sesquiterpenes were attractive to *H. rufipes*, although no bait tested was as attractive as diseased tree controls. Laboratory bioassays with *H. rufipes* were marginally successful. In laboratory bioassays, nine of 14 sequiterpenes identified from active fractions of Porapak extracts elicited significant response from *Scolytus multistriatus* male and female beetles: δ- and γ-cadinene, α-cubebene, γ-muurolene, and β-elemene were most active. However, in field tests, none of the sesquiterpenes alone or in combination significantly attracted *S. multistriatus*, nor did they significantly enhance the attraction of *S. multistriatus* to female-produced pheromone components (4-methyl-3-heptanol [H] and α-multistriatin [M]). In other field tests, α-cubebene (C) significantly enhanced response of *S. multistriatus* to H plus M, but foliage, logs, or chips of healthy elm did not enhance trap catch to HMC.

Key Words—*Hylurgopinus rufipes*, *Scolytus multistriatus*, Coleoptera, Scolytidae, sesquiterpenes, host attractants, Dutch elm disease, kairomone, pheromone, elm bark beetles, *Ulmus americana*.

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INTRODUCTION

Dutch elm disease has ravaged elm populations in North America and Europe. In North America, the principal vectors of the disease are two scolytids: the European elm bark beetle [*Scolytus multistriatus* (Marsham)] and the native elm bark beetle [*Hylurgopinus rufipes* (Eichhoff)]. *S. multistriatus* is the dominant species in most of the United States, and it has been studied extensively (for review, see Gardiner, 1976). In the mid-1970s, the aggregation pheromone complex for *S. multistriatus* beetles was reported (Pearce et al., 1975), and synthetic pheromones are now registered for detection and mass trapping of this beetle (e.g., O'Callaghan et al., 1980; Lanier, 1981; Peacock et al., 1981).

H. rufipes beetles are the principal vector of Dutch elm disease in the northern United States and Canada, where winters are too severe for *S. multistriatus* to survive in significant numbers. To date, attempts to demonstrate the use of pheromones by *H. rufipes* have failed (Peacock, 1979; Gardiner, 1979), and it has been suggested that this insect may aggregate using only host volatiles (Gardiner, 1979; Lanier, 1983). Indeed, several groups have reported, and we have confirmed, that both sexes of *H. rufipes* are attracted to cut elm wood (Martin, 1936), wounded elms (Landwehr et al., 1981), naturally moribund elms (Gardiner, 1979), and especially trees killed with the arboricide cacodylic acid (Gardiner, 1979).

There have been a few reports of volatile compounds produced by the genus *Ulmus* (e.g., Rowe et al., 1972; Rowe and Toda, 1969; Pearce et al., 1975; Peacock et al., 1975; Blight et al., 1978; 1983; Blight, 1981) and of volatiles produced by microorganisms associated with *Ulmus* (French et al., 1984). However, there has been no systematic attempt to isolate and identify the principal volatiles released by elm trees; most previous work with volatile compounds has been directed towards the identification of pheromones produced by *S. multistriatus* and *S. scolytus* (Fab.) (Pearce et al., 1975; Peacock et al., 1975; Lanier et al., 1976; Blight, 1981). Since the pheromone components in these cases were found to be much more powerful attractants than host volatiles, only the sesquiterpene α-cubebene, which synergized the pheromone of *S. multistriatus*, was of more than passing interest. However, Peacock et al. (1984) recently showed that elm logs enhanced attraction of *S. multistriatus* to multilure, and this effect could not be duplicated by increasing levels of α-cubebene. In the case of *H. rufipes*, where no pheromonal activity has been demonstrated, host volatiles are of paramount interest as potential attractants.

The aims of this research were: (1) to isolate and identify the attractive host volatiles produced by moribund elm trees, (2) to test artificially compounded mixtures of attractive chemicals as baits for *H. rufipes*, and (3) to determine whether additional host compounds are involved in the aggregation of *S. multistriatus*.

METHODS AND MATERIALS

Field Bioassays. Experiment 1 (Table 1) tested the hypothesis that *H. rufipes* beetles aggregate on wood suitable for breeding in response to pheromones. *H. rufipes* reared from our laboratory colonies were induced to bore into elm bolts (30 cm long and 15 cm diameter) to produce the following treatments: 20 females in log, 20 males in log, 10 males plus 10 females in log, and log without beetles. The logs were placed on the ground in sticky $\frac{1}{4}$-in. mesh hardware cloth cylinders closed at both ends by plywood squares. Experimental details are as follows: From May 24 to July 30, 1978, three replications were exposed near Ohio, Herkimer County, New York; from July 21 to September 8, 1978 and from September 5 to October 6, 1978, single replications were exposed near Manlius, Onondaga County, New York. *H. rufipes* were collected from the sticky traps at irregular intervals and at the end of the experiment. The Ohio test site was an open grassy area, whereas the Manlius sites were densely wooded with elms and other broad-leaved trees.

Experiment 2 (Table 2) was a field bioassay to measure the relative attractiveness of healthy, diseased, and cacodylic acid-killed trees. White, plastic-coated, 20 × 45-cm paper sheets treated with Stickum Special® remained wrapped around test trees at heights of 1–2 m above ground in Oswego County, New York from April 30 to July 6, 1979. At the end of the test period, the number of beetles caught on each sticky panel was counted. In addition, we assessed the number of beetle attacks on the various trees as measured by the number of insects reaching the xylem per square decimeter of bark surface area.

Experiment 3 (Table 3) employed two bioassay methods to ascertain

TABLE 1. EXPERIMENT 1: FIELD RESPONSE OF *Hylurgopinus refipes* TO BEETLES IN LOGS AND LOGS ALONE[a]

	Test 1	Test 2	Test 3	Total[b]
Log + 20 males	9	29	20	58
Log + 20 females	5	23	21	49
Log + 10 males and 20 females	7	22	13	42
Log	10	14	5	29
Total	31	88	59	178

[a] Test 1, Ohio, New York, May 24–July 30; test 2, Manlius, New York, July 21–September 8; test 3, Manlius, New York, September 5–October 6, 1978. Each test consisted of three replications of each treatment.

[b] Combined catches for three tests differ significantly from equality among treatments ($\chi^2 = 10.09$; $P < 0.05$). The sex ratio for beetles captured was 1:1.2 (♀ : ♂), and there were no significant differences in sex ratios among treatments.

TABLE 2. EXPERIMENT 2: *Hylurgopinus rufipes* ATTRACTED TO AND ATTACKING HEALTHY, DISEASED, AND CACODYLIC ACID-KILLED ELM TREES

	Beetles on sticky bands		Beetle attacks/tree	
	Replicates	Mean[a]	Replicates	Mean[a]
Healthy	8	17.38a	3	0 a
Diseased	4	190.25b	3	1168b
CA-killed	9	259.69b	3	277c

[a]Means followed by the same letter are not significantly different (Student's t test, $P < 0.01$).

TABLE 3. EXPERIMENT 3: FIELD ATTRACTION OF *Hylurgopinus rufipes* TO ELM EXTRACTS

	Date/place	Treatment	Beetles caught
Test 1	May 8–14, 1981	Ethanol extract, 20 μl	71
	Oswego Co., New York	Ethanol	13
Test 2	June 6, 1981	Porapak-Q extract, 20 μl	86
	Syracuse, New York	Hexane, 20 μl	0
		Ethanol extract, 20 μl	3
		Ethanol, 20 μl	0

whether *H. rufipes* was attracted to either an ethanol extract of the bark of cacodylic acid-killed elms or to a pentane extract of Porapak-Q-trapped volatiles from bark of killed elms. In the first test, blower olfactometers (Gara and Vité, 1962) were used to bioassay extracts dispensed from 2-ml polyethylene vials with a pinhole in the cap. In the second test, 10 μl of the extracts were pipetted onto Masonite panels that were fastened immediately above sticky traps (45 × 22 cm white cardboard sheets) placed 1–2 m above ground on healthy elms. The Masonite panels were rebaited and rotated among trap positions at 30-min intervals.

Experiment 4 (Table 4) field bioassayed the response during May to July 1984 of *H. rufipes* to compounds identified from elm. Three test plots were used: one was in a level swampy area in Rutland, Vermont, and the other two were outside Tinmouth, Vermont, on gently sloping hillsides. On all sites, elms were present but were not the most abundant hardwood species. Two replicates of each bait were tested in each plot. Traps were 50 × 25-cm cardboard panels coated with Stickem Special, wrapped around and stapled to trees about 1.5 m above ground level. Traps with baits were placed at least 8 m apart on species

TABLE 4. EXPERIMENT 4: ATTRACTION OF *Hylurgopinus rufipes* TO SESQUITERPENES IN THE FIELD, TINMOUTH AND RUTLAND, VERMONT, MAY 24–JUNE 16, 1984

Bait[b]	Beetles/trap observation[a]	
	Number	Mean ± SE
Diseased elm	12	31.33 ± 7.85a
3	12	11.83 ± 2.72b
4	12	8.16 ± 2.55bc
5	12	7.83 ± 2.23bc
6	12	4.25 ± 1.16cd
2	12	2.58 ± 0.68d
1	12	2.25 ± 1.32de
7	12	1.33 ± 0.62de
Healthy elm	12	0.58 ± 0.26e
8	12	0.42 ± 0.19e

[a] Three plots each with two replications observed twice (May 31, June 16) for 12 trap observations. Means followed by the same letter were not significantly different ($P > 0.05$, Student's t test).
[b] Baits were as follows: 1, β-elemene; 2, α-cubebene; 3, β-elemene, α-cubebene, α-copaene, α-gurjunene, allo-aromadendrene, thujopsene, and β-caryophyllene; 4, same as 3 less β-elemene and α-cubebene; 5, same as 3 less β-elemene; 6, same as 3 less α-cubebene; 7, δ-cadinene; 8, calamenene, γ-muurolene, α-humulene, α-selinene, and α-circumene. Later tests of β-elemene + α-cubebene and other combination yielded no data. Lack of beetles on traps on diseased elms indicated that few beetles were moving to breeding material after late June.

of trees other than elms and on healthy and diseased elm trees (controls). Baits were prepared by absorbing hexane solutions of compounds into grey rubber GC septa (Supelco, 9 mm). Each septum was loaded with 1 mg of each component of the bait. Baits were impaled on a large metal paper clip bent into a U-shape, and the paper clip was hooked over the top edge of the trap panel so that the bait was positioned about 4 cm from the trap surface. The bait was covered with a 2-dr glass shell vial to keep off rain and wind-blown debris. Baits were assigned to trees by randomly drawing vials of baits from a bag.

The same sticky traps were used throughout the test period, but the baits were replaced when the trap catches were counted. All *H. rufipes* were removed when the trap catches were counted at two-week intervals. Traps were examined twice; thus there were 12 observations of each bait (3 plots × 2 replicates/plot × 2 observation periods). Testing was discontinued when the trap catch on diseased control trees fell to less than $\frac{1}{10}$ of the peak season catch.

Experiment 5 (Table 5) examined the effects of elm volatiles on the enhancement of attraction of *S. multistriatus* to its synthetic pheromone, multilure. Multilure was dispensed from Conrel® hollow fibers (Albany International, Needham Heights, Massachusetts) at nominal daily release rates of 274 μg 4-methyl-3-heptanol, 126 μg α-multistriatin, and 369 μg α-cubebene (Cuth-

TABLE 5. EXPERIMENT 5: ATTRACTION OF *Scolytus multistriatus* TO PHEROMONE AND HOST ODORANTS IN THE FIELD, SYRACUSE, NEW YORK

Bait[a]	Beetles/trap observation		Index (mean ± SE)[c]
	Number[b]	Mean	
A. May 25–August 25, 1980			
HMC	22	820.6	46.15 ± 2.78a
HMC + log	22	905.9	45.89 ± 3.89a
22 ♀ ♀ + log	22	58.1	4.88 ± 2.00b
Log	22	41.9	2.97 ± 1.26b
B. June 1–August 14, 1981			
HMC + chips	16	365.5	33.0 ± 2.47a
HMC	16	310.4	25.9 ± 2.67ab
HMC + log	16	265.9	22.2 ± 2.59bc
HMC + foliage	16	200.1	18.8 ± 1.97c

[a] HMC 4-methyl-3-heptanol, α-multistriatin, and α-cubebene dispensed from Conrel hollow fibers.
[b] A, two replications observed 11 times; B, four replications observed four times. Logs, chips, and foliage were changed at each observation.
[c] Catches on each trap were indexed as a percentage of the total catch on a replicate. Total catch 1980, 18,270 beetles; 1981, 40,183 beetles.

bert et al., 1983). Beetles were trapped on white 46 × 66-cm poly-coated cardboard sticky traps affixed 2.5 m above the ground to utility poles at least 40 m apart on streets where there were no elms in a residential section of Syracuse, New York.

In 1980, treatments were: (1) multilure, (2) multilure plus an elm log (25 cm long, 8–10 cm diam), (3) 20 female *S. multistriatus* beetles with an elm log (as a source of natural pheromone), and (4) an elm log alone. Each treatment was represented once on two experimental replicates on different streets. Beetles on traps were counted and logs were changed at weekly intervals from May 25 to August 25. Logs were wrapped in fine mesh copper screening during the experiment and examined for beetle attacks when they were replaced.

In 1981 treatments were: (1) multilure, (2) multilure plus elm log, (3) multilure plus 180 g elm wood and bark chips (produced with a chain saw), and (4) multilure plus 120 g fresh elm foliage. The logs were as described for the 1980 experiment; elm foliage and chips were held in copper wire screen baskets affixed to utility poles. Each treatment was represented once in each of four replicates. Fresh elm materials were provided, and treatments within replicates were rotated at the beginning of four 21-day observation periods between June 1 and August 14.

Experiment 6 (Table 6) investigated the possibility that limonene could be substituted for α-cubebene as the host component of multilure. Methods of bee-

TABLE 6. EXPERIMENT 6: ATTRACTION OF *Scolytus multistriatus* TO PHEROMONE AND COMBINATIONS OF PHEROMONE PLUS α-CUBEBENE OR LIMONENE; SYRACUSE, NEW YORK, AUGUST 1–SEPTEMBER 3, 1980

Bait[a]	Beetles/trap observation		Index (mean ± SE)[c]
	Number[b]	Mean	
HMC	20	511.4	33.10 ± 3.57a
HML−	20	353.0	23.74 ± 3.49ab
HM	20	302.4	21.92 ± 2.92b
HML+	20	290.3	20.99 ± 2.72b
Blank	20	3.5	0.27 ± 0.08c

[a] H = 4-methyl-3-heptanol; M = α-multistriatin dispensed from Conrel hollow fiber; C = α-cubebene; L+ = (+)-limonene; L− = (−)-limonene dispensed from polyethylene vials.
[b] Four replicates observed five times (weekly intervals).
[c] Catches on each trap were indexed as a percent of the total replicate catch during each observation interval. Total beetle catch for the experiment was 29,214 beetles. Means followed by the same letter were not significantly different ($P > 0.05$, Student's t test). The probability level for the difference between HMC and HML− was $<0.10 >0.05$.

tle trapping and release of 4-methyl-3-heptanol and α-multistriatin were the same as those used in experiment 5. Limonene was released from heat-sealed 2-ml polyethylene vials with 2 mg of (+)- or (−)-limonene loaded into each vial. α-Cubebene was released from grey rubber GC septa (9 mm, Supelco) which had been impregnated with 1 mg α-cubebene in hexane solution. Actual release rates of limonene and α-cubebene were not determined. Baits were placed in a cardboard shelter and stapled to the trap substrate. Each of four treatments and a blank was replicated four times and observed at weekly intervals between August 1 and September 3, 1980.

Insects and Laboratory Bioassays. H. rufipes were from colonies maintained as described by Gardiner and Roden (1977). *S. multistriatus* adults used in laboratory bioassays emerged from naturally infested elm wood and were conditioned in glass Petri dishes under 12 hr fluorescent light at 23°C and ambient humidity (Lanier et al., 1977). In early bioassays of host extracts with *S. multistriatus*, the males and females were tested separately, but when no significant differences in response were evident, the sexes were not separated.

The bioassay device used was the Moeck (1970) olfactometer, in which beetles were induced by positive phototaxis to cross an air flow that bore a narrow odor stream. Beetles that turned and walked 2 cm toward the odor source or made at least one 360° turn within the odor stream were judged to have responded positively. Beetles that did not respond to the odorant on the first trial were released once more; the response for the group was the sum of the two trials. This is one of the assays used in the isolation of *S. multistriatus*

pheromone components (Pearce et al., 1975) and in subsequent laboratory tests (Lanier et al., 1977; Lanier and Burns, 1978; Elliott et al., 1979). A walkway olfactometer (Jantz and Rudinsky, 1965) and a dual-choice device (White and Richmond, 1979) were also tried but found unsuccessful for bioassays with *H. rufipes*.

For all laboratory bioassays with *S. multistriatus*, a 5 beetle-hour equivalent of multilure (the synthetic pheromone of *S. multistriatus*) was the standard against which responses to other volatiles were indexed (5 beetle-hour equivalents = 12.5 ng 4-methyl-3-heptanol, 1 ng α-multistriatin, and 25 ng α-cubebene).

Experiment 7 (Table 7) assessed the laboratory response of *S. multistriatus* to the same ethanolic and Porapak extracts of moribund elm tested for the field response of *H. rufipes* (experiment 3).

Experiment 8 (Table 8) bioassayed response of *S. multistriatus* to fractions and subfractions of one of the Porapak extracts (EB43). A pentane extract of Porapak was made following aeration (2 liters/min) from July 16 to 25, 1980, of 5.5 kg bark stripped from a 30-cm-diameter American elm that had been killed by cacodylic acid treatment on June 20. The fractionation procedures are described in the section "Isolation and Identification of Compounds from Elm" (below).

Experiment 9 (Table 9) tested the laboratory response of *S. multistriatus* to compounds (5 ng in 1 μl hexane on filter paper) isolated from the most attractive host extract fractions (1 and 2, Table 8).

Experiment 10 (Table 10) tested individual sesquiterpenes as possible synergists to H + M in attracting *S. multistriatus*. Bioassays were run from

TABLE 7. EXPERIMENT 7: RESPONSE OF *Scolytus multistriatus* TO MULTILURE AND ELM EXTRACTS IN MOECK OLFACTOMETER

Treatment	Index[b]	
	Replicates[a] (*N*)	Mean ± SE
Multilure (pheromone standard)[c]	12	100 ± 0a
Porapak-Q extract of logs	19	44.3 ± 27.4b
Ethanol extract of bark	4	15.9 ± 10.6c
Blank airstream	9	0.7 ± 0.8d

[a] 25 Beetles/replicate.

[b] Response to each stimulus was indexed as a percent of the response to the pheromone standard during a series of tests. Letters indicate significant differences ($P < 0.05$, t test of pairwise comparisons).

[c] 12.5 ng 4-methyl-3-heptanol, 1 ng α-multistriatin, and 25 ng α-cubebene in 5 μl hexane.

TABLE 8. EXPERIMENT 8: RESPONSE OF *Scolytus multistriatus* TO FRACTIONS[a] OF HOST EXTRACT IN MOECK OLFACTOMETER, 1980–1981

Material tested	Replicates	Index (mean ± SE)[b]
Multilure[c]	12	100 ± 0a
Porapak extract	7	77.1 ± 5.71b
Individual fractions of extract		
1 (monoterpenes)	5	44.0 ± 5.79c
2 (sesquiterpenes)	5	57.2 ± 7.95bc
3 (residues)	4	24.2 ± 10.40cd
4 (residues)	4	20.2 ± 7.61cd
Combined fractions of extract		
1 + 2 + 3	5	52.6 ± 6.70c
2 + 3 + 4	5	43.6 ± 6.50c
1 + 3 + 4	5	25.2 ± 2.87d
1 + 2 + 4	5	46.6 ± 6.02c
Air control	16	12.1 ± 3.82c

[a] Fractions 1–4 were collected at 0–24, 24–40, 40–74, and 74–126 min, respectively; 1.5 m × 7 mm ID, 10% Carbowax 20 M on chromosorb W 50/80 N_2 80 cc/min, 100° for 24 min, 100–190° at 6°/min. Fraction 2 was cut into five subfractions collected at 0–22, 22–29, 29–40, 40–47, and 47–66 min, respectively; 2.4 m × 4 mm ID, 4% Apiezon L on chromosorb G 60/80, N_2 60 cc/min, 150°. Indices of response to five subfractions of fraction 2 ranged from 8.5 to 20.5 for individual subfractions and from 11.0 to 22.0 for the various combinations of four of the five subfractions.

[b] Response to each stimulus was indexed as a percent of the response to the pheromone standard during a series of tests. Letters indicate significant differences ($P < 0.05$, t test of pairwise comparisons).

[c] 12.5 ng 4-methyl-3-heptanol, 1 ng α-multistriatin, and 25 ng α-cubebene in 5 μl hexane.

August 15 to September 12, 1984, in residential Syracuse, New York. Traps were set up as described in experiment 7. H + M were released from Conrel fibers (experiment 7), and sesquiterpenes were released from grey rubber GC septa (Supelco, 9 mm) loaded with 1 mg of test compound. Each of six sesquiterpenes, α-cubebene, β-elemene, thujopsene, α-copaene, allo-aromadendrene, and α-gurjunene, were tested alone and in combination with α-multistriatin and 4-methyl-3-heptanol (HM).

Traps with large number of beetles were removed and replaced to facilitate counting the trap catch. When trap catches were large (>500 beetles, estimated) beetles in 10 circles (4.5 cm diam) printed on the trap were counted, and the total catch was estimated by the regression equation $y = 41.8 + 12.5x$, where y is estimated total catch and x is the number of beetles on the circles.

Experiment 11 (Table 11) tested mixtures of sesquiterpenes as possible synergists to H + M in attracting *S. multistriatus*. Traps and baits were prepared as described in experiment 9. Sesquiterpene treatments were prepared with 1 mg of each sesquiterpene per septum. All *S. multistriatus* on the traps

TABLE 9. EXPERIMENT 9: RESPONSE OF *Scolytus multistriatus* TO SESQUITERPENES IN MOECK OLFACTOMETER, 1983–1984

Material tested[a]	Replicates[b]	Index (mean ± SE)[c]
Pheromone standard[d]	30	100.0a
δ-Cadinene	8	65.0 ± 9.02b
γ-Cadinene	3	63.3 ± 5.25b
α-Cubebene	7	51.9 ± 12.09bc
γ-Muurolene	3	44.0 ± 11.62bc
β-Elemene	16	43.2 ± 5.05bc
±-Calamenene	3	41.7 ± 19.24bc
α-Selinene	7	34.4 ± 7.78c
α-Muurolene	4	29.2 ± 14.05cd
±-Cyclosativene	5	20.2 ± 4.83cd
α-Gurjunene	6	9.8 ± 3.35de
Thujopsene	6	9.5 ± 3.72de
β-Caryophyllene	6	8.4 ± 3.39de
Allo-aromadendrene	6	5.9 ± 1.96de
α-Copaene	6	5.9 ± 3.10de
Blank	13	6.3 ± 1.88e

[a] 5 ng/μl hexane on filter paper.
[b] Twenty-five unsexed *S. multistriatus*/replicate.
[c] Response to each stimulus was indexed as a percent of the response to the pheromone standard during a series of tests. Letters indicate significant differences ($P < 0.05$, t test of pairwise comparisons).
[d] 12.5 ng 4-methyl-3-heptanol, 1 ng α-multistriatin, and 25 ng α-cubebene in hexane solution totaling 5 μl.

were counted four times at weekly intervals. The experiment was run from July 6 to September 6, 1984, in residential Syracuse, New York.

Isolation and Identification of Compounds from Elm. American elm logs (wood + bark) were obtained from an urban area of Syracuse, New York. Logs were either sawn into thin disks or chipped with an industrial chipper. When required, logs were peeled so that wood and bark could be extracted separately. Ethanol extracts were made by steeping macerated bark (25 g) in 200 ml of 95% ethanol at room temperature. The resulting solutions were filtered and concentrated by removal of ethanol through a fractional distillation column.

For preparation of Porapak-Q extracts, an aeration apparatus was made from a thoroughly cleaned 55-gallon steel drum, fitted with a charcoal trap at the inlet, and a Porapak-Q trap (20 cm × 2.5 cm ID) at the outlet. A vacuum pump was used to pull air through the apparatus (2–9 liters/min). Aerations were continued for a period of one week, after which the material being aerated and the Porapak-Q trap were changed. Porapak-Q traps were stripped of volatiles by percolating 100 ml of glass-distilled pentane through the trap. The re-

TABLE 10. EXPERIMENT 10: ATTRACTION OF *Scolytus multistriatus* TO PHEROMONE AND INDIVIDUAL SESQUITERPENES IN THE FIELD[a]

	Beetles/trap observation		Index[c]	
Bait[b]	Number	Mean	Number	Mean ± SE[d]
HME	6	827.8	6	36.7 ± 9.4a
HMT	6	752.7	6	34.7 ± 19.7a
HM	30	675.4	30	35.9 ± 14.0a
HMCu	30	675.4	30	31.8 ± 13.4a
HMA	6	543.2	6	31.3 ± 11.5a
HMCo	6	402.3	6	26.0 ± 17.4a
HMG	6	528.3	6	23.8 ± 3.1a
XS_i	30	10.6	30	1.0 ± 1.3b
Blank	30	6.9	30	0.8 ± 1.1b

[a] Individual sesquiterpenes were presented in two replications, observed three times. Other baits were presented in each replicate with each of the five sesquiterpene (two replicates × three observations × five experiments = 30).

[b] H, 4-methyl-3-heptanol; M, α-multistriatin dispensed from Conrel hollow fibers. The following sesquiterpenes were dispensed from rubber septa: E, β-elemene; T, thujopsene; Co, α-copaene; A, allo-aromadendrene; Cu, α-cubebene; G, α-gurjunene; XS_i are sesquiterpenes that were presented individually but pooled in this table.

[c] Catches on each trap were indexed as a percentage of the total catch on traps in each replicate during each observation interval. Total beetle catch for the experiment was 70,725.

[d] Means followed by the same letter were not significantly different ($P > 0.05$, Student's t test).

sulting solution was concentrated to 200–300 μl with a fractional distillation column.

The crude extract was then fractionated with a series of preparative GC columns (Table 12). Preparative GC was done with a Varian 1700 Aerograph GC, equipped with an FID detector, and using nitrogen carrier gas. GC fractions were collected with a thermal gradient collector (Brownlee and Silverstein, 1968), using 1.7- or 3-mm OD glass capillaries. All preparative columns used were glass, packed with 60/80 mesh AW-DMCS Chromosorb G or W. Generally, injector and detector temperatures were 170° and 200°, respectively. A Varian 3700 capillary GC equipped with an FID detector and using helium carrier gas was used for analytical GC work in the splitless mode. Fused silica columns used were FFAP (50 m × 0.21 mm ID, 35° for 1.67 min, programmed at 4°/min to 170°, held for 30 min) or OV-101 (50 m × 0.21 mm ID, 35° for 1.67 min, programmed at 4°/min to 190°, held for 30 min), with injector and detector temperatures of 150° and 210°, respectively. A Finnigan 4000 capillary GC-MS was used in splitless mode with EI (70 eV). Columns were fused silica FFAP (50 m × 0.21 mm ID) and DB-1 (30 m × 0.21 mm ID) temperature programmed from 50° to 150° at various rates. Proton NMR spectra were

TABLE 11. EXPERIMENT 11: ATTRACTION OF *Scolytus multistriatus* TO PHEROMONE AND COMBINATIONS OF SESQUITERPENES IN THE FIELD; SYRACUSE, NEW YORK, JULY 6–SEPTEMBER 6, 1984

	Beetles/trap observation[b]		Index[c]	
Bait[a]	Number	Mean	Number	Mean ± SE
HMS 16	10	6.88	4	27.81 ± 7.54a
HMS 13	13	26.38	4	14.57 ± 3.20a
HMS 15	9	33.55	4	13.46 ± 3.39a
HMS 18	10	24.50	4	12.25 ± 2.30a
HMS 14	10	20.60	4	9.68 ± 2.63a
HM	20	21.80	4	9.28 ± 1.84a
HM 17	10	18.20	4	8.50 ± 2.60a
XS_i	32	0.84	18	0.37 ± 0.04b
Blank	11	0.73	3	0.28 ± 0.08b

[a] H, 4-methyl-3-heptanol; M, α-multistriatin dispensed from polyethylene vials. S 13–18 are combinations of sesquiterpenes dispensed from rubber septa. Sesquiterpene baits were as follows: 13, β-elemene, α-cubebene, α-copaene, α-gurjunene, allo-aromadendrene, thujopsene, and β-caryophyllene; 14, baits 13 + 17; 15, baits 13 + 18; 16, baits 17 + 18; 17, γ-cadinene, δ-cadinene, α-muurolene; 18, α-selinene,*trans*-calamenene, α-humulene, α-curcumene, α-calacorene.

[b] Sesquiterpenes were initially presented twice with HM and twice without HM (except HMS 13 appeared thrice). HM and blank were presented four times. During the fourth and final observation period HM was added to previously unaccompanied sesquiterpene and blanks. Two traps (blank and HMS 15) were down during one observation period.

[c] Each treatment was indexed as a percent of the total catch during an observation period. Means followed by the same letter are not significantly different ($P < 0.05$, Student's t test).

obtained with a Varian XL-100 spectrometer, using 5-mm or cavity tubes. In cases where high resolution was required, 360 MHz spectra were recorded on a Bruker WM-360 spectrometer. Solvents used were $CDCl_3$ (100% D, Sigma Chemical Co.) for nonaromatic samples, and CD_2Cl_2 (100% D, Sigma Chemical Co.) for aromatic samples. Optical rotations were measured on a Perkin-Elmer model 141 polarimeter, using a 1 ml cell (1 dm path length) and chloroform solvent at 589 nm (Na "D" line).

Individual compounds were isolated from the crude pentane extracts of Porapak-Q-trapped volatiles by using the sequences of columns shown in Table 12. Isolated compounds were identified by comparison of their spectra, and Kovats indices with published data (Davies and Bignall, undated; Pearce, 1975; Andersen and Falcone, 1969; Swigar and Silverstein, 1981; Heller and Milne, 1978; Yukawa and Ito, 1973) and with those of authentic samples (Table 13). Confirmation of structures was obtained by coinjection of our isolated compounds with authentic compounds on capillary columns. Where the quantity of isolated material permitted, the sign of the optical rotation and, in two instances, the specific rotation were determined.

Authentic samples of compounds were obtained from a variety of sources. Monoterpenes, with the exception of sabinene (Firmenich, S.A., Geneva), and 2-methylnaphthalene were obtained from Aldrich Chemical Co. Sesquiterpenes were obtained from sources listed in Table 13.

Isolation of individual sesquiterpenes from essential oils was in general carried out as follows. The fraction with a boiling point of 81° at 15 mm Hg to 150° at 0.1 mm Hg was separated. The hexane-soluble portion of this material was loaded onto a slurry-packed (hexane) column of alumina (neutral, activity I, 60/80 mesh; 1 g hexane-solubles per 25 g alumina) and eluted with three column volumes of hexane under gravity flow. The eluate was concentrated under reduced pressure and loaded onto a flash chromatography column of 15% $AgNO_3$ on silica gel (230/400 mesh) with a loading of no more than 1 g of material to 50 g of silica. Columns were eluted with hexane followed by increasing concentrations of ether in hexane. Where necessary, final purifications were done by preparative GC, using a 4-m × 1-cm-OD glass column packed with 20% FFAP on AW-DMCS Chromosorb G (60/80 mesh).

β-Elemene was prepared by dehydration of elemol. Since several attempts to purify crude elemol by fractional crystallization failed, the crude material (a viscous syrup), was used directly. Crude elemol (Firmenich, SA) refluxed with a fivefold excess of acetic anhydride for 48 hr gave a 9:1 ratio of β-elemene and an unidentified sesquiterpene olefin. Pure β-elemene was isolated in approximately 50% yield by concentration of the crude reaction mixture followed by fractional distillation through a 30-cm × 1-cm-ID column of glass helices, using a distillation head with a split ratio of 20:1, bp 28–32° at 0.05 mm Hg.

Racemic α-curcumene was synthesized by cuprous iodide-catalyzed reaction of the Grignard reagent of *p*-bromotoluene with the tosylate of 5-hepten-1-ol (Aldrich) in THF. Racemic calamenene, as a mixture of the *cis* and *trans* isomers (30:70 *cis–trans*), was synthesized by treatment of racemic α-curcumene with polyphosphoric acid at 80–90°, as previously described (Anderson et al., 1972).

α-Cadinene was prepared as a minor product of the dehydrohalogenation of cadinene dihydrochloride with $AgNO_3$ in DMSO (Mehta, 1971). Cadinene dihydrochloride was itself prepared from treatment of cubeb oil distillation fractions rich in α-copaene and δ-cadinene with dry HCl in ether at 0° (Herout and Sykora, 1958), followed by recrystallization of the crude product from ether at −70°.

RESULTS

Biology. The numbers of *H. rufipes* captured on traps baited with beetle-infested logs were low (Table 1) in spite of considerable beetle activity in the test areas; for example, unbaited sticky bands on utility poles and elms in the

TABLE 12. COMPOUNDS ISOLATED FROM ELM VOLATILES FROM ENTRAINED AIR COLLECTED ON PORAPAK-Q

Identified compound	Isolation sequence[a]	Identification[b]	Kovats' index: Carbowax 20 M, column A[c]		Kovats' index: Apiezon-L, column B[c]		Ratio
Decane	A[d]	MS, coinject					minor
(+)-α-Pinene	A, B	MS, coinject, NMR, rotation					major
(−)-β-Pinene	A, B	MS, coinject, NMR, rotation					major
Sabinene	A[d]	MS, coinject					trace
Undecane	A[d]	MS, coinject					trace
Myrcene	A[d]	MS, coinject					trace
Limonene	A[d]	MS, coinject					trace
p-Cymene	A[d]	MS, coinject					trace
(−)-α-Cubebene	A, B, C	MS, KI, NMR, coinject, rotation	(1481) 1482	132° 132°	(1368) 1368	155° 155°	minor
(+)-Cyclosativene	A, B, C	MS, KI, NMR, coinject, rotation	(1549) 1551	165° 165°	(1412) 1410	155° 155°	major
α-Copaene	A, B, C[e]	MS, KI, NMR, coinject	(1551) 1550	165° 165°	(1410) 1408	155° 155°	minor
(−)-α-Gurjunene	A, B, C	MS, KI, NMR, coinject, rotation	(1558) 1553	132° 132°	(1413)[f] 1408	130° 130°	minor
(−)-β-Elemene	A, B, C	MS, KI, NMR, coinject, rotation	(1608) 1603	132° 132°	(1410) 1406	155° 155°	major
(+)-β-Caryophyllene	A, B, C	MS, KI, NMR, coinject, rotation	(1618) 1620	132° 132°	(1452) 1449	155° 155°	minor
Thujopsene	A, B, C	MS, KI, coinject	(1684) 1679	165° 165°	(1676) 1676	155° 155°	trace
(−)-Allo-aromadendrene	A, B, C, D, C	MS, KI, NMR, coinject, rotation	(1700)[g] 1700	172° 172°	(1495)[g] 1500	161° 161°	minor

α-Humulene	A, B, C, D, C	MS, KI, NMR, coinject	(1719) 1716	165° 165°	(1487) 1485	155° 155°	minor
γ-Muurolene	A, B, C, B	MS, KI, NMR, coinject	(1724) 1728	165° 165°	(1506) 1503	155° 155°	minor
(−)-α-Muurolene	A, B, C, D, C	MS, KI, NMR, coinject, rotation	(1753) 1753	165° 165°	(1548) 1548	190° 190°	minor
(+)-α-Selinene	A, B, C, D, B	MS, KI, NMR, coinject, rotation	(1751) 1745	150° 150°	(1534) 1532	155° 155°	minor
(+)-δ-Cadinene	A, B, C, E	MS, KI, NMR, coinject, rotation	(1784) 1790	165° 165°	(1562) 1564	190° 190°	major
(+)-γ-Cadinene	A, B, C, E	MS, KI, NMR, coinject, rotation	(1792) 1797	165° 165°	(1574) 1573	190° 190°	minor
α-Cadinene	A, B[d]	MS, coinject	(1819)[h] 1819	165° 165°	(1602)[h] 1602	190° 190°	trace
(−)-*trans*-Calamenene	A, B	MS, KI, NMR, coinject, rotation	(1839) 1835	150° 150°	(1568) 1569	190° 190°	major
2-Methylnaphthalene	A, B	MS, NMR, coinject					minor
α-Calacorene	A, B	MS, KI, NMR, coinject	(1926) 1933	165° 165°	(1590) 1586	190° 190°	minor
Cadalene	A, B	MS, KI, coinject	(2581)[h,i] 2581	200° 200°	(1733) 1732	195° 195°	minor

[a] Column used in isolation procedure: A: 10% carbowax 20 M, Chromosorb W, 2 m × 9 mm ID, 100°/23.3 min, 6°/min, 190°/45 min. B: 4% APL, Chromosorb G, 2 m × 4 mm ID, 152°. C: 10% FFAP, Chromosorb W, 7 m × 4 mm ID, 110°, D: 4% TCEP, Chromosorb G, 3.2 m × 4 mm ID, 110°. E: 5% APL, Chromosorb G, 7 m × 4 mm I.D., 180°.

[b] Monoterpene coinjections done on FFAP and OV-101 capillary columns; sesquiterpene coinjections on FFAP capillary column. Details in Methods and Materials. Only the signs of rotation were measured. KI = Kovats' index.

[c] Literature values are in parentheses (Davies and Bignall, undated).

[d] Pure compounds not isolated.

[e] Isolated material contained 10–20% impurity.

[f] Kovats' index on 5% SE-30, on Chromosorb W, 1.5 m × 10 mm OD.

[g] Literature values from Pearce (1975).

[h] Calculated Kovats' index for authentic compound.

[i] Kovats' index on a 5% DEGS on Chromosorb G, 2 m × 4 mm ID.

TABLE 13. SOURCES OR AUTHENTIC SESQUITERPENE STANDARDS

Compound	Source
(−)-Allo-aromadendrene	Gurjun Balsam (Fritzche, Dodge, and Olcott, Inc.)
Cadalene	Carnegie-Mellon University, API Standard Reference Materials
α-Cadinene	Synthesized
(+)-δ-Cadinene	Cubeb oil (Fritzche, Dodge, and Olcott, Inc.)
(+)-γ-Cadinene	Swedish sulfate turpentine
α-Calacorene	Roure Bertrand Dupont, Grasse, France
(±)-Calamenene	Synthesized
(−)-β-Caryophyllene	Fluka Chemical Corp.
(−)-α-Copaene	Cubeb oil (Fritzche, Dodge, and Olcott, Inc.)
(−)-α-Cubebene	Cubeb oil (Fritzche, Dodge, and Olcott, Inc.)
(+)-Cyclosativene	Dr. Edward Piers, University of British Columbia
(−)-β-Elemene	Synthesized
(−)-α-Gurjunene	Gurjun balsam (Fritzche, Dodge, and Olcott, Inc.)
α-Humulene	Fluka Chemical Corp.
(−)-α-Muurolene	Swedish sulfate turpentine (Dr. Torbjörn Norin, Kungl. Teknisha Högskolan, Stockholm, Sweden)
(+)-γ-Muurolene	Swedish sulfate turpentine
(±)-α-Selinene	Rosewood oil (J. Mannheimer, Inc.)
(−)-Thujopsene	Fluka Chemical Corp.

general area of test 1 caught 366 *H. rufipes*, while the hardware cloth sticky traps containing elm logs with or without *H. rufipes* boring in them caught an aggregate of only 31 beetles. During the spring, when *H. rufipes* beetles were colonizing elm for breeding, uninfested logs were as attractive as material that contained boring *H. rufipes*. However, in the late summer and fall, when new adults move to live elms in which they feed and overwinter, the beetle-infested log treatment (summed for tests 2 and 3) attracted significantly more *H. rufipes* than the uninfested log.

H. rufipes were clearly more strongly attracted to diseased elms, and to elms killed with cacodylic acid than to healthy elms (Table 2). This confirmed Gardiner's (1979) observations and indicated that volatile chemical attractants were produced by the dead or dying elm tree. Even through more *H. rufipes* were captured on cacodylic acid-killed elms, a significantly greater number of beetles bored into the bark of diseased elms. Treatment with cacodylic acid had

obviously rendered the trees less acceptable for breeding than trees dying naturally, as had also been noted with *Scolytus scolytus* and *S. multistriatus* in England (O'Callaghan et al., 1984).

Ethanol extracts of elm bark and pentane extracts of Porapak-Q-trapped volatiles from elm logs proved to be more attractive than solvent controls (Table 3). In the second test, a Porapak-Q extract was more attractive than the ethanol extract. In this test, one of us (GNL) observed flying *H. rufipes* following the Masonite panel to which the Porapak-Q extract had been applied as the treatments were being carried for rotation among trap positions. Due to the apparent potency of the Porapak extract, and because only volatile materials were obtained from Porapak-Q aerations, this type of extract was chosen for GC fractionation and isolation of attractive components.

In the laboratory, *H. rufipes* exposed to a variety of conditioning treatments gave no indication of response to elm extracts presented in a walkway olfactometer (Jantz and Rudinsky, 1965) or in a dual-choice device (White and Richmond, 1979). A few *H. rufipes* beetles gave a classic klinotactic response to stimuli presented in a Moeck (1970) forced-air olfactometer. However, the overall percent response was very low (4.9% and 8% to Porapak and ethanol extracts, respectively), and it was not deemed feasible or reliable to test individual isolated compounds for activity by this method.

Having no laboratory bioassay to test possible attractants for *H. rufipes*, all further bioassays were conducted in the field. Faced with a considerable number of identified compounds to bioassay, it was decided to test initially several combinations of compounds and, with nothing else to guide us, we decided to prepare mixtures based on the results of laboratory bioassays of the isolated compounds as attractants for *S. multistriatus* (Tables 7–9). We do not mean to imply that *H. rufipes* and *S. multistriatus* beetles are necessarily attracted by the same compounds, but *S. multistriatus* was attracted in lab assays to the extracts that attracted *H. rufipes* in the field, and it seemed possible that they might have some host attractants in common. Several points emerge from the field bioassays (Table 4). First, no bait tested was as attractive as a diseased elm tree. However, five of the eight baits tested were significantly more attractive than the healthy elm controls. In particular, a seven-component mixture (bait 3) was the most attractive bait in most of the replicates and its overall catch was approximately 40% of that of a diseased tree. Deleting α-cubebene (bait 6), β-elemene (bait 5), or both of these compounds (bait 4) appeared to reduce response to the residual mixture. α-Cubebene alone (bait 2) was more attractive than a healthy elm while β-elemene (bait 1) was not. Very low attraction to δ-cadinene (bait 7) and the combination of calamenene, γ-muurolene, α-humulene, α-selinene, and α-curcumene (bait 8) indicated that these compounds were of little interest to *H. rufipes*.

With these preliminary data in hand, different combinations of the sesquiterpenes were made up and tested. Unfortunately, by early July when new baits

were placed in the field, beetle flight to breeding material had virtually ceased (very low trap catches even on diseased trees), and no further useful data were obtained.

The first bioassays with *S. multistriatus* were designed to detect enhancement of pheromone baits by host plant material, but no evidence was found to indicate that the attractiveness of multilure (4-methyl-3-heptanol + α-multistriatin + α-cubebene) dispensed from Conrel fibers was enhanced by odors from plant material (Table 5). Experiments that extended through most of the *S. multistriatus* flight seasons in 1979 and 1980 caught more than 18,000 and 40,000 beetles, respectively, but no treatment caught significantly more beetles than the multilure bait alone. In fact, beetle catch on traps baited with multilure plus wilting foliage was significantly lower than that on multilure alone. Surprisingly, virgin females boring in logs (1980, Table 5) did not significantly enhance the attraction to logs. Removal of the bark revealed sterile (no larvae) beetle tunnels in all logs with which 20 female *S. multistriatus* were confined ($\bar{x}$ = 11.8 tunnels/log); no feral beetles had attacked the untreated logs.

Bioassays to test limonene as a substitute for α-cubebene, as had been reported by Blight et al. (1983), were inconclusive despite the large numbers of beetles caught. Addition of α-cubebene to a mixture of 4-methyl-3-heptanol (H) and α-multistriatin (M) significantly increased trap catches, but no significant differences could be demonstrated between either HM and [HM + (−)-limonene] or [HM + α-cubebene] and [HM +(−)-limonene]. Addition of (+)-limonene to HM baits had no discernible effect.

The pattern of response by *S. multistriatus* to Porapak extracts and ethanol extracts of elm tissue in laboratory bioassays (Table 7) was similar to that of *H. rufipes* in field tests (Table 3). The aeration-collected volatiles were clearly more active than the solvent extract, but both evoked response above the level of the air control in all tests.

A Porapak extract of elm bark was divided into four fractions by preparative GC (1, monoterpenes; 2, sesquiterpenes; 3 and 4, longer-retention-time compounds). Laboratory bioassays of individual GC fractions with *S. multistriatus* showed that the sesquiterpenes (fraction 2) could account for 74% of the response to the whole extract (Table 8). When fractions were presented in combinations, deletion of the sesquiterpene fraction significantly reduced response. Bioassay of subfractions of the sesquiterpene fraction evoked only low responses from *S. multistriatus;* recombining subfractions increased responses, but the interaction seemed additive rather than synergistic. Since further fractionation and bioassay seemed impractical, we proceeded to identify the principal components in the active fraction and to bioassay synthetic or purified samples of these compounds. Results of the chemical analysis are given in the following section.

When 14 sesquiterpenes isolated from the most attractive fraction of the elm extracts were bioassayed with *S. multistriatus* in the Moeck olfactometer,

nine compounds evoked a response significantly higher than the blank (Table 9). These were 20–65% as attractive as the pheromone standard. The most attractive compounds were δ-cadinene, γ-cadinene, α-cubebene, γ-muurolene, β-elemene, and calamenene. Of these, only α-cubebene had been previously identified as an attractant component for *S. multistriatus* (Pearce et al., 1975).

In field bioassays, traps baited with sesquiterpenes, either individually or in combinations, did not catch significantly more *S. multistriatus* than blank traps (Tables 10 and 11). Mean catches on traps baited with *S. multistriatus* pheromone components H and M plus sesquiterpenes were not significantly different from those with HM alone. Despite lack of significance of differences, some field activity of sesquiterpenes is suggested because combinations of sesquiterpenes with HM generally attracted more beetles than HM alone (Table 11), and the ranked order of attractiveness of HM plus individual sesquiterpenes (Table 10) was similar to the order projected by laboratory bioassays (Table 9).

Chemistry. As was the case with similar aerations of *Ulmus americana* logs infested with *S. multistriatus* (Pearce et al., 1975; Peacock et al., 1975), and with extracts of several species of elm infested with *S. scolytus* (Blight et al., 1983), our Porapak-Q extracts consisted primarily of mono- and sesquiterpene hydrocarbons. A representative chromatogram is shown in Figure 1. The total amount of material obtained from any one large-scale aeration in the 55-gallon drum varied from 3 to 20 mg. The maximum amount of any one compound obtained was 4 mg for sesquiterpenes, and 10 mg for monoterpenes. The major portion of the volatiles of debarked and chipped or intact elm logs was sesquiterpenoid, while bark volatiles were primarily monoterpenes. Compounds isolated from the Porapak-collected elm volatiles are listed in Table 12 and

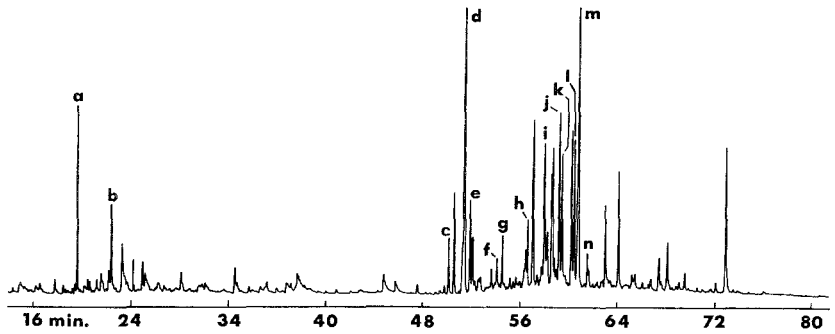

FIG. 1. Gas chromatogram of Porapak-Q-trapped volatiles from an elm log (0V-101 column 50 m × 0.21 mm ID, initial column temp. 35° for 1.67 min then programmed at 2°/min to 190°. He flow rate = 3 ml/min, detector 250°, injector 150°C): a, α-pinene; b, β-pinene; c, α-cubebene; d, cyclosativene; e, α-copaene; f, α-gurjunene; g, β-caryophyllene; h, α-huumulene; i, γ-muurolene; j, α-selinene; k, α-muurolene; l, γ-cadinene; m, δ-cadinene; n, *trans*-calamenene.

structural formulae of the principal compounds are illustrated in Figure 2. There was considerable variation in the relative and absolute amounts of compounds obtained from different extracts. This is not surprising, as no attempts were made to standardize the age, season, or condition of elm logs used for aerations; all of these factors could affect the secondary metabolite composition of the elm tree.

Because of the small amount of each compound isolated, it was not feasible to measure accurately the optical rotation; thus the sign of the rotation was the only value recorded (Table 12), with the exception of α- and β-pinene. These two monoterpenes were isolated in multimilligram quantities from several combined bark extracts. Isolated α-pinene gave a specific rotation of $[+25.2°]_D^{21}$ (c 5.5, $CHCl_3$), while a standard sample (Aldrich) gave $[+45.5°]_D^{21}$ (c 3.0, $CHCl_3$). The specific rotation of isolated β-pinene was $[-15.2°]_D^{21}$ (c 3.0, $CHCl_3$), lit. value $[-21°]_D^{21}$ (neat).

Authentic samples of sesquiterpenes that were not commercially available were isolated from essential oils or synthesized (Table 13). In particular, it was

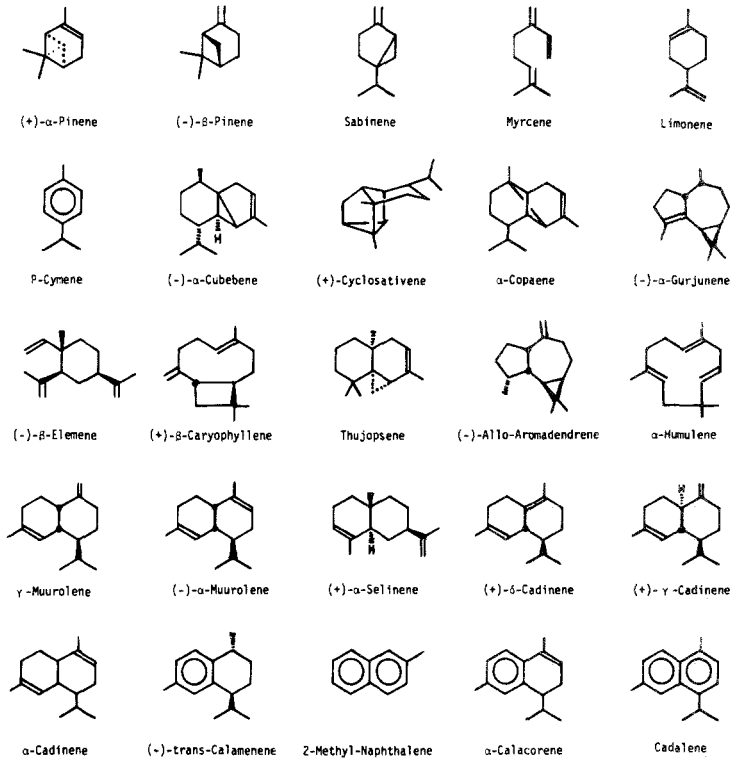

FIG. 2. Structures of terpenes and sesquiterpenes identified from elm volatiles.

found that flash chromatography of essential oil distillation fractions on silica gel–15% $AgNO_3$ that had been activated by heating to 80° under high vacuum for 12 hr was very effective, providing excellent separations of double-bond isomers on a several-gram scale on elution with stepwise gradients of hexane–ether. There was no detectable isomerization even of such acid-labile compounds as α-cubebene, as had been reported in the older literature.

The dehydration of elemol to β-elemene has been previously reported (Thomas, 1974). However, since the published method gives a 3:2 mixture of β- and δ-elemene, respectively, other dehydration methods were surveyed. It was found that refluxing crude elemol in acetic anhydride for 48 hr gave a 9:1 mixture of β-elemene and an isomer that could be cleanly separated by fractional distillation under reduced pressure.

α-Curcumene was synthesized in moderate yield. A major by-product was 4,4′-dimethylbiphenyl, the dimerization product from the Grignard reagent used. It is recommended that a published one-pot procedure giving a 90% yield be followed instead (Hall et al., 1975). α-Curcumene was then cyclized to calamenene by polyphosphoric acid treatment (Andersen et al., 1972).

Dehydrohalogenation of cadinene dihydrochloride gave a mixture of predominantly δ- and β-cadinene, with α-cadinene present as a minor component. The three isomers were separable in milligram amounts by careful preparative GC on a Carbowax 20 M column.

DISCUSSION

The literature has established that several types of elm materials are attractive to the two North American vectors of Dutch elm disease, *S. multistriatus* and *H. rufipes*. Before this study, the only host compound actually implicated as an attractant was the *S. multistriatus* pheromone synergist, α-cubebene (Pearce et al., 1975; Gore et al., 1977; Lanier et al., 1976). The results presented here strongly support the hypothesis that *H. rufipes* beetles locate moribund elms in which to breed principally by attraction to host volatiles. Several sesquiterpenes, including α-cubebene, appear to play a role in host-finding by *H. rufipes*.

Like Gardiner (1979) and Peacock (1979), we found no indication that *H. rufipes* beetles use a pheromone to aggregate on breeding material. Nevertheless, during the late summer and fall, when newly emerged adults were moving to healthy elms in which to overwinter, beetle-infested material seemed to be more attractive than logs alone. However, our data do not permit distinction among possibilities that this enhancement was produced by a pheromone, an increased release or different blend of host compounds, or a combination of these factors.

Moribund elms have a sweet and spicy apple-like odor that, to humans, is

quite distinct from the odor of healthy elms. Whether the difference is mainly qualitative or quantitative remains to be determined, but a previous investigation (Gore et al., 1977) found that the quantity of α-cubebene released by elm increases as the tissues degrade. The important point is that *H. rufipes* beetles seem to have little difficulty in finding moribund elms among a background of odors, and therefore no pheromone seems to be required for aggregation. Like dung flies and carrion beetles, *H. rufipes* can be quite assured of finding mates and breeding sites by following odor trails to decomposing elm trees. The fact that most studies of scolytid chemical ecology have focused on aggressive tree-killing species of bark beetles (see Borden, 1982) probably has created a biased impression of the importance of aggregation pheromones in the family as a whole. The majority of the scolytid species are scavengers that, like *H. rufipes*, may rely on host volatiles alone to locate breeding sites.

The most attractive sesquiterpene mixture caught only 40% of the number of *H. rufipes* (Table 4) as did the diseased elm controls. However, several important points must be made. First, since ratios varied from one extract to another, the sesquiterpene mixtures were arbitrarily made up with equal amounts of each sesquiterpene (1 mg/bait), so that the ratios in which the compounds occur naturally in elms were not duplicated. Thus, the attractiveness of baits might be increased by varying the ratios of the components in the most attractive bait. Secondly, there are probably other components, which, when added to the currently most attractive mixture, may increase the attractiveness of the baits either additively or synergistically. In addition, the compounds listed in Table 12 that were not identified in time for field trials in 1984 will be evaluated as attractants in the next series of bioassays.

It should also be borne in mind that there is a tremendous difference between using a whole diseased tree as a source of attractants and a point source of attractants that a septum bait represents. This may well account for much of the difference between trap catches of diseased trees versus sesquiterpene baits.

There is obviously a great deal more work to be done in refining bait mixtures. The data already in hand clearly demonstrate that *H. rufipes* beetles will respond to sesquiterpene baits and that it should be possible to use sesquiterpene-baited traps as a monitoring tool for *H. rufipes*, even with the nonoptimized baits used to date. In fact, sesquiterpenes could probably be added to multilure baits so that the same bait and trap could be used for the two North American vectors of Dutch elm disease.

H. rufipes and *S. multistriatus* beetles behaved quite differently toward sesquiterpenes in bioassays; *H. rufipes* responded poorly in the laboratory but well in the field, whereas *S. multistriatus* was attracted in the laboratory but not in the field. The problem with our laboratory bioassay of *H. rufipes* may be that this insect does not readily display the type of klinotaxis developed as a criterion of response in bioassays of *S. multistriatus* and *Ips* species. On the other hand, our field experiments on attraction of *S. multistriatus* always included the pher-

omone components 4-methyl-3-heptanol and α-multistriatin in some of the positions. The pheromone may have overwhelmed the beetles' propensity to respond to host compounds. One of our experiments (Table 6) strongly corroborates previous work (Pearce et al., 1975; Lanier et al., 1977) that showed α-cubebene to be a synergist of the pheromone components H and M, but other experiments provided only equivocal support for the host–synergist hypothesis. There are several factors (position effects, inappropriate release rates) that might be invoked to explain the apparent lack of enhancement by host volatiles on attraction of *S. multistriatus* to HM, but low beetle catches are not among them since over 250,000 *S. multistriatus* beetles were caught in these experiments. Our results (Table 5) do not corroborate the enhancement of HMC by host material as was reported by Peacock et al. (1984), but enhancement might have been confounded by the high concentration of pheromone released by the commercial baits used in our tests.

Blight et al. (1978) presented equivocal evidence that (−)-limonene synergized response of *S. scolytus* to 4-methyl-3-heptanol in Europe. Our data (Table 6) demonstrate that this is not the case with *S. multistriatus*, and we would not recommend substitution of (−)-limonene for α-cubebene in baits for *S. multistriatus*. Limonene and other monoterpenes were present in crude extracts from aerations, but the GC fraction containing them was only slightly attractive to *S. multistriatus* in laboratory bioassays.

Finally, specific rotations obtained for α- and β-pinene that were considerably less than those of pure enantiomers indicate that the trees produce enantiomeric mixtures. This also suggests that some of the other compounds isolated may not be enantiomerically pure, which could, of course, introduce a whole new dimension of complexity into the bioassay of artificially formulated baits. At present, there does not seem to be a simple solution to determining the chiral purity of the 20 or so hydrocarbon compounds obtained in small amounts.

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CROSS-ATTRACTION BETWEEN TWO SPECIES OF *Matsucoccus*[1] Extraction, Bioassay, and Isolation of the Sex Pheromone

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Abstract—In laboratory bioassays, males of *Matsucoccus resinosae* from *Pinus resinosa* in New York and *Matsucoccus* n. sp. from *Pinus thunbergiana* in Korea were strongly attracted to crude hexane extracts of *M. resinosae* females, and *M. resinosae* males responded strongly to extracts of *Matsucoccus* n. sp. females. Males of the two species responded similarly to gas chromatographic fractions and subfractions of a pentane extract of *M. resinosae* females. Sex pheromones of these two species appear to be the same or very similar.

Key Words—Sex pheromone, interspecific attraction, red pine scale, *Matsucoccus resinosae*, *Matsucoccus* n. sp., *Pinus resinosa*, *Pinus thunbergiana*, Homoptera, Coccoidea, Margarodidae.

INTRODUCTION

Scale insects of the margaroid genus *Matsucoccus* (about 30 known species) feed exclusively on pines (genus *Pinus*) (Ray, 1982). *M. matsumurae* (Kuwana), the type species for the genus, was first described from Japan. It was also reported from Korea (Kanda, 1941), and currently devastates pine forests of eastern mainland China (McClure et al., 1983). *M. resinosae* Bean and Godwin (red pine scale) attacks *P. resinosa* (red pine) in the northeastern United

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States, where it is presumed to have been imported (Bean and Godwin, 1955; Anderson et al., 1976; Grimble and Miller, 1976).

M. matsumurae was also known as *M. liaoningensis* Tang in China until Young (1979) proposed synonymy based on similarities in morphology and life histories of the two species. McClure (1983) suggested that *M. resinosae* was a junior synonym of *M. matsumurae*. This opinion was reiterated by Young et al. (1984), but synonymy has not been formally proposed.

M. resinosae was the first coccoid for which evidence of a female-released sex attractant pheromone was reported (Doane, 1966). Young and Qi (1983) used reduced cross-attractiveness and asynchrony of life cycles of *M. matsumurae* and *M. massonianae* Young and Hu to support the validity of these species. Conversely, strongly cross-attractive sex pheromones, along with similarity of morphology and life history, suggested synonymy of *M. resinosae* under *M. matsumurae* (Young et al., 1984).

An apparently undescribed *Matsucoccus* species has recently been damaging forests of *Pinus thunbergiana* (Japanese black pine) on the southeastern Korean peninsula, about 200 km west of the nearest known *M. matsumurae* infestation. This previously unknown entity is morphologically distinct, although it closely resembles *M. matsumurae* and *M. resinosae* (Miller, personal communication) and is univoltine rather than bivoltine as are *M. matsumurae* and *M. resinosae*.

We report experiments on the cross-attractiveness of sex pheromones of the unnamed species from Korea and *M. resinosae* from New York. We also report isolation of the principal active components of the *M. resinosae* sex pheromone.

METHODS AND MATERIALS

Insect Sources and Laboratories. M. resinosae used for pheromone preparations and bioassays were collected in Putnam County, New York. *M. resinosae* female pheromone extracts were fractionated and bioassayed with *M. resinosae* males at Syracuse, New York. Laboratory rearing and tests with males of the Korean *Matsucoccus* n. sp. were performed at the Forest Research Institute in Seoul, and field collections were made in Posung-gun of Chollanam Province.

Preparation of Crude Pheromone Extracts for Bioassay and Fractionation. M. resinosae were obtained by beating infested branches of sapling red pines between 7 AM and 1 PM on September 11 and 12, 1982, late August 1983, and on June 4, 1985. Adult females were collected with forceps from a white cotton drop cloth. A crude hexane extract was prepared from the 1982 collection by placing 100 female adults into a screw-cap glass vial (ca. 1 cm in diameter, 3.5 cm in height, fitted with a Teflon gasket) containing 300 μl of hexane at 9

AM on September 11, 1982. After three days at 25°C, the crude pheromone extract was serially diluted with hexane to 1%, 0.1%, 0.01%, and 0.001% of the original concentration. The crude pheromone extract dilutions (CPEDs) were stored in glass vials with screw caps lined with Teflon gaskets. All CPEDs were stored at -8 ± 3°C. Crude extract of *Matsucoccus* n. sp. was made from females collected from Korea in April 1984. Extract of 400 females in 1 ml of hexane was diluted 100 times for bioassay of attractiveness to *M. resinosae* males.

In 1982, an extract was prepared for GC fractionation from 3500 female *M. resinosae* placed in five vials; four contained 200 μl pentane and one contained 200 μl hexane. After three cycles of freeze-sonication, each with replacement of fresh pentane, the combined extracts were dried over Na_2SO_4 and concentrated to 0.4 ml through a 20-cm packed (glass beads) column. The concentrated extract was fractionated on a 1.5 m × 7 mm ID glass column, 10% Carbowax 20 M on Chromosorb W, 50–80, N_2 80 cm^3/min, 150° isothermal. Three fractions were collected: F1, 0–42 min, F2, 42–60 min; and F3, 60–132 min. Each fraction was diluted to 0.1 female equivalent/μl hexane for bioassays. F3 (the most active fraction) was further fractionated on a 3-m × 4-mm-ID glass column, 3% OV-101 on Chromosorb G, 60–100, N_2 60 cm^3/min, 150° isothermal: F3-1, 0–6 min; F3-2, 6–18 min; F3-3, 18–24 min; F3-4, 24–42 min; and F3-5, 42–90 min. Each fraction was diluted to 0.1 female equivalent/μl hexane. F3-5 (the active fraction) was further fractionated on the same OV-101 column under the same conditions into four fractions: F3-5-1, 0–42 min; F3-5-2, 42–54 min; F3-5-3, 54–62 min; and F3-5-4, 62–94 min.

Similar procedures were used to isolate the principal compound in F3-5-4 from 17,000 *M. resinosae* females collected during August 1983 in Putnam County, New York.

Preparation of Males for Testing. In the United States branches of red pine containing cocoons of *M. resinosae* were collected in the field and put into an emergence cage at 23 ± 2°C under natural photophase. The emergence cage consisted of a cardboard box with a glass jar (ca. 300 ml) attached at one side. Because the males are positively phototactic, they moved into the glass jar, from which they were easily removed.

In Korea, saplings (5–8 years) of Japanese black pine infested by *Matsucoccus* n. sp. were transplanted into vinyl pots and were reared in the laboratory at 20 ± 3°C under natural photophase. Newly spun cocoons were removed from the saplings with forceps. They were stored in Petri dishes, and the males emerging therein were used for bioassay.

General Bioassay Background and Procedures. Doane (1966) stated that the males of *M. resinosae* emerge during the night and fly most actively in the early morning hours. He also said that by 10 AM many males were able only to walk about or make uncoordinated attempts to launch themselves in flight, but that they were able to mate with the adjacent females and fertilize them.

We found that, after the flight period, the males of both species of *Matsucoccus* began to walk about continuously, moving at random in the absence of the pheromone. A few hours thereafter, they became sluggish and weak-legged and walked erratically with a limping motion. Males that walked continuously were more consistently attracted by pheromone than those that showed an erratic limping motion or those that made intermittent unsuccessful attempts at flight in tests we conducted by puffing very dilute pheromone vapors at them as they walked on a sheet of white paper. Males that stopped moving or paused to groom their antennae did not respond well to pheromones, so the bioassay was delayed until walking resumed.

Bioassays were done under fluorescent light (700 lux) at 25 ± 2°C and 60 ± 10% relative humidity between 9 AM and 1 PM. Bioassays of the CPEDs and fractions with *M. resinosae* males were run in September 1982, on the 20th for the CPEDs and from the 22nd through the 26th for the fractions. For the males of *Matsucoccus* n. sp., tests of the CPEDs were run on February 1, 1984, and tests of the fractions were run on February 7 and 8. Bioassays of *M. resinosae* male response to CPED of *Matsucoccus* n. sp. and purified pheromone of *M. resinosae* were conducted from June 6 to 16, 1985. Each male was kept under a numbered, inverted, transparent plastic cup (ca. 20 ml) on a piece of white paper until he was tested. After each test, the male was covered with the same cup and kept for the next test.

Attraction of males was tested by puffing pheromone vapors from a medicine dropper with an inside diameter of ca. 1.7 mm at the tip. Micropipets were used to place 1 μl of CPED or fraction inside a medicine dropper, about 1 cm from the tip. To expel the evaporating hexane, the rubber bulb of the medicine dropper was pressed 10 times in 10 sec. Then the tip of the dropper was positioned 8 ± 2 mm from the male and pointed at his antennae. Puffs of air were then forced from the medicine dropper by gently pressing the bulb at 1.5-sec intervals; about 1 ml of air was expelled at each puff.

If a male turned toward the dropper tip and began to move toward it, the tip was moved so that its distance from the advancing male would remain roughly constant. If a male followed the retreating medicine dropper tip, the latter was moved in a straight line for 4 cm, and then the direction of its course was altered by 120° for a second 4-cm distance. If the male followed the dropper tip for another full 4 cm, the direction was again altered by 120° so that it was moving toward the starting point. Thus, the path of the dropper tip and the following male would have described a 4-cm-sided equilateral triangle. Altering the path of the dropper tip in this manner precluded its haphazard coincidence with the path of the male. For each test, males completing one or more sides of the test triangle were given scores from 1 to 3, equal to the number of triangle sides completed; males responding to the pheromone but following the dropper tip for less than 4 cm were given a score of 0.5, and males that failed to respond to the point of following were given a score of 0.

Bioassays of Crude Pheromone Extracts. The order for testing CPEDs was: control, 0.001%, 0.01%, 0.1% and 1%. Ten males were used in successive tests in a standard order (i.e., 1–10) so that each male would rest the maximum interval between tests.

Bioassays of Pheromone Extract Fractions. The males of both *Matsucoccus* species have a very short life-span (less than 24 hr), and they move towards the pheromone source for only a few hours. Therefore, in order to give males the maximum resting time between each test, and to minimize the effect of senescence of the males, the sequence in which the males were tested was set as a Latin square matrix. Because the fractions inside the medicine dropper continuously evaporate during the experiment, each fraction was newly applied to the medicine dropper every 5 min. A new medicine dropper was used at each additional application to avoid accumulation. In each experiment, hexane was used as control, and the 1% CPED was used as the standard unfractionated pheromone extract.

RESULTS AND DISCUSSION

Males of both *M. resinosae* and *Matsucoccus* n. sp. were attracted by the CPED of *M. resinosae* at dilutions of 0.1% and 1% (Table 1). Every male tested was attracted by the 0.1% CPED, but attraction was strongest to the 1% CPED. Males of *M. resinosae* were attracted to the CPED of *Matsucoccus* n. sp., although significantly less than to purified pheromone from *M. resinosae* females (Table 2).

Males of both *Matsucoccus* species responded to the third of three fractions (F3) of the *M. resinosae* extract (Table 3). Bioassays of five subfractions of F3 showed clearly that all biological activity resided in F3-5. This fraction, further divided into four parts, produced two equally active fractions, F3-5-3 and F3-

TABLE 1. MEAN RESPONSES OF *Matsucoccus resinosae* AND *Matsucoccus* N. SP. MALES TO CRUDE EXTRACT OF *M. resinosae* FEMALES[a]

		Dilution (%)[b]				
	Number	0	0.001	0.01	0.1	1.0
M. resinosae	10	0	0.05	0.45	2.30	2.80
Matsucoccus n. sp	10	0.05	0.05	0.55	2.35	2.90

[a] Response scores: 0 = failed to respond to point of following; 0.5 = followed dropper tip, but for less than one full test-triangle side; scores 1, 2, and 3 equal the numbers of complete test triangle sides males followed dropper tip. Means with the same underline are not significantly different ($P < 0.05$, Student's t test).

[b] Relative dilution when 100 females per 300 μl of hexane was 100%.

TABLE 2. MEAN RESPONSES OF *Matsucoccus resinosae* MALES TO *M. resinosae* PURIFIED PHEROMONE AND CRUDE EXTRACT OF *Matsucoccus* N. SP. FEMALES[a]

	Number	Mean response[b]
Matsucoccus resinosae pheromone[c]	54	2.98
Matsucoccus n. sp. crude extract of females[d]	54	2.76
Hexane control	54	0.07

[a]Response scores: see footnote in Table 1.
[b]All means are significantly different ($P < 0.01$, Student's t test).
[c]Equivalent to 3-5-4 in Table 3.
[d]1% concentration of 400 females in 1 ml hexane; approximately equivalent to 1.0% dilution of *M. resinosae*, Tables 1 and 3.

TABLE 3. MEAN RESPONSES OF *Matsucoccus resinosae* AND *Matsucoccus* N. SP. MALES TO FRACTIONS OF EXTRACT OF *M. resinosae* FEMALES[a]

	Number Tested	Hexane	Fractions of Extract[b] 1	2	3	Crude extract[c]
M. resinosae	5	0	0	0.10	2.60	2.60
Matsucoccus n. sp.	5	0.10	0.10	0.20	2.60	3.00

		Second fractions of F3[d]					
		Hexane	3-1	3-2	3-3	3-4	3-5	Crude extract[c]
M. resinosae	7	0.07	0.14	0	0.07	0	2.86	3.00
Matsucoccus n. sp.	7	0	0.14	0.07	0.14	0	2.71	2.71

		Third fractions of F3-5[e]				
		Hexane	3-5-1	3-5-2	3-5-3	3-5-4	Crude extract[c]
M. resinosae	6	0	0.08	0.08	3.00	2.83	2.83
Matsucoccus n. sp.	6	0.17	0	0.08	2.67	3.00	3.00

[a]Response scores: see footnote in Table 1. Means with the same underline are not significantly different ($P < 0.05$, Student's t test).
[b]Concentration of fractions from the extract = 175 females/1500 μl hexane.
[c]1% Concentration of 100 females in 30 μl hexane.
[d]Concentration of fractions of F3 = 875 females in 150 μl hexane.
[e]Concentration of fractions of F3-5 = 300 females in 15 μl hexane.

5-4. Another test with *M. resinosae* using the original three fractions at tenfold lower dilutions (i.e., more concentrated solution) showed that F2 also had biological activity. Bioassays of subfractions of F2 showed that F2-4 was active. Comparisons by GC showed that the activity in F2-4 probably corresponded to that in F3-5.

Since patterns of response for *Matsucoccus* n. sp. and *M. resinosae* males are identical, we believe that the pheromones are also largely or entirely identical. The slight, but significant, difference in response of *M. resinosae* to materials from conspecific and *Matsucoccus* n. sp. was probably a result of the difference in the nature of the stimuli (purified pheromone of *M. resinosae* vs. CPED of *Matsucoccus* n. sp.).

Pheromones of coccoids studied prior to *Matsucoccus* had proven to be species specific, as with pairs of closely related citrus scales within the genera *Aonidiella* (Moreno et al., 1972) and *Planococcus* (Rotundo and Tremblay, 1975). Even reciprocal hybrid *Planococcus citri* (Risso) × *ficus* (Singoret) males reacted positively only to the pheromone of the maternal species (Rotundo and Tremblay, 1982); this is in contrast to bark beetles in the genus *Ips* for which Lanier (1970) reported abolition of pheromone specificity in species hybrids. Tremblay and Rotundo (1976) speculated that pheromonal specificity might be a mechanism for sympatric speciation in scale insects.

Young et al. (1984) suggested that strong cross-attractiveness between *M. matsumurae* and *M. resinosae* supports their conspecificity, but they are studying other characteristics before formally proposing synonymy. While lack of pheromone specificity is clearly a valid criterion for taxonomic decisions, our tests, together with moderate cross-attractiveness between *M. matsumurae* and *M. massonianae* reported by Young and Qi (1983), indicate that cross-attraction does not necessarily demonstrate conspecificity in the genus *Matsucoccus*.

There is evidence that the pheromone of *M. resinosae* females is attractive to flying males. Males were caught in the field by sticky paper flight-traps to which crushed *M. resinosae* females had been attached (Park, unpublished data), and, in flight chamber tests, Doane (1966) observed flying *M. resinosae* males being attracted to females. Although testing the walking males with a medicine dropper was an ideal way of bioassaying fractions available only in extremely small amounts, further studies with crude pheromones and larger amounts of pheromone fractions would be needed for determining what components of the pheromonal system effect male flight behavior or if other components are involved besides those that affect walking behavior.

In a personal communication cited by Doane (1966), Kring stated that some aphids must go through a preliminary flight period before they become attracted to colored panels; it is conceivable that an analogous behavior will be found in newly emerged *Matsucoccus* males. Inbreeding would be discouraged if *Matsucoccus* males take flight and become attracted by the pheromone only after a preliminary period of sustained flight.

The host range of most *Matsucoccus* species is rather narrow. All *Matsucoccus* species feed only on pines; of the 31 named species of *Matsucoccus*, none is known to feed on more than one subgenus of *Pinus*. Rieux (1975) stated that there is a close relationship between certain groups of *Pinus* and those of *Matsucoccus*, which suggests concomitant speciation in *Matsucoccus* and hosts. Rieux also devised a classification of *Matsucoccus* based on host preference. Because both *Pinus resinosa* and *P. thunbergiana* belong to the subsection *Sylvestres* (Critchfield and Little, 1966) and they appear very similar, the relationship between *M. resinosae* and the Korean *Matsucoccus* n. sp. may be equally close. It might be expected that cross-attraction would not be shown by species of *Matsucoccus* with hosts in more distantly related groups of pines. If so, cross-attraction experiments with *Matsucoccus* species could prove to be an effective way of studying the phylogeny of the genus.

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STEREOSELECTIVE SYNTHESIS OF (*Z*,*E*)-3,5-TETRADECADIENYL ACETATE: Sex Attractant for Carpenterworm Moth, *Prionoxystus robiniae* (Peck) (Lepidoptera: Cossidae) and Effect of Isomers and Monounsaturated Acetates on Its Attractiveness[1]

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Abstract—A new efficient stereoselective synthesis for (*Z*,*E*)-3,5-tetradecadienyl acetate (*Z*3,*E*5–14 : Ac) a potent male sex attractant for the carpenterworm, *Prionoxystus robiniae* (Peck), was developed. The effects of the other three isomers (*Z*,*Z*; *E*,*Z*; *E*,*E*) on the field attractiveness of the *Z*,*E* isomer toward male *P. robiniae* were determined. The *Z*,*Z* isomer inhibited attraction, the *E*,*E* isomer synergized attraction, and the *E*,*Z* isomer had no effect on attraction. Seven monounsaturated 14-carbon acetates were evaluated for their effect on the attractiveness of *Z*3,*E*5–14 : Ac. (*E*)-3-Tetradecenyl acetate enhanced attraction and (*Z*)-9- and (*E*)-5-tetradecenyl acetate reduced trap captures. (*Z*,*E*)-3,5-Tetradecadien-1-ol also reduced the attractiveness of *Z*3,*E*5–14 : Ac.

Key Words—*Prionoxystus robiniae*, Lepidoptera, Cossidae, Carpenterworm, sex attractant, (*Z*,*E*)-3,5-tetradecadienyl acetate, inhibition, synergism.

INTRODUCTION

The sex pheromone of the adult female carpenterworm, *Prionoxystus robiniae* (Peck), a destructive borer in the trunks of southern hardwood trees, was first

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reported by Solomon and Morris (1966) and partially characterized by Solomon et al. (1972). (*Z,E*)-3,5-Tetradecadienyl acetate (*Z*3,*E*5–14 : Ac) was found to be a powerful attractant for male carpenterworms (Doolittle et al., 1976a) through a combination of electroantennogram (EAG) investigations of standard chemicals and synthesis of candidate chemicals possessing the biologically active functionalities. Although this chemical is a strong field attractant, its presence in the insect has never been established.

The original synthesis (Doolittle et al., 1976b) involved a Wittig condensation between (*E*)-2-undecenal and the phosphorane prepared from 3-hydroxypropyltriphenylphosphonium iodide and two equivalents of strong base. Unfortunately this reaction produces a mixture of the *E,E* (*E*3,*E*5–14 : Ac) and *Z,E* (*Z*3,*E*5–14 : Ac) isomers in a ratio of about 3 : 1 *E,E/Z,E* along with a small (<2%) amount of *Z,Z* isomer. Under carefully controlled conditions, it is possible to increase this ratio to about 1 : 1. Commercial preparations are commonly the 3 : 1 ratio (Doolittle).[3] The *Z,E* isomer content can be increased to about 90% by spinning band distillation, but this is a tedious process and a large amount of the *Z,E* isomer is lost. Initially formation of the *E,E* isomer was advantageous because this isomer is a powerful attractant for another destructive cossid, the aspen carpenterworm, *Acossus centerensis* (Litner) (Doolittle et al., 1976b). Interestingly, neither isomer inhibited the attraction of either species to the other isomer and, indeed, there seemed to be some indication that the attraction of *P. robiniae* to the *Z,E* isomer was enhanced by the *E,E* isomer (Solomon et al., 1978). This did not overcome the inherent inefficiency of the original synthetic procedure when applied to the commercial production of this attractant.

The initial EAG experiments (Doolittle et al., 1976a) indicated the presence of a second active component in female extracts. Based on its gas chromatographic retention time, this component was most likely a monounsaturated 14-carbon acetate. With this in mind and in consideration of the necessity of developing a new, more efficient synthesis for the *Z,E* isomer, we investigated the stereoselective synthesis of all four isomers of 3,5-tetradecadienyl acetates and the effect of the *E,Z*; *Z,Z*; and *E,E* isomers and the *Z*- and *E*-3, *Z*- and *E*-4, *Z*- and *E*-5, and *Z*-9 tetradecenyl acetates (*Z*3–14 : Ac, *E*3–14 : Ac, *Z*4–14 : Ac, *E*4–14 : Ac, *Z*5–14 : Ac, *E*5–14 : Ac, and *Z*9–14 : Ac) on the attractiveness of *Z*3,*E*5–14 : Ac. These monoenes had given the strongest responses in the initial EAG investigations. In light of the reported (Tumlinson et al., 1972) inhibitory effects of alcohols, the diene alcohol *Z,E*-3,5-tetradecadien-1-ol (*Z*3,*E*5–14 : OH) was also tested for its effect on the attractiveness of *Z*3,*E*5–14 : Ac toward male *P. robiniae*. The results of this investigation are the subject of this report.

[3] Farchan Laboratories, Gainesville, Florida has attempted commercial preparation of this compound and has produced ratios of from 4 : 1 to 2 : 1 *E,E/Z,E*.

METHODS AND MATERIALS

Instrumentation. Instrumentation used in the chemical synthesis included a Perkin Elmer model 1420 infrared spectrophotometer (infrared spectra were recorded as 2% w/v solutions in CCL_4), Varian Aerograph models 1400 and 2100 gas chromatographs equipped with user-designed all-glass capillary split-inlet systems with carrier gas (He) linear flow velocities of 18 cm/sec and a split ratio of 100:1. Four columns were used: (A) 50 m × 0.20 mm ID coated with SP-1000 (175°C); (B) 15.2 m × 0.20 mm ID coated with 0.1% cholesteryl-*p*-chlorocinnamate (Heath and Doolittle, 1983) (when this column was operated below 150°C, it was in a supercooled state); (C) 14 m × 0.20 mm ID coated with 0.15% cholesteryl-*p*-nitrocinnamate (170°C) (Heath and Doolittle, 1983); and (D) 14 m × 0.25 mm ID fused silica (Quadrex Corp. "007" series) coated with 0.25 μm of SP2340 (140°C).

High-performance liquid chromatography was done at a flow rate of 9.9 ml/min using a Waters Associates M6000 solvent delivery system, a Rheodyne 905-19 injector for sample introduction, and a Waters Associates R401 differential refractometer detector. Mass spectra were recorded on a Finnigan 1015S chemical ionization mass spectrometer using isobutane as reagent gas. [^{1}H]Magnetic resonance spectra were recorded in $CDCl_3$ on a Nicolet 300-MHz spectrometer. Chemical shifts are in parts per million from tetramethylsilane and the coupling constants are in Hertz.

Chemical Synthesis of Z*3,*E*5-14:Ac and* E*3,Z5-14:Ac.* The synthetic routes used are outlined in Figure 1, scheme A. This method of synthesis is a modification of that described by Zweifel and Backlund (1978) with details as follows for the *Z,E* isomer: a 1 M solution of diborane in THF (0.1 mol, 100 ml) was added dropwise to 80 ml of a 2.5 M solution of cyclohexene in THF (0.200 mol) held at −15°C. Tetrahydrofuran (THF) was dried by distillation from lithium tetrahydroaluminate. Alternatively, borane methyl sulfide (10 ml, 0.10 mol) was diluted to 100 ml with THF to generate the borane solution, after which the solution was stirred at 0–5°C for 1 hr. Then a solution of 1-decyne (14 g, 0.10 mol) in 25 ml of THF was added dropwise at 0–5°C, and the mixture stirred at 0–5°C for 2 hr during which time the white slurry dissolved. The tetrahydropyranyl ether of 3-butyn-1-ol, prepared from 3-butyn-1-ol and dihydropyran by the method of Miyashita et al. (1977), bp 96°C/23 mm, had spectral characteristics (IR, NMR, MS) consistent with its chemical structure.

This ether (15.4 g, 0.10 mol) was placed in a flask with 80 ml of THF and treated dropwise at −60°C with 0.10 mol (77 ml of a 1.3 M soln) of ethereal methyl lithium. After addition of the methyllithium, the solution was brought to −15°C for 0.5 hr and then was transferred via cannula to the borane reagent which was held at −25°C. The solution was allowed to warm to room temperature, held there for 1 hr, and chilled again to −15°C at which time 30 ml (0.11 mol) of distilled tri-*n*-butyltin chloride was added. The reaction mixture

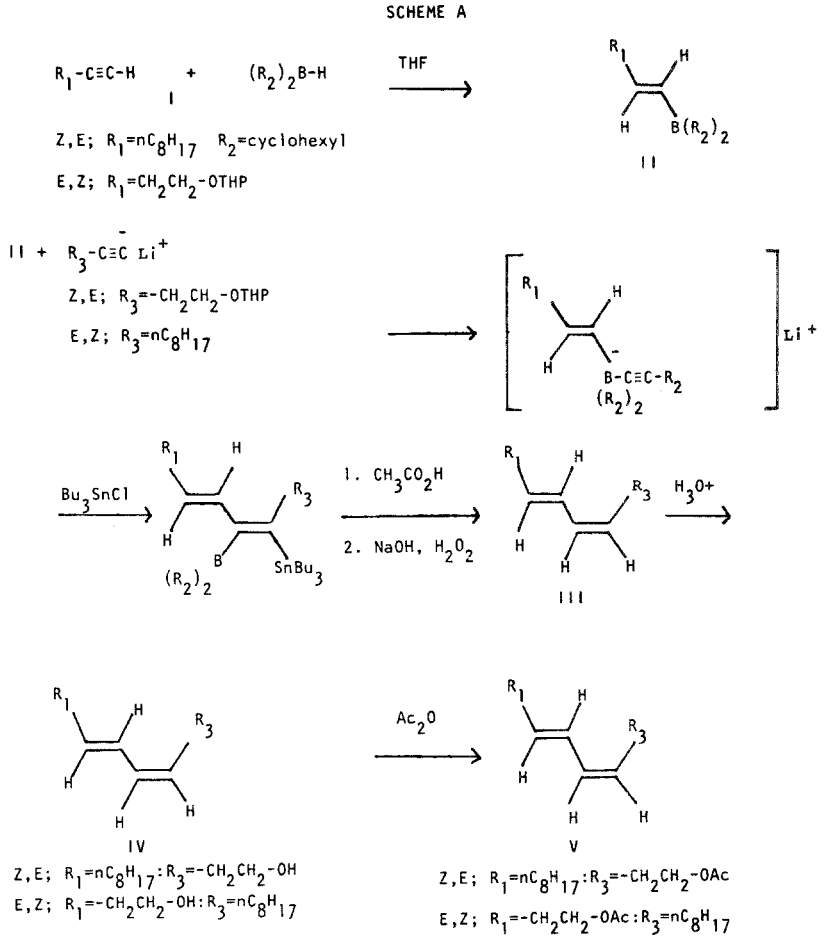


FIG. 1. Synthesis of three of the four isomers of 3,5-tetradecadienyl acetate.

was brought to room temperature over 15 min and stirred for 1 additional hr, then 23 ml of glacial acetic acid was added and the mixture was heated at 50–60°C for 1 hr. The reaction was cooled to about 30°C, 100 ml of methanol and 80 ml of 6 N sodium hydroxide were added, and the mixture was oxidized with 24 ml of 30% H_2O_2 at 35–40°C. After stirring for 1 hr at room temperature, the mixture was diluted with water and extracted several times with ether and hexane.

The combined organic extracts were washed several times with water, saturated brine, and dried over Na_2SO_4. After removal of the drying agent and evaporation of the solvent, the residual oil was chromatographed on a column of Florisil to remove the $Bu_3SnOSnBu_3$. For this scale reaction, a minimum of

SCHEME B

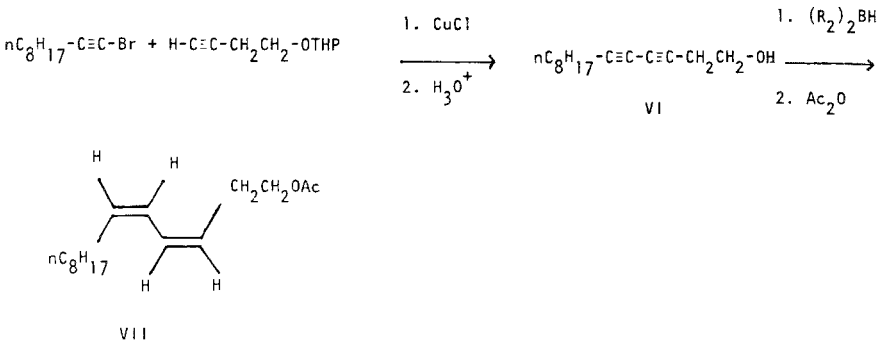


FIG. 1. Continued.

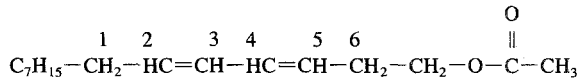
400 g of Florisil was used and the product eluted with 3% ethyl acetate in hexane. Hydrolysis by stirring overnight in 100 ml of methanol containing 1 ml conc. HCl; dilution with water; extraction several times with ether; washing the combined ether extracts with water, saturated $NaHCO_3$, and saturated brine; drying over Na_2SO_4; and removal of drying agent and solvents produced crude alcohol IV, $R_1 = nC_8H_{17}$; $R_3 = CH_2{-}CH_2OH$, containing approximately 1% *E,Z* isomer and 1.5% *E,E* isomer (col B). This alcohol was recrystallized from pentane at Dry-Ice temperatures to yield 16.84 g (76% yield) of 98% pure alcohol which was acetylated with acetic anhydride and triethylamine in either in the usual fashion and vacuum distilled (bp 107°C/0.03 mm) to give V, $R_1 = nC_8H_{17}$, $R_3 = -CH_2CH_2OAc$ containing <2% *E,E* and <1/2% of the other two isomers (cols B, C, and D).

The spectral data are as follows: IR cm^{-1} 3020 (olefinic C-H), 1745, 1235, 1035 (acetate), 980, and 947 (*Z,E* conjugated diene); [^{1}H]NMR 0.88 (3H, t), 1.27 (12H, m), 2.04 (3H, s), 4.09 (2H, t), remaining proton assignments in Table 1. MS *m/z* (rel. abundance) 193 (100) (M+1−60), 252 (11) (M), 253 (45) (M+1).

E,Z Isomer. The *E,Z* isomer V, $R_1 = CH_2CH_2OAc$; $R_3 = nC_8H_{17}$ was synthesized in an analogous fashion by reversing the order in which the acetylenic starting materials were used. It was prepared in 75% yield and from 97 to 99% isomeric purity after recrystallization and acetylation. The spectral data are as follows: IR cm^{-1} 3020 (olefinic C-H), 1745, 1235, 1032 (acetate) 980, and 950 (*E,Z* conjugated diene); [^{1}H]NMR 0.88 (3H, t), 1.27 (12H, m), 2.05 (3H, s), 4.11 (2H, t), remaining proton assignments in Table 1. MS *m/z* (rel. abundance) 193 (100) (M+1−60), 252 (2) (M), 253 (47) (M+1).

Synthesis of Z3,Z5-14:Ac, VII. This synthesis is outlined in Figure 1, scheme B, and consists of a (Chodkiewicz and Cadiot, 1955) type of reaction followed by reduction with dicyclohexylborane (Zweifel and Polston, 1970) as

TABLE 1. DIAGNOSTIC [^{1}H]NMR CHEMICAL SHIFTS FOR ISOMERIC 3,5-TETRADECADIENYL ACETATES[a]



Proton	Chemical shifts[b] (ppm)			
	Z,E	*E,Z*	*E,E*	*Z,Z*
1	2.11	2.15	2.04	2.17
2	5.72	5.37	5.62	5.52
3	6.27	5.95	6.00	6.22
4	6.06	6.39	6.09	6.38
5	5.26	5.60	5.51	5.41
6	2.49	2.43	2.38	2.52

[a]Other chemical shifts are listed in materials and methods. The chemical shift values in the table were assigned from spin-spin decoupling experiments.
[b]The signals of all protons gave correct integration values. Signals were all AA'B_2XY patterns.

follows: 3-butyn-1-ol (0.1 mol, 7.0 g), *n*-propylamine (0.17 mol, 14 ml), CuCl (1 mmol, 0.1 g), hydroxylamine hydrochloride (7 mmol, 0.5 g), and 125 ml of methanol were placed in a flask under nitrogen and chilled to 15°C. 1-Bromodecyne (0.1 mol, 21.72 g), prepared by the method of Brandsma (1971) dissolved in 25 ml of methanol was added dropwise, maintaining the temperature between 15 and 22°C. After addition, the reaction was stirred at this temperature range for several hours, adding small amounts of hydroxylamine hydrochloride whenever the mixture turned blue or blue-green. When the reaction mixture remained yellow and any tendency for the temperature to rise ceased, it was poured into water and extracted several times with ether.

The combined ether extracts were washed with water, saturated brine, and dried over Na_2SO_4. After removal of the drying agent and solvent, the residual oil was vacuum distilled (bp 132–136°C/0.001 mm). The distilled product was chromatographed on a column of 150 g of silica gel to remove the C_{20} diyne hydrocarbon that gas chromatography–mass spectrometry indicated was present. This is a common impurity in Cadiot coupling reactions, arising from self-coupling of the bromoalkyne. The diyne alcohol eluted with 10% ethyl acetate–hexane to give 10.9 g (53%) of pure product VI. The diyne alcohol was converted to its tetrahydropyranyl (THP) ether by the aforementioned procedure used for 3-butyn-1-ol, and the crude ether was reduced to the diene as follows: a 0.55 molar solution of dicyclohexylborane (0.04 mol) in THF was prepared as described (Zweifel and Polston, 1970), 4.36 g (0.015 mol) of the THP ether

in 20 ml of THF was added at 0°C, and the mixture was stirred at room temperature overnight.

Acetic acid (9 ml) was added, and the mixture was heated at 65°C for 5 hr, cooled to room temperature and 31 ml of 6 N NaOH was added. It was then oxidized at 35–40°C with 9.5 ml of 30% H_2O_2, stirred at room temperature, diluted with water, extracted and dried in the usual fashion, and the residual oil remaining after removal of the solvent was hydrolyzed with 50 μl of conc. HCl in 100 ml of methanol for 2 hr. The diene alcohol isolated from the hydrolysis by the usual extraction procedure was treated with acetyl chloride in ether with triethylamine as acid scavenger, worked up in the usual fashion, and vacuum distilled (bp 108–109°C/0.01 mm) to yield 2.11 g (55.4%) of *Z,Z* acetate VII which contained <1% each of the *Z,E* and *E,Z* isomers and no detectable amount of *E,E* isomers by GC analysis on column C.

The spectral data are as follows: IR cm^{-1} 3020 and 3005 (olefinic C-H), 1745, 1235, 1035, (acetate); [^{1}H]NMR 0.88 (3H, t), 1.27 (12H, m), 2.04 (3H, s), 4.09 (2H, t), remaining proton assignments in Table 1. MS *m/z* (rel. abundance), 193 (100) (M+1−160), 252 (14) (M), 253 (52) (M+1).

E*3*, E*5–14:Ac. Pure E,E* isomer was obtained by saponification of material that had been synthesized by the original route (Doolittle et al., 1976a) and purified via spinning band distillation to 95% *E,E* content, followed by low-temperature crystallization of the alcohol from pentane to >99% purity (determined on columns A and B) and reacetylation. This gave the *E,E* isomer in >99% isomeric purity. Spectral data were as follows: IR cm^{-1} 3010 (olefinic C-H), 1745, 1235, 1020 (acetate), 985 (*E,E* conjugated diene); [^{1}H]NMR 0.88 (3H, t), 1.26 (12H, m), 2.04 (3H, s), 4.09 (2H, t), remaining proton assignments in Table 1. MS *m/z* (rel. abundance) 193 (100) (M+1−60), 252 (17) (M), 253 (43) (M+1).

Z*3–14:Ac,* E*3–14:Ac,* Z*4–14:Ac,* E*4–14:Ac,* Z*5–14:Ac,* E*5–14:Ac, and* Z*9–14:Ac.* These monoene acetates were either purchased from commercial sources or synthesized via established well-documented routes (Sonnet, 1984; Mori, 1979, 1981; Henrick, 1978; Rossi, 1977) and were purified by high-performance liquid chromatography (HPLC) on silver-nitrate-impregnated silica gel as described by Heath et al. (1977). Gas chromatographic analysis on columns A, B, and C gave isomeric purities >99%.

Field Tests. Field studies were conducted during the carpenterworm flight season from mid-May to mid-July of 1982 and 1983 in the Delta Experimental Forest, Stoneville, Mississippi (a mixed hardwood forest). In 1982, the diene acetates *E*3,*Z*5–14 : Ac, *Z*3,*Z*5–14 : Ac; and *E*3,*E*5–14 : Ac; the monoene acetates *Z*3–14 : Ac, *E*3–14 : Ac, *Z*4–14 : Ac, *E*4–14 : Ac, *Z*5–14 : Ac, *E*5–14 : Ac, and *Z*9–14 : Ac; and the diene alcohol *Z*3,*E*5–14 : OH were tested alone and in blend with the active diene acetate *Z*3,*E*5–14 : Ac to determine their effects upon attractiveness to male *P. robiniae*. The *Z,E* isomer was prepared by dis-

solving 350 μg $Z3,E5$–14 : Ac (the active isomer) in 200 μl nanograde hexane. A 10 : 1 ratio of Sustane 6 was added as an antioxidant/preservative. Each of the three diene acetates, seven monoene acetates, and diene alcohol was blended with the Z,E isomer ratios of 0, 1, 3, 10, and 25%.

In 1983, additional field tests were conducted to further define the effects of the diene acetate, $E3,E5$–14 : Ac, and the monoene acetate, $E3$–14 : Ac, upon the active isomer, $Z3,E5$–14 : Ac. Test samples were prepared in the same manner as in 1982, except that ratios of 0, 0.5, 2, 5, 12, and 15% of E,E isomer and ratios of 0, 0.5, 1, 2, 3, and 5% of $E3$ isomer to Z,E isomer were tested.

Each test sample was micropipetted onto a cotton dental roll (2 × 1 cm) dispenser. After the solvent evaporated, charged dispensers were placed in platform sticky traps (Solomon et al., 1978). Traps were suspended from tree branches 1.5 m above the ground and randomly assigned to sites 110–160 m apart in the forest. Traps were rerandomized weekly to new sites, and male catches were recorded daily except weekends, for as long as the baits remained attractive. The total catch for each trap over the entire test period was recorded. There were four replications (baited traps) per treatment in 1982 and eight replications per treatment in 1983. The data from the acetate mixtures (Figures 2–6) were subjected to regression analysis (Steel and Torrie, 1960), and the data from the mixtures of $Z3,E5$–14 : Ac and $Z3,E5$–14 : OH were subjected to analysis of variance and Duncan's new multiple-range test at the 0.05 level.

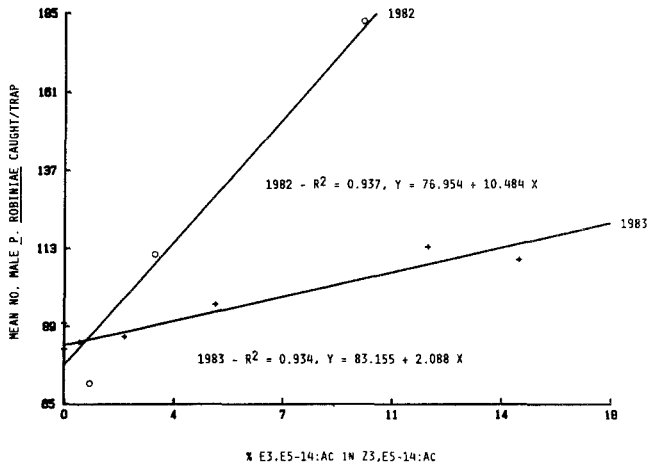


FIG. 2. Attraction of mixtures of (E,E)- and (Z,E)-3,5-tetradecadienyl acetates ($Z3,E5$- and $E5,E5$–14 : Ac) toward male carpenterworms (*P. robiniae*) in 1982 and 1983, Stoneville, Mississippi.

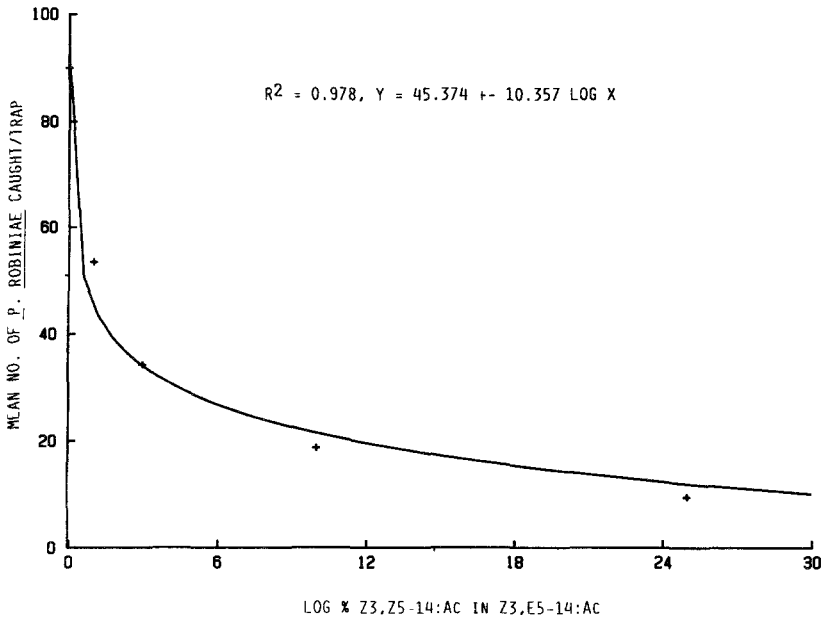


FIG. 3. Effect of (*Z*,*Z*)-3,5-tetradecadienyl acetate (*Z*3,*Z*5–14 : Ac) on the attractivenss of (*Z*,*E*)-3,5-tetradecadienyl acetate (*Z*3,*E*5–14 : Ac) toward male *P. robiniae* in 1982, Stoneville, Mississippi.

RESULTS AND DISCUSSION

Chemical Synthesis. The *Z*,*E* and *E*,*Z* isomers of 3,5-tetradecadienyl acetate were synthesized stereoselectively via scheme A of Figure 1. This procedure produced these isomers in high stereopurity and good yield. Traces of isomeric impurities could be conveniently removed by low-temperature crystallization of the alcohols from hydrocarbon solvents. For example, this technique is especially efficient at removing the *E*,*Z* and *Z*,*Z* isomers from a sample of *Z*,*E* since the *E*,*Z* and *Z*,*Z* isomers are the more soluble ones. Recrystallization was very efficient for the removal of isomers from a sample of *E*,*E* alcohol, since this isomer is the least soluble of the four, but would not be as effective for the removal of the *E*,*E* isomer from one of the other three since it would have a strong tendency to cocrystallize.

Allowing the reaction mixture to remain at room temperature overnight after the addition of the *n*-Bu_3SnCl but before addition of the acetic acid (see Methods and Materials) produced some startling results. In the case of the *E*,*Z* isomer, varying conditions in this fashion in three preparations produced from

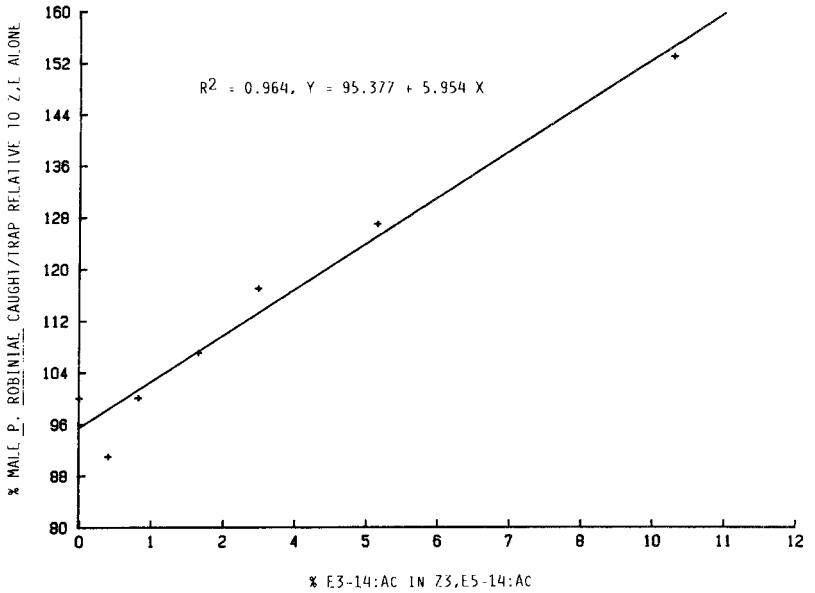


FIG. 4. Attraction of mixtures of (*E*)-3-tetradecenyl acetate (*E*3–14 : Ac) and (*Z*,*E*)-3,5-tetradecadienyl acetate (*Z*3,*E*5–14 : Ac) toward male carpenterworms (*P. robiniae*) in 1982 and 1983, Stoneville, Mississippi.

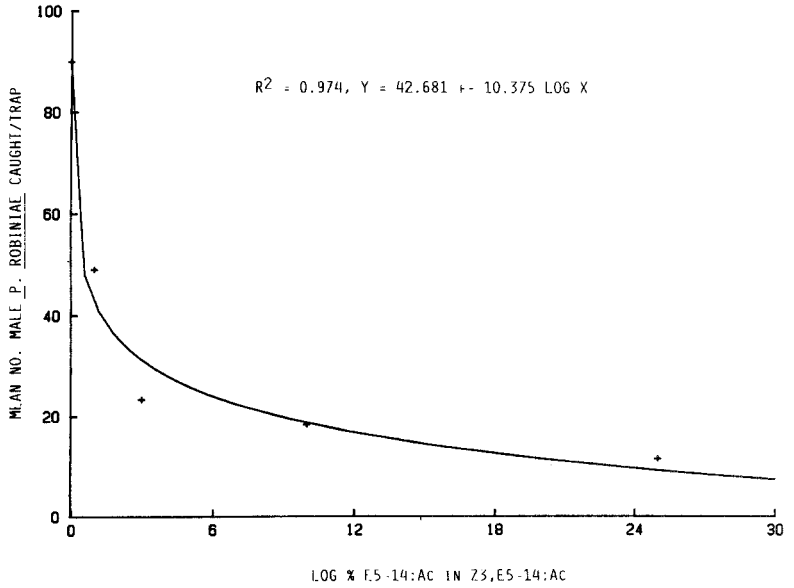


FIG. 5. Effect of (*E*)-5-tetradecenyl acetate (*E*5–14 : Ac) on the attractiveness of (*Z*,*E*)-3,5-tetradecadienyl acetate (*Z*3,*E*5–14 : Ac) toward male carpenterworms (*P. robiniae*) in 1982, Stoneville, Mississippi.

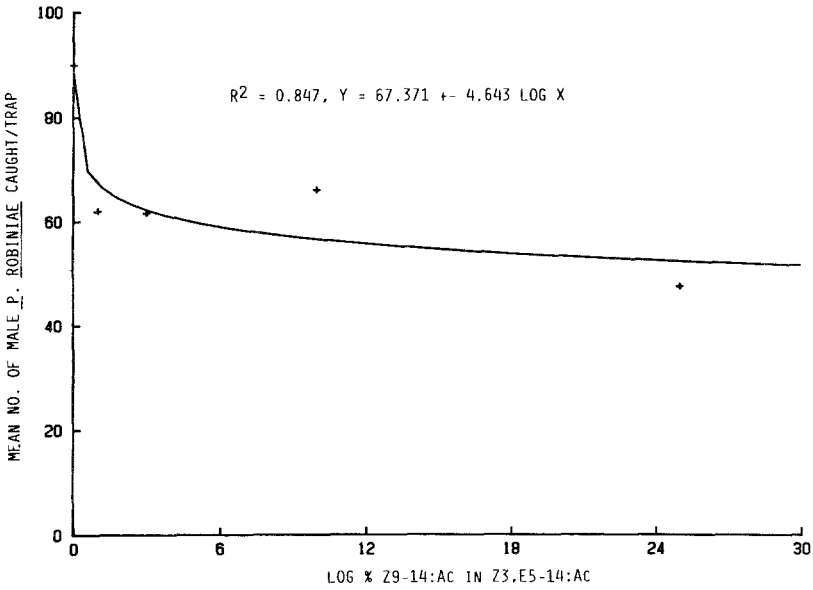


FIG. 6. Effect of (*Z*)-9-tetradecenyl acetate (Z9–14 : Ac) on the attractiveness of (*Z*,*E*)-3,5-tetradecadienyl acetate (*Z*3,*E*5–14 : Ac) toward male carpenterworms (*P. robiniae*) in 1982, Stoneville, Mississippi.

10 to 37% of the *Z*,*E* isomer but <6% of the *E*,*E* isomer and only traces of the *Z*,*Z* isomer. Allowing the mixture to remain at room temperature the prescribed 1 hr produced the *E*,*Z* isomer containing <2% of the *Z*,*E* isomer. In one preparation of the *Z*,*E* isomer, 19% *E*,*Z* was produced when the reaction mixture was allowed to stand at room temperature overnight. This reversal of stereochemistry of both bonds is unprecedented and emphasizes the drastic changes that can occur in stereochemistry with a rather minor change in reaction conditions.

The *Z*,*Z* isomer of 3,5-tetradecadienyl acetate was synthesized by the route outlined in Figure 1, scheme B, which produced this isomer in good yield with high stereoselectivity. The stereochemically pure *E*,*E* isomer was prepared by repeated recrystallization of a sample of the *E*,*E* alcohol available from previous work, followed by acetylation. The stereopurity of these isomers was assessed by capillary gas chromatography on a variety of columns. Chromatograms of all four isomers on liquid crystal and isotropic phases are presented in Figure 7. Resolution between the *Z*,*E* and *E*,*Z* isomers is the smallest, but it is sufficiently high on the liquid crystal column (col C) for the determination of small amounts of one isomer in the presence of the other. Note the reversal of the elution order for the *Z*,*E* and *E*,*Z* isomers on the two columns.

The [^{1}H]NMR chemical shift values for the olefinic and allylic protons as

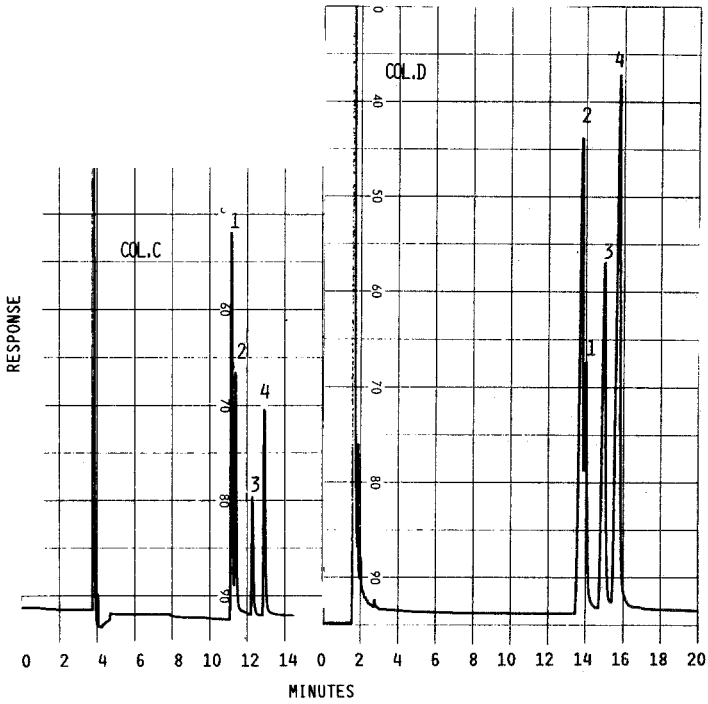


FIG. 7. Chromatograms of the four isomers of 3,5-tetradecadienyl acetate. 1 = *E,Z*; 2 = *Z,E*; 3 = *Z,Z*; 4 = *E,E*. Col. C:40 m × 0.20 mm ID fused silica capillary coated with 0.15% cholesteryl-*p*-nitrocinnamate (180°C). Col. D: 14 m × 0.25 mm ID fused silica capillary coated with 0.25 μm SP 2340 (140°C).

determined by a series of spin–spin decoupling experiments is presented in Table 1. The monounsaturated acetates were purified and analyzed as described in Methods and Materials.

Field Tests. The *E,Z*; *Z,Z*; and *E,E* isomers of 3,5–14 : Ac, as well as the seven monoene acetates when tested alone, did not attract male *P. robiniae*. *E*3,*Z*5–14 : Ac, *Z*3, *Z*4, and *E*4 and *Z*5–14 : Ac had no significant effect on the attractancy of *Z*3,*E*5–14 : Ac toward male *P. robiniae*.

Addition of *E*3,*E*5–14 : Ac to the *Z,E* isomer did increase the attractiveness of the *Z,E* isomer to male *P. robiniae* (Figure 2) as had been reported (Doolittle et al., 1976a; Solomon et al., 1978). The intensity of response of male moths to the mixtures appears to be somewhat different for the two years. However, data for 1983 should be considered the more reliable because of the large number of replications. In any case, there is a pronounced positive effect from the *E,E* isomer.

*Z*3,*Z*5–14 : Ac has a pronounced inhibitory effect on the attractiveness of *Z*3,*E*5–14 : Ac toward male *P. robiniae* (Figure 3). Addition of *E*3–14 : Ac to *Z*3,*E*5–14 : Ac resulted in increased trap captures similar to the effect of the *E*3,*E*5–14 : Ac (Figure 4).

Both *E*5 and *Z*9–14 : Ac caused a reduction in the number of male carpenterworms captured by *Z*3,*E*5–14 : Ac when added to this attractant (Figures 5 and 6). The effect of the *Z*9–14 : Ac is not as marked as with the *E*5–14 : Ac, but nonetheless reduction in trap captures did occur. The effect of *E*5–14 : Ac was similar to that of the *Z*3,*Z*5–14 : Ac.

It was found that the diene alcohol *Z*3,*E*5–14 : OH had a moderate inhibitory effect on trap captures by *Z*3,*E*5–14 : Ac (Table 2).

These results confirm and further define the effect that *E*3,*E*5–14 : Ac has on the attraction of *Z*3,*E*5–14 : Ac toward male *P. robiniae*. The presence of small quantities (up to 10%) of this isomer in samples of the attractant is not only acceptable but desirable. When designing a synthetic route to the *Z*,*E* isomer, those reactions that tend to produce the *Z*,*Z* isomer or the free alcohol as an impurity should be avoided. The synthesis herein described produced no detectable amount of *Z*,*Z* isomer and, when carefully controlled, produces only very minor quantities of the *E*,*Z* and *E*,*E* isomers, one of which is innocuous and the other desirable.

The inhibitory effects of the *E*5 and *Z*9–14 : Ac are difficult to explain and attempts to do so would be speculative. The enhancement of attraction caused by *E*3–14 : Ac strongly indicates that the pheromonal system probably consists of a blend of chemicals which is apparently becoming the rule rather than the exception with lepidopterous sex pheromones (Steck et al., 1980, 1982; Klun et al., 1979).

TABLE 2. RESPONSES OF MALE *P. Robiniae* TO MIXTURES OF (*Z*,*E*)-3,5-TETRADECADIEN-1-OL AND (*Z*,*E*)-3,5-TETRADECADIENYL ACETATE (STONEVILLE, MISSISSIPPI, 1982)

Ratio of *Z*3,*E*5–14:Ac to *Z*3,*E*5–14:OH	Mean number males captured per trap[a]
100:0	203.8a
99:1	113.0b
97:3	65.0b
90:10	97.5b
75:25	62.8b
0:100	0.5

[a]Means followed by the same letter are not significantly different at the 0.05 level as judged by Duncan's new multiple-range test.

CONCLUSION

The new stereoselective synthesis of $Z3,E5$-14:Ac herein described provides an economical route for the efficient production of reasonably large quantities of this attractant free of deleterious isomers. The synthesis should make possible field investigations into the possible application of this chemical for monitoring and control programs.

The data from the effects of monounsaturated acetates on the attractiveness of $Z3,E5$-14:Ac should provide some starting points for the unraveling of the pheromone system of this insect and perhaps other members of the family Cossidae.

Acknowledgments—We thank E.R. Mitchell for his invaluable assistance with the statistical analysis of the field data.

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DETOXIFICATION FUNCTION OF GEOPHAGY AND DOMESTICATION OF THE POTATO

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Abstract—Detoxification as the adaptive function of geophagy is demonstrated from field and historical data associating clay consumption with the domestication of potentially toxic potatoes. In vitro analyses showed that the glycoalkaloid, tomatine, was effectively adsorbed by four classes of edible clays over a range of simulated gastrointestinal conditions. These results, in conjunction with reports of geophagy by nonhuman primates, suggest geophagy as a solution to the impasse chemical deterrents pose to the process of domestication and to chemical constraints on plant exploitation by non-fire-using hominids. The inorganic component of the chemical environment deserves increased attention from chemical ecologists.

Key Words—Geophagy, hominid, primate, detoxification, glycoalkaloids, tomatine, domestication, potatoes, clay-organic interactions.

INTRODUCTION

Selection for changes in secondary chemical constituents has been an essential aspect of the domestication of food plants (Rindos, 1980). However, it is problematic as to how the wild progenitors of staple cultigens with appreciable levels of toxins would have been acceptable initially for human consumption, and therefore subject to genetic manipulation. Cooking has been suggested as the major technological mechanism for making plant foods available to humans (Stahl, 1984; Leopold and Ardrey, 1972). However, heat-stable toxins would remain serious deterrents to foragers, including those with controlled fire. The heat-stable, water-insoluble glycoalkaloids of the potato, which are considered

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in the present study, are not destroyed by cooking. I present evidence associated with the domestication of the potato to show that geophagy was crucial to this process.

I suggest that geophagy is the most basic human detoxification technique with behavioral antecedents that are prehominoid. Soil consumption has been observed in many animals, including invertebrates (Shachak et al., 1976) and reptiles, birds, and mammals (Marlow and Tollestrup, 1982; Smith 1979). Although clays may (or may not) be important nutritionally, they may at least have a detoxification function in mammals. Geophagy in rats has been interpreted to have evolved in relation to the gastrointestinal illness that occurs after eating toxic substances (Mitchell et al., 1976; Burchfield et al., 1977).

The role of geophagy in the detoxification of alkaloids, tannins, and quinones is suggested by several cases of geophagy recorded in the anthropological literature (Laufer, 1930; Anell and Lagercrantz, 1958). In nonhuman primates, the nature of geophagy has led primate ecologists to suggest that a detoxification function in relation to plant secondary compounds is being served (Hladik, 1977).

Interpretation of the adaptive significance of geophagy associated with potato consumption and domestication is decidedly less problematic than with other cases where geophagy and organic toxins have been linked, in that detoxification is the explicit intent of those who practice it. Indians of the American Southwest and adjacent Mexico consume clays with the wild potato species, *S. jamesii* Torr. and *S. fendleri* Gray (Laufer, 1930; Whiting, 1939). The expressed reason for the consumption of specific clays is their effectiveness in eliminating bitterness and in preventing stomach pains or vomiting that result from eating large quantities of these foods without clay. Similar reports of Andean native consumption of specific clays with potatoes date from the 16th-century chroniclers (Weiss, 1953). Specific clays are still consumed with frost-resistant potato cultigens (*Solanum* × *juzepczukii* Buk. and *S.* × *curtilobum* Juz. et Buk.) by the inhabitants of the Central Andean altiplano, with the stated rationale of eliminating the bitter taste characteristic of these potatoes.

Clays are obtained from locally well-known sites in both North and South America. In Peru and Bolivia, I have seen excavations for edible clays extending two or three meters below the surface. Edible clays may be used medicinally, but they are distinguished from clays used for other purposes such as pottery making and for whitewash. Remnants of the historical trade in edible clays persist among the Quechua and Aymara of southern Peru and Bolivia (Rowe, 1946; Tschopik, 1946). Andeans distinguish quality of edible clays largely on the basis of texture. Of the two Peruvian clays, *ch'aqo* and *p'asalla* (Table 1), the former is judged superior because of the relative ease with which it breaks up in water. Andeans consume unseasoned potatoes as the sole or dominant constituent of a meal. Bitter potatoes are dipped in a thick slurry of

TABLE 1. ORIGIN OF FIELD-COLLECTED CLAYS

Clay (language)	Geographical origin and collection data
P'asa (Aymara)	Achocalla, Prov. Murillo, Dept. La Paz, Bolivia; June 22, 1983; purchased in Villa Remedios, Prov. Ingavi.
P'asalla (Aymara, Quechua)	Tincopalca, Prov. San Roman, Dept. Puno, Peru; July 14, 1981; purchased in Mercado Mancocapa, Juliaca, Prov. Puno.
Ch'aqo (Aymara, Quechua)	Acora, Prov. Puno, Dept. Puno, Peru; July 15, 1981; purchased in Puno, Prov. Puno.
Dleesh (Navajo)	Chinlee area, Navajo Reservation, Arizona, U.S.A.; July 1, 1981; collected by Wendy Wolfe.

clay. Indians of the American Southwest consumed potatoes both cooked and raw. Clays were reportedly added during cooking (Bailey, 1940) or were taken with each mouthful of potato (Whiting, 1939).

Solanine, the bitter glycoalkaloid common in most potatoes, is considered to be toxic to humans in quantities above 20 mg/100 g fresh weight (Gregory, 1984; Jadhav et al., 1981), although cases of solanine poisoning are rare. Glycoalkaloid poisoning involves gastrointestinal and neurological disturbances similar to those caused by the related classes of steroid glycosides, saponins and cardiac glycosides (Jadhav et al., 1981). Tuber-bearing species of *Solanum* vary in the identity and quantity of the glycoalkaloids contained (Gregory et al., 1981; Osman et al., 1978; Johns, 1985). The pharmacokinetics of clay–organic interactions have been studied under gastrointestinal conditions (White and Hem, 1983), and adsorption is generally either through ion-exchange and/or physical adsorption (Theng, 1974). Naturally occurring alkaloids have been shown to be effectively bound by clays (Hendricks, 1941; Wai and Banker, 1966; Ridout, 1967; Barr and Arnista, 1957). The degree of binding depends on reaction conditions (White and Hem, 1983) and on the nature of the clays (Barr and Arnista, 1957; McGinity and Hill, 1975).

In this study the detoxification potential of geophagy was estimated by measuring the adsorptive capacity for tomatine of four potato clays that were recognized as distinct by native practitioners. Qualitative mineral composition was determined with X-ray diffraction.

METHODS AND MATERIALS

Materials. All of the four clays used with potatoes (see Table 1 for sources) were obtained as dry solid pieces. In preparation for adsorption assays, clays obtained from the field were pulverized in a mortar. Field samples and standards were elutriated with 15 times their weight of deionized water for 1 h. Samples were decanted to remove large particles of clay and sand. The decanted portion was centrifuged at 15,000*g* for 10 min, and the pellet was dried at 80°C, repulverized, and stored in a desiccator until used. Kaolin and bentonite were purchased from Sigma Chemical Co.

X-Ray Diffraction. Clays were characterized by powder pattern X-ray diffraction. Air-dried and glycolated preparations were analyzed by M. Cosca, Department of Geology, University of Michigan.

Determination of Adsorptive Capacity. Adsorption of alkaloids on well-characterized clays such as kaolin has been shown to follow an isotherm represented by the Langmuir equation (Barr and Arnista, 1957; Ridout, 1967). The adsorption of a representative and commercially available glycoalkaloid, tomatine, by four sample clays, *ch'aqo*, *p'asalla*, *p'asa*, and *dleesh*, and by commercially available clays, kaolin and bentonite, was determined using the linear form of the Langmuir adsorption isotherm:

$$\frac{C}{X/M} = \frac{1}{ab} + \frac{1}{\mathrm{b}}C$$

where C is the equilibrium concentration of tomatine in solution, and X/M is the amount of tomatine adsorbed per gram of adsorbant. Constants are designated as a and b, with b being equal to the monolayer capacity of the adsorbant.

Adsorptive capacity was determined by stirring 6-mg samples of clay with tomatine (concentration 2–10 mg/10 ml) in 10 ml of buffer (ammonium acetate, pH 5.5) at room temperature for 1 hr. Equilibrium concentrations (C) of tomatine were determined. All tubes were centrifuged for 10 min at 14,500*g*, and a 5-ml aliquot of the supernatant was freeze-dried. The aglycone, tomatidine, was obtained by hydrolyzing samples in 10 ml of 2 NH_2SO_4 heated at 100°C for 2 hr. Aglycones were precipitated with concentrated NH_4OH and were extracted with benzene. Known amounts of tomatine in four concentrations were taken through the lyophylization, hydrolysis, and extraction procedures in each experiment, and served as standards. The quantity of tomatidine present was determined using a titration method (Fitzpatrick and Osman, 1974). The inverse of the slope ($1/b$) of a linear regression of $C/(X/M)$ on C for each clay sample was used to determine the adsorptive capacity (b).

Adsorptive Capacity under Physiological Conditions of pH and Ionic Strength. The effect of pH on tomatine adsorption was determined for *p'asa* and bentonite using the method described above. Solutions were buffered over

a pH range of pH 5.5–1.5, using 0.1 M ammonium acetate (pH 5.5 and 4.5) and 0.1 M ammonium formate (pH 3.5 and 1.5). Adsorptive capacities under stomach conditions were simulated at pH 2.0 and 0.075 M for *p'asa*, bentonite, and *dleesh*.

Determination of the adsorptive capacity at reduced ionic strength was not possible at pH 5.5 or above because of the limited solubility of tomatine (cf. pK_a of α-solanine = 6.6 (Windholz, 1976)). The effect of ionic strength on the adsorption of tomatine was determined at pH 4.5 for *p'asa* and bentonite using concentrations of ammonium acetate varying from 0.15 to 0.01 M. Adsorption under unbuffered conditions was determined for *p'asa* and bentonite using a starting pH of 2.5. Determinations at pH 5.5 and ionic strength of 0.15 were used to approximate sorption in the intestinal tract for *p'asa*, bentonite, and *dleesh*.

RESULTS AND DISCUSSION

Adsorptive Capacities of Potato Clays. Qualitative characterizations of the four field-collected clays are recorded in Table 2. The sample of *p'asa* showed high interlayer swelling in water. Many of its properties were similar to those of bentonite.

Values of the adsorptive capacities for tomatine for each of the clay samples at pH 5.5 and 0.1 M ammonium acetate are shown in Table 2. These determinations are representative of binding under oral conditions. Significant differences ($P < 0.05$) in slopes are seen between all clays tested (analysis of covariance). Assays with kaolin showed experimental variation that was difficult to correct with the experimental methods used. The steep slope of the Langmuir isotherm for kaolin as compared to those of montmorillonite clays such as bentonite and *p'asa* is consistent with the weak cation-exchange capacity of kaolin (Barr and Arnista, 1957; Meshali, 1982).

The adsorption of tomatine by the six clay samples and standards follows a pattern consistent with the known cation-exchange capacities and intercalation properties of different clay groups (Theng, 1974). With an adsorptive capacity of 0.50 g/g (pH 5.5, 0.1 M ammonium acetate), the Bolivian clay, *p'asa*, is superior to bentonite (adsorptive capacity: 0.37 g/g), one of the best commercially available adsorbants of organic compounds. At pH 4.5 and 0.01 M buffer concentration, *p'asa* and bentonite have capacities of 0.68 and 0.65 g/g, respectively. For unbuffered samples, adsorptive capacities of 0.61 g/g and 0.64 g/g, respectively, were recorded for the two clays. Under these conditions of low ionic strength, tomatine adsorption by bentonite is comparable on a milligram basis to that of previously studied pharmaceutical agents (Wai and Banker, 1966).

Of the four clay samples, the weakest adsorption was shown by *dleesh*,

TABLE 2. CHARACTERIZATION OF CLAYS AND COMPARISON OF DETOXIFICATION POTENTIALS AS REPRESENTED BY ADSORPTIVE CAPACITIES FOR TOMATINE

Clay	Characterization	Adsorptive capacity [g/g (r^2)]
P'asa	Predominantly smectites, mixed-layered with illite; quartz. High interlayer swelling.	0.50 (0.99)
P'asalla	Mixed-layer of smectites and illite; quartz.	0.28 (0.94)
Ch'aqo	Predominantly illite, mixed-layered with smectites, chlorite, kaolinite; quartz.	0.19 (0.98)
Dleesh	Predominantly illite, mixed-layered with smectites, kaolinite, chlorite; quartz	0.14 (0.97)
Bentonite		0.37 (0.98)
Kaolin		0.05 (0.77)

obtained from the Navajo reservation. Its adsorptive capacity of 0.14 g/g is, nonetheless, approximately three times greater than Kaolin under similar conditions.

Effects of pH and Ionic Strength on Adsorptive Capacity. The adsorptive capacities of tomatine by *p'asa* and bentonite show no significant ($P > 0.05$) differences between pH 5.5 and 1.5 (Table 3). The lack of appreciable effects due to increasing proton concentration is consistent with other studies that have considered the adsorption of organic bases onto clays (Meshali, 1982; Browne et al., 1980). The pK_as of the alkaloid portion of glycoalkaloids favor the protonated form under experimental conditions.

The increased binding of tomatine as ammonium acetate concentration is decreased from 0.15 to 0.01 M is highly significant ($P < 0.0001$) for both *p'asa* and bentonite (Table 4). The nature of the competition for negatively charged sites between tomatine and other cations will depend on the exchangeable cations present on the surface of the clays and the particular cations present in solution (McGinity and Hill, 1975).

Sorption of high-molecular-weight cations has been shown to involve both ion-exchange and physical adsorption and to reach adsorption levels that exceed the cation-exchange capacity of the clays (Wai and Banker, 1966). At low ionic strength, 0.66 meq/g of tomatine were adsorbed per gram of *p'asa*, while ben-

TABLE 3. EFFECT OF pH ON ADSORPTIVE CAPACITY OF P'ASA AND BENTONITE

pH	Ionic strength (M)	Adsorptive Capacity [g/g (r^2)]		
		P'asa	Bentonite	Dleesh
5.5	0.10	0.50 (0.99)	0.37 (0.98)	
4.5	0.10	0.45 (0.98)	0.41 (0.96)	
3.5	0.10	0.50 (0.97)	0.42 (0.97)	
2.5	0.10	0.50 (0.99)	0.46 (0.98)	
1.5	0.10	0.48 (0.99)	0.44 (0.98)	
2.0	0.075	0.49 (0.97)	0.43 (1.00)	0.11 (0.93)

TABLE 4. EFFECT OF IONIC STRENGTH ON ADSORPTIVE CAPACITY OF P'ASA AND BENTONITE

Ionic strength (M)	pH	Adsorptive Capacity [g/g (r^2)]		
		P'asa	Bentonite	Dleesh
0.15	5.5	0.40 (0.99)	0.30 (0.98)	0.03 (0.98)
0.15	4.5	0.45 (0.97)	0.33 (0.99)	
0.10	4.5	0.45 (0.98)	0.41 (0.96)	
0.05	4.5	0.58 (0.99)	0.46 (1.00)	
0.01	4.5	0.68 (0.99)	0.65 (0.99)	
Unbuffered	2.5 (initial)	0.61 (0.99)	0.64 (0.99)	

tonite adsorbed 0.63 meq/g. On a molar basis, the measured values of tomatine are, in fact, less than the average meq/g reported for montmorillonite clays (0.8–1.5 meq/g). The failure of tomatine to occupy all sites available for cation exchange most likely represents a "cover-up effect" (Hendricks, 1941). The large size of the tomatine molecule (mol wt 1034) exceeds the area available per exchange site.

Detoxification under Physiological Conditions. Adsorption studies over the range of conditions encompassing gastrointestinal pH and ionic strength (Lentner, 1981) demonstrate the ability of these edible clays to bind tomatine and hence neutralize the bitterness and eliminate the gastrointestinal irritation associated with glycoalkaloid ingestion (Jadhav et al., 1981). The quantities of clay necessary to reduce glycoalkaloids from a level of 100 mg/100 g fresh weight, typical of wild potatoes (Gregory, 1984), to a recognized nontoxic level of 20 mg/100 g (Gregory, 1984) are minuscule in comparison to the amounts that are consumed at an actual meal (Whiting, 1939). Culinary practices of

Andeans and the Hopi, as described above, should facilitate glycoalkaloid adsorption.

The determinations of adsorptive capacity made at pH 5.5 and 0.1 M ammonium acetate account for the decrease in bitterness reported by native peoples. Soluble molecules of glycoalkaloids available to interact with oral receptors are also those available for clay adsorption.

The overall detoxification of the clays is most dependent on adsorption in the stomach. Determinations of adsorptive capacity at pH 2.0 and 0.0075 M monovalent cation concentration (ammonium ion) reflect binding during the gastric residence period. Under these conditions, only 271 mg of the least adsorbant of the clays, *dleesh*, would reduce a toxic level of 50 mg tomatine in 100 g of potatoes to the recognized nontoxic level of 20 mg/100 g TGA. *P'asa*, the most effective adsorbant would provide the same detoxification with only 60 mg of clay.

Desorption at the increased pH and ionic strength of the intestines could reduce the effective detoxification of clays. At pH 5.5 and 0.15 M ammonium acetate, the adsorptive capacities of *p'asa*, bentonite, and *dleesh* are the lowest in comparison to all the conditions tested. The reduced solubility of glycoalkaloids at pHs above 5.5 would be expected to reduce adsorption under normal intestinal pHs of 6–8 (Lentner, 1981). Previous work (White and Hem, 1983) has shown that in vivo, in the presence of clays, pK_as are effectively higher. Such an effect would facilitate clay–tomatine binding in vivo. Even if clay adsorption of glycoalkaloids is reduced in the intestine, their insolubility under basic conditions should minimize adsorption by the intestine and decrease the resultant toxic effects.

Evolutionary Significance of Geophagy by Humans. Wild potatoes, as weedy annuals occurring in abundance on sites of human disturbance, meet the classic criteria for a domesticable plant (de Wet and Harlan, 1975). However, weakly toxic glycoalkaloids, while not eliminating the possibility of consumption, would limit the use of potato tubers to a casual occurrence or as an emergency food. Geophagous practices associated with the gathering of potatoes from the wild (Laufer, 1930), and with the cultivation of wild potatoes (Whiting, 1939; cf. Pennington, 1963) and primitive domesticates (Weiss, 1953) link this detoxification phenomenon with the process of human-directed evolution leading to plant domestication. Association of the detoxification function of geophagy with stages in the domestication of the world's premier vegetatively propagated cultigen (FAO, 1983) has important implications, as well, for the domestication of many crops where wild relatives contain toxic levels of secondary compounds.

The clay–potato association provides insight into the general phenomenon of geophagy. Potato clays are used habitually with a resource which otherwise is only modestly exploitable because of the presence of weak but quantitative

toxins. The use of clays in this situation corresponds to geophagous practices of at least eight species of primates which include at least some leaves in their diet (Hladik, 1978; Wrangham, personal communication). Nonlethal quantities of secondary compounds limit the exploitation of particular abundant resources by generalist herbivores (Freeland and Janzen, 1974) and constrain primate food choice (Clutton-Brock, 1977; McKey et al., 1981). Specific clays, often from termite mounds, are eaten daily, sometimes seasonally, by chimpanzees (Uehara, 1982; Wrangham, 1977; Hladik and Gueguen, 1974).

If early human foraging was analogous to the feeding of modern nonhuman primates, then food procurement by humans must have been subject to the constraints imposed by plant defensive compounds. Geophagy is a behavior with antecedents that are certainly prehominoid, and higher primates, including humans, have apparently maintained it as a mechanism for dealing with naturally occurring toxins.

Rats consume clay when subjected to chemical toxins in laboratory settings (Mitchell et al., 1976, 1977a). Mitchell et al. (1977b) demonstrated that if a rat develops a conditioned taste aversion to a novel (and nontoxic) flavor, it will respond to this conditioned stimulus by consuming clay, even if clay was not administered during the original conditioning episode.

The conditioned aversion response has been extensively studied in rats and other animals (Garcia et al., 1974; Riley and Baril, 1976) and has provided a model for considering human taste aversions and preferences (Garb and Stunkard, 1974; Garcia et al., 1974; Bernstein, 1978). The well-studied model linking toxicosis and conditioned responses, mediated through gastrointestinal upset, can be extended to include geophagy as part of an integrated "psychological" response to environmental toxins. This model may be extended to include geophagy as an adaptive behavioral response for generalist primates as well as rats.

Widespread medicinal uses of clays by humans to treat diarrhea (Vermeer and Ferrell, 1985) and other gastrointestinal ailments may relate to the detoxification function of geophagy either directly or indirectly via the "psychological" response to gastrointestinal upset.

Periodic deficiencies in food availability are a fundamental stress facing humans and other primates (Stahl, 1984). Wild potatoes are typically exploited by humans during famines or periods of seasonal scarcity (Laufer, 1930; Whiting, 1939). Geophagy accompanying the use of famine foods is a widely documented behavior of traditional agriculturalists around the world (Laufer, 1930; Solien, 1954). Here geophagy is a response to gastrointestinal stress in the form of hunger (Solien, 1954) or poisoning by ingestion of barely tolerable wild plants or bacterially contaminated foods. The most appropriate responses to this stress are maintained and transmitted through human culture and institutions. Modern cultural attitudes towards geophagy are clearly important in encour-

aging or restricting this behavior. Maintenance of geophagy as a cultural trait may be a relic of its historical significance (Laufer, 1930; Anell and Lagercrantz, 1958; Hunter, 1973).

Crises in food production are an inherent component of many models of the development of agriculture (Rindos, 1980). The transition of geophagy from a general response to toxin-related stress to a more specialized detoxification technique can be interpreted as an important step allowing expanded resource exploitation and the domestication of particular plants. Wild potatoes were presumably a minor dietary constituent or a famine resource under primitive conditions of exploitation, but eventually provided a subsistence base for complex prehistoric civilization in the Central Andes. By analogy, wild plants were probably more available to early hominids than has been previously supposed (Leopold and Ardrey, 1972).

The functional significance of geophagy suggests that the interaction of animals with the inorganic component of the chemical environment deserves increased attention from chemical ecologists. Animals ingest exogenous substances for nutritional, digestive (Martin and Martin, 1978), defensive, and communicative purposes (Harborne, 1982). Clearly they ingest exogenous substances for their detoxification properties as well.

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ISOLATION OF FEEDING DETERRENTS AGAINST ARGENTINE STEM WEEVIL FROM RYEGRASS INFECTED WITH THE ENDOPHYTE *Acremonium loliae*

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Abstract—Infection of ryegrass (*Lolium perenne*) by an endophytic fungus (*Acremonium loliae*) confers resistance against the Argentine stem weevil (*Listronotus bonariensis*). Extracts from ryegrass clones, infected and uninfected with *A. loliae*, were compared in a feeding choice bioassay, and several fractions were identified which affected stem weevil feeding behavior. One stem weevil feeding deterrent, peramine $C_{12}H_{17}N_5O$, has been isolated from infected ryegrass and partially characterized as a basic indole derivative. Extracts from cultured *A. loliae* had no effect on stem weevil feeding behavior nor was peramine detected in the fungal cultures examined. Peramine and the other active substances are hydrophilic in contrast to the lipophilic properties reported for the neurotoxic lolitrems also isolated from ryegrass infected with *A. loliae* and associated with causing ryegrass staggers disorder in livestock. It is suggested that ryegrass staggers and stem weevil feeding deterrency may arise by different biochemical mechanisms.

Key Words—Perennial ryegrass, *Lolium perenne*, Gramineae, Argentine stem weevil, *Listronotus bonariensis*, Coleoptera, Curculionidae, *Acremonium loliae*, *Acremonium coenophialum*, endophyte, bioassay, induced resistance, feeding deterrent, peramine.

INTRODUCTION

Perennial ryegrass (*Lolium perenne L.*), the dominant New Zealand pasture grass, can be severely damaged by Argentine stem weevil [*Listronotus bonariensis* (Kuschel), Coleoptera: Curculionidae]. In the spring, diapausing adults respond to increasing daylength and begin laying eggs in the leaf sheath at the

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base of ryegrass tillers. The larvae penetrate the stem and develop through four instars during which time they mine and kill up to four tillers. There are two generations per year, with populations peaking in the spring in the South Island and in the summer in the North Island. The adult weevils feed on the tips of leaves but do little damage to mature plants.

Perennial ryegrasses resistant to stem weevil have been identified (Kain et al., 1982a,b), and this resistance has been linked to the presence of an endophytic fungal infection in the resistant plants (Prestidge et al., 1982). The resistance of endophyte-infected ryegrass derives in part from selective feeding by the adult weevils. Gaynor and Hunt (1983) showed that there was less feeding by adult weevils on plants infected with endophyte, and this led to fewer eggs and larvae and to less damage. The endophytic fungus associated with stem weevil resistance of ryegrass, *Acremonium loliae* Latch, Christensen and Samuels, is seed transmitted and closely related to *Acremonium coenophialum* Morgan-Jones and Gams, found in tall fescue, *Festuca arundinaceae* (Schreber) (Morgan-Jones and Gams, 1982; Latch et al., 1984). A second, less common, *Gliocladium*-like endophyte has also been isolated from ryegrass (Latch et al., 1984) but, when ryegrass was artificially infected with this fungus, no resistance to stem weevil was detected (Gaynor et al., 1983).

Endophytic infection of ryegrass has been reported to be associated with resistance to eight species of sod webworms (*Crambus* spp.) (Funk et al., 1983a), to billbugs (*Sphenophorus* sp.) (Funk et al., 1983b) in the United States, and to pasture mealy bug, *Pseudantonina poae* (Mask.), in New Zealand (W.D. Pearson, DSIR, Lincoln, personal communication). Tall fescue infected with *A. coenophialum* has been shown to be resistant to stem weevil (Barker et al., 1983) and to oat aphid, *Rhopalosiphum padi* (L.) (Latch et al., 1985). Endophyte-infected ryegrass, however, is not resistant to *R. padi*. Endophytic fungi have also been reported from Chewings fescue (*F. rubra* L. *sensu lato*), Bermuda grass [*Cynodon dactylon* (L.) Pers.] and a number of other species (Latch et al., 1984). However, only for Chewings fescue are there indications of endophyte-associated insect resistance (Funk et al., 1983b).

The presence of *A. loliae* in ryegrass is also associated with a specific livestock disorder known as ryegrass staggers (Fletcher and Harvey, 1981). This disorder occurs sporadically in stock grazing ryegrass pasture infected with endophyte and is characterized by tetanic muscle spasms and a hypersensitivity to external stimuli. Ryegrass staggers is of considerable importance to agriculture in Australia and New Zealand (Cunningham and Hartley, 1959; Gallagher et al., 1984). The presumed causative agents of ryegrass staggers, a series of tremorgenic neurotoxins named lolitrems, have recently been isolated from extracts of "toxic" pasture and seed (Gallagher et al., 1981, 1982). The lolitrems are structurally similar to the other known tremorgenic mycotoxins such as the penitrems and the janthitrems (Gallagher et al., 1984). Infection of tall fescue

pasture with *A. coenophialum* in the United States is also associated with a complex of stock disorders referred to as fescue foot, fat necrosis, and summer slump or fescue toxicity (Johnson, 1983).

Recent research suggests that endophyte-infected ryegrass and tall fescue is less palatable and may also adversely affect growth and reproduction of stock (Johnson, 1983; Fletcher, 1983; Fletcher and Barrell, 1984). We decided to identify the biochemical mechanisms by which endophytic infection confers stem weevil resistance to the infected plant. This should indicate the feasibility of producing insect-resistant ryegrasses which have no associated animal toxicity and also suggest possible approaches to this goal, e.g., via plant selection or reinfection with mutant endophytes.

METHODS AND MATERIALS

The preference of adult weevils for endophyte-free plants, coupled with the consequent increase in larval damage, led us to develop a bioassay sufficiently sensitive to detect adult weevil feeding preferences between extracts from endophyte-infected and uninfected ryegrasses. "Grasslands Nui" perennial ryegrass was used throughout, and clones free of *A. loliae* were obtained by treating infected ryegrass with benomyl fungicide using the method of Latch and Christensen (1982). The plants were repotted several months prior to testing and checked for endophyte infection microscopically and by an ELISA technique (Musgrave, 1984). For large-scale extraction, the plants were subdivided and grown in the field for several months as spaced plants.

Feeding Choice Bioassay. Ryegrass from either infected or uninfected clones was harvested using an electric sheep-shearing handpiece to collect all material above ground level and stored at −20°C until required. Frozen grass was chopped into 2-cm lengths, blended twice in 95% ethanol, and the resulting extract filtered through Celite and evaporated to dryness under reduced pressure. This extract was redissolved in chloroform–ethanol–water (5:2:1), and a known volume was added to cellulose powder (Whatman CC31) to give a concentration of 2.5 g extract/4 g of cellulose powder. The cellulose powder was dried on a rotary evaporator at 40°C and then on a vacuum pump at 40°C for 1 hr.

Four grams of this cellulose powder was stirred rapidly into a hot mix made from agar (4 g), sucrose (5 g), and water (90 ml). After cooling, 35–40 agar disks were cut with a cork borer (1 cm diameter × 3 mm thickness) and used in a feeding choice bioassay to determine the feeding preference of adult weevils. Subsequent fractions isolated after solvent partitioning or chromatography were assayed at concentrations proportional to their concentration in the 95% ethanol extract.

For the feeding choice bioassay, one adult weevil in an 8-cm-diameter Petri dish was presented with two agar disks, one containing extract from an endophyte-infected ryegrass and the other extract from an uninfected clonal replicate. Dishes were replicated a minimum of 35 times and maintained at 16°C with 16 hr daylength for 72 hr. The amount of feeding on each disk was assessed by scoring blind on a 0–3 scale the amount of frass produced by the weevil (0, having no frass; 1, having 1–2 small clumps of frass; 2, having 4–10 clumps of frass; 3, having large areas of the disk covered in frass). The difference in feeding scores between the two agar disks of each Petri dish was calculated and the significance of the deviation of these differences from zero (i.e., no difference in feeding) was tested using the Wilcoxon signed rank test (Siegel, 1956). The mean difference in feeding scores (MFS) was determined for all the Petri dishes in the test. A positive MFS value indicates more feeding on the disks containing extract from uninfected ryegrass, while a negative MFS value indicates more feeding on the disks containing extract from infected plants.

Feeding Deterrent Bioassay. Activity in the feeding choice bioassay could be the result of either the presence of a feeding deterrent in the extract from the infected ryegrass or higher levels of some feeding stimulant in the extract from uninfected ryegrass. To distinguish between these possibilities, a change was made in the preparation of the cellulose powder used in the feeding choice bioassay. The 95% ethanol extract from uninfected ryegrass was partitioned between 80% ethanol and petroleum ether. The 80% ethanol phase was evaporated to dryness, weighed, redissolved in chloroform–ethanol–water (1 : 1 : 1), and adsorbed onto cellulose powder at 2 g of extract/4 g of cellulose powder. To this cellulose powder was added either sufficient purified extract of an active fraction from an infected plant to give a normal plant concentration (plus solvent, 15 ml total volume), or just solvent (15 ml). The resulting two suspensions were evaporated to dryness and the two portions of cellulose powder used to prepare the two agar disks for the feeding choice bioassay. This was then used to determine whether the previously active fraction still deterred feeding when incorporated back into the ethanolic extract, thus suggesting the presence of a feeding deterrent. To check whether an imbalance of feeding stimulants was involved, the above procedure was repeated but using instead the corresponding extract from a clonal plant uninfected with *A. loliae*.

Phase Partitioning. The 95% ethanol extract, prepared as previously described, was partitioned between chloroform–methanol–water (1 : 1 : 1), then further partitioned between *n*-butanol–water (1 : 1), or petroleum ether–80% ethanol (1 : 1) following the sequences outlined in Figures 1 and 2. All phases were backwashed with the appropriate solvents. The backwashes were partitioned against each other and recombined with their parent phases.

Chromatography. The fractions obtained from the above partitioning were further separated by chromatography following the sequences outlined in Fig-

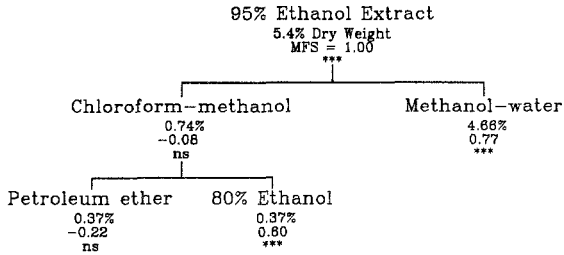

FIG. 1. Phase separation of ryegrass extract showing partitioning of mass (% dry weight), mean difference of stem weevil feeding scores when offered a choice of extracts from infected or uninfected ryegrass (MFS), and the significance of the difference (ns = not significant, *** = $P < 0.001$). A positive MFS value indicates more feeding on the agar disks containing extract from uninfected ryegrass.

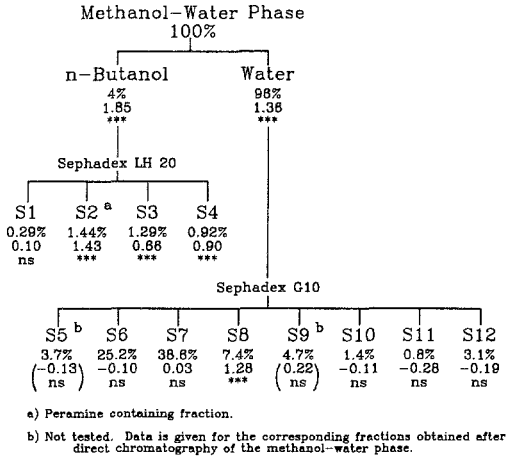

FIG. 2. Phase separation and chromatography of the methanol–water extract showing for each fraction: partitioning of mass (as a % of methanol–water extract), mean difference of stem weevil feeding scores when offered a choice of extracts from infected or uninfected ryegrass (MFS), and the significance of the differences (ns = not significant, *** = $P < 0.001$).

ures 2 and 3. Chromatographic fractions were routinely examined by silica gel thin-layer chromatography (TLC) using a vanillin–sulfuric acid spray reagent, and fractions containing similar components were bulked together for the bioassay. Extracts from both infected and uninfected plants were chromatographed separately, under, as far as possible, identical conditions.

For Sephadex G-10 chromatography (Figure 2), the sample (2.8 g) was eluted from a 25 × 920-mm column of Sephadex G-10 (V_0 180 ml) using 1%

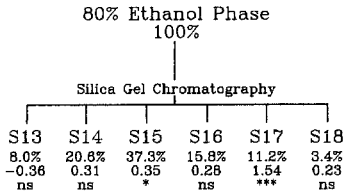

FIG. 3. Fractionation of 80% ethanol extract showing for each fraction: partitioning of mass (as a % of 80% ethanol extract), mean difference of stem weevil feeding scores when offered a choice of extracts from infected and uninfected ryegrass (MFS), and the significance of the differences (ns = not significant, * = $P < 0.05$, *** = $P < 0.001$).

n-butanol–water. The flow rate was 60 ml/hr, and 10-ml fractions were collected. Fractions eluting between 160 and 200, 200 and 330, 330 and 450, 450 and 580, and 580 and 1000 ml were bulked on the basis of their UV adsorption at 280 nm.

For Sephadex LH-20 chromatography (Figure 2), a ratio of 1 g sample to 55 g Sephadex LH-20 was used. The column was eluted with 80% ethanol.

For silica gel chromatography (Figure 3), a sample of the 80% ethanol phase (12.2 g) was applied to a column of silica gel (400 g) packed in 50% petroleum ether–chloroform. The column was eluted successively with chloroform (1.3 liters), chloroform–ethanol (9:1) (6.3 liters, two fractions collected); chloroform–ethanol (8:2) (3.5 liters); chloroform–ethanol–95% ethanol (7:3:3) (3 liters); then (1:2:2) (2.3 liters); then 95% ethanol (1.5 liters).

Isolation of Peramine. The stem weevil feeding deterrent, which we have named peramine, was detected in fraction S2 (Figure 2) as a blue staining spot ($R_f \sim 0.3$) after TLC and spraying with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde–hydrochloric acid). TLC was performed on aluminum-backed silica gel-60 using the lower phase of a chloroform–methanol–water (7:13:8) partition.

Fraction S2 was rechromatographed on Sephadex LH-20 with 80% ethanol until no significant further purification could be achieved. Further chromatography on Sephadex LH-20 (100:1 w/w) using 95% ethanol–dichloromethane (1:9) was used to remove pigments. Fractions containing peramine were eluted using a gradient of 95% ethanol–dichloromethane (2:8 to 1:1). This material was dissolved in 0.01 M ammonium bicarbonate buffer, pH 7.8, containing 2% *n*-butanol and applied to a column of CM-Sephadex in the same buffer. The column was washed with ammonium bicarbonate buffer until the last yellow band had eluted. Peramine was then eluted with 1% acetic acid, pH 4.6, containing 2% *n*-butanol.

Purification of peramine by high-performance liquid chromatography (HPLC) was achieved on a 5-μm C-18 Rad-Pak 8 × 100-mm column fitted with a RCSS C-18 Guard-Pak insert. A linear solvent gradient from 60% to

90% methanol–water over 20 min was used. Both solvents were 0.005 molar in sodium *n*-heptanesulfonate. The flow rate was 1 ml/min with UV detection at 230 and 280 nm. Peramine eluted as the single major peak after 27 min. To remove the bulk of the sodium *n*-heptanesulfonate prior to mass spectrometry, the material was partially desalted using G-10 Sephadex, eluting with water. Subsequent elution with methanol gave peramine.

Flatbed paper electrophoresis was run at pH 10.8 using a 0.1 M sodium carbonate buffer and 5-hydroxy- and 5-methoxytryptamine as standards.

Acetylation of Peramine. Peramine (~0.5 mg) was dissolved in acetic anhydride (0.5 ml) and pyridine (0.5 ml) and kept at room temperature for 6 hr until TLC indicated no peramine remained. Excess reagents were removed with toluene under reduced pressure, and the residue was taken up in dichloromethane and filtered. Preparative silica gel TLC using acetone–dichloromethane (1:3) gave diacetyl-peramine as the major product: electron ionization mass spectrometry (EIMS) *m/z*: 331(M^+), 272, 247, 230, 213, 205, 188, 175 (100%).

Bioassay with Cultured Endophyte. Mycelium from a liquid potato–dextrose culture of *A. loliae* endophyte was blended in 95% ethanol, filtered, evaporated, and made up as a standard solution as for the plant extracts. The hyphal extract was added to cellulose powder onto which had been adsorbed extract from endophyte-free ryegrass as described for the feeding deterrent bioassay above. Agar disks containing hyphal extract plus extract from endophyte-free ryegrass were compared with disks containing only extract from endophyte-free ryegrass. Bioassay concentrations corresponded to 0.7, 4, 7, and 28 mg fungal mycelium/g agar.

RESULTS

Crude Extracts. When adult stem weevils were presented with a choice of agar disks containing 95% ethanol extract either from ryegrass infected with *A. loliae* or from an uninfected clonal replicate, there was significantly less feeding (MFS = 1.00) on the disks containing the extract from infected ryegrass (Figure 1). When this extract was solvent partitioned with chloroform–methanol–water, activity was found in the more polar methanol–water phase (MFS = 0.77). When the inactive chloroform–methanol phase (MFS = −0.08) was further partitioned between 80% ethanol and petroleum ether (Figure 1), activity was found in the 80% ethanol phase (MFS = 0.60), suggesting a masking effect by inactive petroleum-soluble substances.

Isolation of Peramine. The methanol–water phase was partitioned between *n*-butanol and water (Figure 2), and activity was found in both phases. The *n*-butanol phase was chromatographed on Sephadex LH-20 and yielded three active fractions S2, S3, and S4. Fractions from both infected and uninfected plants

were compared by TLC using a variety of spray reagents. The only observed difference between the extracts from infected and uninfected plants was a blue-staining compound detected with Ehrlich's reagent only in fraction S2 from infected ryegrass. This blue-staining material, which we have named peramine, was purified by repeated Sephadex LH-20 chromatography and further purified on Sephadex LH-20 using a gradient of 95% ethanol in dichloromethane. This purified material was active in the feeding choice bioassay of 10 ppm (MFS = 1.13; $P < 0.001$) and 1 ppm (MFS = 0.50; $P < 0.001$). It was also tested in the feeding deterrent bioassay and was found to deter weevil feeding at 10 ppm (MFS = 0.49; $P < 0.001$). The concentration of this fraction in fresh plant tissue from infected plants was estimated to be approximately 8 ppm.

Peramine, homogeneous by TLC and essentially pure by HPLC, was obtained from the partially purified material by ion-exchange chromatography using CM-Sephadex. This material was tested in the feeding choice bioassay by incorporation into agar–cellulose powder disks containing sucrose as the only stimulant (sucrose agar) and was active at 1.3 ppm (MFS = 1.45, $P < 0.001$, concentration based on UV adsorption at 285 nm; ϵ taken as 10,000 based on indole). Ammonium acetate, a coeluant with peramine from the CM-Sephadex column, was also tested at a nominal 18 ppm against sucrose agar in the feeding choice bioassay and was inactive (MFS = 0.18).

A final purification of peramine was achieved by reversed-phase HPLC which removed a number of minor UV adsorbing impurities. A phase modifier, sodium *n*-heptanesulfonate (0.005 M), was added to the mobile phase in order to obtain retention of peramine on the C-18 stationary phase. The phase modifier proved difficult to separate from the small quantities of peramine present, reducing the preparative value of this separation step. Peramine, obtained after HPLC, was desalted on Sephadex G-10 and showed identical UV, low-resolution mass spectra, and chromatographic behavior on TLC to material obtained after CM-Sephadex chromatography.

Peramine showed UV maxima at 232 and 285 nm, suggestive of a 2,3-disubstituted indole (de Jesus et al., 1983). Paper electrophoresis indicated peramine was a strong base, retaining a positive charge at pH 10.8. Peramine gave a positive reaction with Dragendorff's reagent (1-μg sample on TLC) but no reaction with ninhydrin (8-μg sample size). High-resolution electron ionization mass spectrometry (EIMS) established $C_{12}H_{17}N_5O$ as the formula for the highest mass ion (*m/z* 247.1404, $C_{12}H_{17}N_5O$ requires 247.1432). A second high mass ion, measured as $C_{11}H_{15}N_3O$ (*m/z* 205.1214, $C_{11}H_{15}N_3O$ requires 205.1214) corresponded to the product of a cyanamide loss. Attempts made to obtain the FAB-mass spectrum of peramine gave only ions arising from the matrix used (glycerol, glycerol HCl, thioglycerol, PEG). The molecular formula of peramine was confirmed as $C_{12}H_{17}N_5O$ by acetylation which gave a diacetyl derivative *m/z* 331 (M^+, $C_{16}H_{21}N_5O_3$). Characteristic acetamide (*m/z* 272) and ketene (*m/z* 247, double ketene) losses were also observed.

Activity of Water Phase. The water phases from both infected and uninfected plants were chromatographed on Sephadex G-10, and the fractions obtained compared in the choice bioassay (Figure 2). Fraction S8, from within the included volume of the Sephadex column, was the only active fraction. Fractions from both infected and uninfected plants were examined by TLC, but no differences were observed under UV irradiation or with ninhydrin, vanillin, Ehrlich's, fast blue, or sulfuric acid as spray reagents. No peramine was observed. When methanol was added to aqueous solutions of S8, inorganic salts were precipitated. The remaining methanol-soluble components were predominantly glucose and fructose as determined by ^{13}C nuclear magnetic resonance spectroscopy ([^{13}C]NMR). No major differences in the [^{13}C]NMR spectra were observed for samples obtained from either infected or uninfected plants. Samples of S8 were consistently inactive in the feeding deterrent bioassay. The activity in the choice bioassay may result from a higher concentration of feeding stimulants in the extract from uninfected ryegrass. In light of these bioassay results and since no chemical differences were detected between extracts from infected and uninfected plants, this fraction was not further examined.

Activity of 80% Ethanol Phase. Silica gel chromatography of the 80% ethanol phase yielded two active fractions (S15 and S17) (Figure 3). Fraction S15 appeared to have marginal activity and was not further investigated. Fraction S17, obtained from endophyte-infected ryegrass, was an active feeding deterrent (MFS = 0.83, $P < 0.001$), while the corresponding fraction from an uninfected plant was inactive (MFS = −0.07). Fraction S17 from the infected plants was further purified by silica gel, Sephadex LH-20 (80% ethanol) and CM-Sephadex ion-exchange chromatography. TLC revealed the presence of peramine, suggesting that the activity of the 80% ethanol phase may be due to the same components as occur in the *n*-butanol phase, indicating an incomplete initial chloroform–methanol–water partition.

Bioassay of Cultured Acremonium. Extracts of mycelium of *A. loliae* grown in culture did not affect weevil feeding in the feeding deterrent bioassay at concentrations corresponding to 0.7, 4.0, 7.0, and 28.0 mg hyphae per gram of agar. The MFS values were, respectively, 0.03, −0.10, 0.19, and −0.10.

DISCUSSION

The results obtained with the feeding choice bioassays show that ryegrass infected with *A. loliae* contains extractable substances which affect the feeding behavior of adult Argentine stem weevil. Activity is concentrated in the more polar phases and can be further resolved by chromatography. A comparison of chromatographic fractions from plants infected and uninfected with *A. loliae* revealed an active feeding deterrent in the *n*-butanol phase. This feeding deterrent, which we have named peramine, was only found in extracts from ryegrass

infected with *A. loliae*. It was not detected in extracts from uninfected plants or in liquid cultures of *A. loliae* (D.D. Rowan, unpublished results). Peramine, $C_{12}H_{17}N_5O$, has been isolated and shows UV adsorptions and chromogenic reactions typical of a substituted indole derivative. Peramine deterred feeding by adult Argentine stem weevil at 1.3 ppm.

Peramine is chemically different from the tremorgenic lolitrems also isolated from ryegrass infected with *A. loliae* and which are suspected of causing sporadic outbreaks of ryegrass staggers in livestock. The molecular formula of lolitrem A ($C_{42}H_{55}NO_8$) and the structures of lolitrems B ($C_{42}H_{55}NO_7$) and C ($C_{42}H_{57}NO_7$) have been published (Gallagher et al., 1981, 1984). Lolitrems are nonpolar, neutral compounds containing a highly substituted indole ring system and are structurally related to the known aflatrem, penitrem, and janthitrem mycotoxins (Gallagher et al., 1984). Their biological activity against insects has not yet been reported. Based on the solvent partitioning studies of Gallagher et al., (1977) and on the HPLC retention data for lolitrem B (Gallagher et al., 1985), we would expect lolitrems to elute in fractions S13 or S14 (Figure 3). Fractions S13 and S14 were not active in the stem weevil choice bioassay, nor could the characteristic mass fragments for lolitrems (*m/z* 348) (Gallagher et al., 1981) be detected in either of the active fractions, S15 and S17. We therefore suggest that the neurotoxic lolitrems, if present in our extracts, are not important as feeding deterrents.

The amount of endophyte in ryegrass varies between 5 and 200 mg/g, with typical concentrations ranging from 30 to 50 mg/g (Musgrave, 1984). Extracts from cultured *A. loliae* were not active against Argentine stem weevil at concentrations corresponding to 28 mg hyphae/g agar. However, as bioassays on ryegrass extracts show activity even at one tenth the natural concentration of plant material, any feeding deterrent activity in the cultured endophyte should be detectable at the levels used in these tests. Neither peramine nor the lolitrem neurotoxins (R.T. Gallagher, personal communication) have yet been detected in liquid cultures of *A. loliae*. This suggests that peramine, the lolitrems, and other stem weevil active substances are not constitutive in the fungal mycelium. However, they might still be produced by *A. loliae* when growing endophytically or under as yet undefined culture conditions. Alternatively, they may be plant metabolites produced in response to the endophyte.

As peramine and the other stem weevil active fractions appear to differ chemically from the lolitrems, it may be possible to produce an endophyte-infected ryegrass resistant to stem weevil but which does not produce ryegrass staggers in livestock. An understanding of the mechanisms whereby endophytic infection of ryegrass confers resistance to infected plants may also be relevant to research on the insect resistance of other grasses infected with endophyte. Finally, the stem weevil resistance of ryegrass infected with endophyte can be seen as another example of a plant resistance to a pest arising from the effects of a previous fungal challenge.

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MALE MOTH SENSITIVITY TO MULTICOMPONENT PHEROMONES: Critical Role of Female-Released Blend in Determining the Functional Role of Components and Active Space of the Pheromone

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Abstract—In the present study male redbanded leafroller (*Argyrotaenia velutinana*), cabbage looper (*Trichoplusia ni*), and Oriental fruit moths, (*Grapholita molesta*), were tested in a flight tunnel to (1) the major pheromone component, (2) the *Z/E* pheromone component mixtures for Oriental fruit moth and redbanded leafroller, (3) and the female-released blends, over a series of dosages. Experiments were designed to test the hypothesis that male response downwind of a female is initiated by the major component and that minor components function only to elicit behaviors close to the female during close-range approach and courtship. The results did not support this hypothesis, but rather showed that males initiated upwind flight in significantly higher percentages to the complete blends of components, at all dosages, compared to single components or partial blends. Addition of minor components also signficantly enhanced male perception of the major component at lower dosages, resulting in completed flights to dosages of the major component that alone did not elicit any upwind flight. Our results support the concept that minor components function to enhance male sensitivity to the pheromone, and the specificity of the signal. Our results also support the hypothesis that the active space of the pheromone is a function of the upper and lower concentration thresholds for the blend of components, and not simply for the major component.

Key Words—Sex pheromone, active space, *Trichopulsia ni*, Lepidoptera, Noctuidae, *Argyrotaenia velutinana*, *Grapholita molesta*, Tortricidae, multicomponent pheromones, sustained-flight tunnel.

INTRODUCTION

Recently, in our lab, the sex pheromone of the cabbage looper (CL), *Trichoplusia ni* (Hubner), and the redbanded leafroller (RBLR), *Argyrotaenia velutinana* (Walker), were reinvestigated (Bjostad et al., 1984a,b; Linn et al., 1984), as part of a series of studies concerned with sex pheromone biosynthesis (Roelofs and Brown, 1982; Bjostad and Roelofs, 1983; Roelofs and Bjostad, 1984). Several new compounds were identified for each species from sex pheromone gland extracts and airborne collections, as predicted from the proposed biosynthetic routes. Subsequent behavioral tests in a sustained-flight tunnel revealed two important points: the female-released blend was superior to the previously identified pheromones (Roelofs et al., 1975; Bjostad et al., 1980), and the blend enhanced all aspects of the male response, both quantitatively and qualitatively. The results of these tests indicated that previously characterized behavioral functions of the components were artifacts (Baker et al., 1976; Linn and Gaston, 1981a,b), the result of analysis based on observations of male behavior with partial or incorrectly identified blends. Our results were also in agreement with those reported previously for the Oriental fruit moth (OFM), *Grapholita molesta* (Busck) (Baker and Cardé, 1979).

The present work was initiated to expand on the results of the studies with OFM, CL, and RBLR. We tested male moths in the sustained-flight tunnel to a dosage series of the major (or most abundant) pheromone component, of the *Z/E* mixture for OFM and RBLR, and of the full blend for each species. Our objective was to determine if males are significantly more responsive to the female-released blend of components, and if they are more sensitive to the full blend compared to only the major component(s) at low dosages, simulating conditions downwind from a female. The results are discussed with respect to current ideas concerning male perception and the active space of the pheromone.

METHODS AND MATERIALS

Insects. RBLR and CL were reared on semisynthetic diet (Shorey and Hale, 1965), and OFM were reared on small green thinning apples, at 25°C, 16:8 light–dark photoperiod. The sexes were separated as pupae, and adult males were segregated daily by age and kept under conditions similar to those during rearing, in chambers separated from females.

Chemicals. For RBLR, the (*Z*)-11-tetradecenyl acetate (*Z*11–14:OAc) isomer (Farchan) was collected by GLC [2m × 2mm glass column packed with 10% XF-1150 (50% cyanoethyl methyl silicone) on 100–120 mesh chromosorb W-AW-DMCS], to ensure purity and was found to be > 99.9% pure. The *E*11–14:OAc isomer (Farchan) was added to the *Z* isomer to make a 92:8 *Z/E* mix,

as shown by capillary GLC (45-m Carbowax 20 M column). The ratio of components in the seven-component blend (Bjostad et al., 1984a) was as follows: 12:OAc (7.5), Z9-12:OAc (1.2), E9-12:OAc (2.5), 11-12:OAc (3.6), 14:OAc (4.6), Z11-14:OAc (100), E11-14:OAc (8.1). The ratio was checked by capillary GLC with all components $\pm 1\%$ of the desired blend.

For OFM the Z8-12:OAc isomer (Farchan) was purified by HPLC (Baker et al., 1981) and shown by capillary GLC to be $> 99.9\%$ pure. The mixture of Z8- and E8-12:OAc (6% E) was prepared and checked by capillary GLC, as was the three-component blend (6% E in Z8-12:OAc with 10% Z8-12:OH added, Cardé et al., 1979; Linn and Roelofs, 1983).

For CL the Z7-12:OAc isomer (Farchan) was shown by capillary GLC to be $> 98\%$ pure with no detectable E7-12:OAc or Z7-12:OH present (Linn et al., 1984). The synthetic chemicals for the six-component mix were the same as in Linn et al. (1984). The proportions of each compound were as follows: 12:OAc (5.6), Z5-12:OAc (7.7), Z7-12:OAc (100), 11-12:OAc (1.9), Z7-14:OAc (0.8), and Z9-14:OAc (0.6). A solution of this blend was prepared in Skelly B and checked on capillary GLC.

Chemical Sources. For RBLR and OFM, the single components Z11-14:OAc and Z8-12:OAc, the respective Z/E mixes, and the seven- and three-component blends were prepared in Skelly B (predominantly *n*-hexanes) and applied in 100-μl amounts to rubber septa (red, 5 × 9 mm, A.H. Thomas Co., Philadelphia, Pennsylvania) to achieve dosages of 3, 10, 30, 100, and 300 μg/septum for RBLR, and 0.001, 0.01, 0.1, 1, and 10 μg/septum for OFM.

For the CL, Z7-12:OAc and the six-component solution in Skelly B were applied to polyethylene caps (OS-6 closures, American Scientific Products, McGaw Park, Illinois) from a 1, 10, or 100 μg/μl solution to achieve dosages of 0.1, 0.3, 1, 3, and 10 mg/cap.

Test Procedures. Moth behavior was observed in the flight tunnel of Miller and Roelofs (1978). Each species was tested independently, in the order CL, OFM, RBLR. For RBLR and OFM, males were placed in the room housing the tunnel 1 h prior to testing (2 h prior to the initiation of scotophase), to acclimate to photophase temperature and light intensity: 21–22°C, 350 lux. Male CL were placed in the tunnel room at the initiation of scotophase, 4 h prior to testing (the fifth and sixth hours of the 8-h scotophase). Flight tunnel temperature and light intensity were 25°C, 0.3 lux. Relative humidity was 50–70% and wind speed was 50 cm/sec for all three species.

The procedures and apparatus for handling and testing males were as previously described (Linn and Roelofs, 1983; Linn et al., 1984). Males were scored for three key behaviors in the flight sequence: taking flight (TF), initiation of upwind flight (UP), and source contact (SC). During each 2-h test period, three treatments were tested, with ten males tested with each treatment. Treatments were always tested in the order of increasing blend complexity. Analysis was made of the number of males exhibiting each behavior in the

sequence, based on the total tested to each treatment ($N = 70$ for CL, 50 for OFM, and 50 for RBLR). Statistical comparison of treatments was based on χ^2 2×2 test of independence with Yates' correction (Sokal and Rohlf, 1969), or the method of adjusted significance levels for proportions (Ryan, 1960, $P < 0.05$).

RESULTS

For all three species, at all dosages, males took flight (TF, Figure 1) in significantly higher percentages to the complete blend of components when compared to single components or the *Z/E* mix (with the exception of the highest dosage tested for RBLR and CL, Figure 1). However, the effect of the blend on male behavior was even more dramatic at the initiation of upwind flight (UP, Figure 1) phase of the response, with significantly higher percentages of males responding to the complete blends at all dosages tested. Male sensitivity, evidenced by the lowest dosage at which complete flights to the source occurred (SC, Figure 1), was also greater, by at least an order of magnitude, to the complete blends. Thus, addition of minor components at all dosages, and particularly at the lower dosages tested, significantly enhanced male sensitivity and recognition of the pheromone signal.

The enhancement of male response brought about by the addition of minor components, while in evidence for each species, varied considerably among species. For RBLR, males exhibited very little behavior with the Z11–14 : OAc isomer alone. The lower levels of activity to 3, 10, 30, and 100 μg were not significantly different from spontaneous levels of activation, and so it is questionable whether or not the observed activations were pheromone induced. Response levels to the *Z/E* mix were significantly enhanced over that with Z11–14 : OAc alone, with some males completing the flight at two dosages (30 and 100 μg) at which males did not initiate upwind flight to Z11–14 : OAc alone. With the full seven-component blend, male response was not only increased significantly with the 30-, 100-, and 300-μg dosages, but males also initiated upwind flight and reached the source with the 3- and 10-μg dosages.

With OFM, the taking flight response to Z8–12 : OAc alone was greater at the higher dosages when compared with RBLR, but addition of the *E* isomer did not significantly enhance this initial behavior. A low percentage of males initiated upwind flight to the *Z/E* mix, but the three-component blend was essential for complete flights at all dosages tested. Similar to the RBLR results, the full blend at the 0.001- and 0.01-μg dosages resulted in complete flights to the source, whereas these dosages did not elicit any upwind flight behavior with the *Z/E* mix.

For the CL, males took flight in higher percentages to all dosages tested, compared to OFM and RBLR. For male CL, blend enhancement of activity

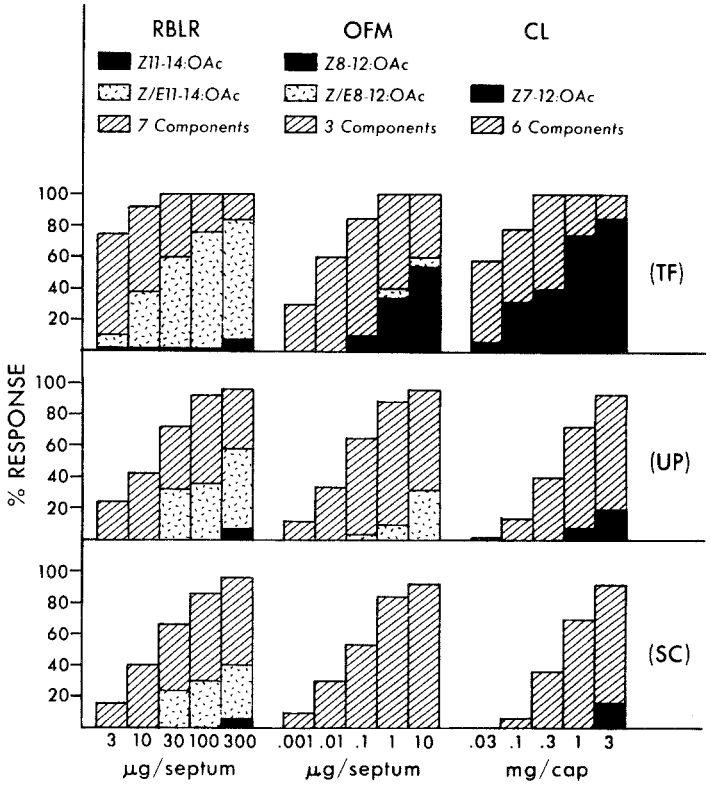

FIG. 1. Percentage of male RBLR, OFM, and CL exhibiting three key behaviors in the sustained flight tunnel (taking flight, TF; upwind flight over a 1.5-m distance, UP; and source contact, SC). Responses for each species are to the major component alone (solid area), the *Z/E* mixture (stippled area), and the complete blends (hatched area). Dosages represent the amount of the major component applied to the source, with the other components added to achieve the correct proportions (see Methods and Materials). $N = 70$ for CL, 50 for RBLR, and 50 for OFM to each blend–dosage combination.

was most evident in the upwind flight phase of the sequence. The lowest dosage of the main component to elicit upwind flight activity was 1 mg, whereas the lowest full blend dosage for this activity was 0.03 mg.

DISCUSSION

The results of the present study support the hypothesis that male moths are more sensitive to the female-released blend of components over a dynamic range

of release rates compared to the major component alone. In each case, the addition of the full complement of minor components not only enhanced male response over that observed to the single major component, but also resulted in completed flights to dosages of the major component that, when presented alone, did not elicit upwind flight activity. We conclude from this that the observations made with OFM by Baker and Cardé (1979) concerning the role of the blend do not constitute a specialized case, but rather that blend enhancement of male response represents an important general principle.

Pheromone Components and Behavioral Functions. The success of the present study and the previous reinvestigations was critically dependent on the fact that observations were made using the female-released blend of components (Baker et al., 1980; Bjostad et al., 1984b), rather than a partial blend (for CL, Linn and Gaston 1981a,b), or exaggerated ratios of components derived from field-screening trials (for RBLR, Baker et al., 1976; for OFM, Cardé et al., 1975a,b). It is clear from earlier results obtained with RBLR, OFM, and CL that certain minor components, when presented in exaggerated ratios, or when added singly to the major component, can significantly enhance trap catch and also give the appearance of controlling the expression of a specific aspect of the flight response. With RBLR, for example, field trials showed that addition of 12:OAc to the previously identified mixture of *Z*- and *E*11–14:OAc (8% *E*) (Roelofs and Arn, 1968; Klun et al., 1973) in a ratio of 1–1.5 times the level of *Z* isomer significantly increased trap catch (Roelofs and Comeau, 1968). Chemical analysis confirmed that 12:OAc was present in female glands, but at a ratio of only 3–5% of the *Z* isomer (Roelofs et al., 1975). At this ratio, however, 12:OAc did not enhance male activity in the field, and it was concluded on the basis of field trapping studies and crude airborne collections that females must be rapidly synthesizing and releasing larger amounts of 12:OAc.

Further observations of male RBLR flight behavior in the lab and the field (using only three of the recently identified seven components), showed that the *Z/E* isomer mix was responsible for upwind anemotactic flight and that 12:OAc significantly enhanced close-range approach, landing, and attempted copulations (Baker et al., 1976). Our most recent evidence indicates, however, that this is incorrect and that when 12:OAc is presented at the appropriate female-released ratio (3–5% of the *Z*11–14:OAc isomer), it does not enhance the response to the *Z/E* mix (Bjostad et al., 1984b). Rather, 12:OAc is one of seven pheromone components (12:OAc, *Z*9–12:OAc, *E*9–12:OAc, 11–12:OAc, 14:OAc, *Z*11–14:OAc, and *E*11–14:OAc) that act as a unit to enhance male sensitivity and flight response.

Similarly with OFM, 12:OH significantly enhanced trap catch when in a 3:1 ratio with the major component *Z*8–12:OAc (6% *E*) (Roelofs et al., 1969, 1973). As with RBLR, field and laboratory studies suggested that the *Z/E* mix was responsible for upwind anemotactic flight and that the relatively high ratio of 12:OH enhanced close-range behavior, including the dramatic hairpencil

courtship display (Cardé et al., 1975a,b). At this time the important blend constituent, Z8–12:OH, was not included in the tests. Subsequent studies showed, however, that Z8–12:OH is an important pheromone component, and that 12:OH, when presented at the appropriate ratio (<10% of the Z isomer and an equal or lower amount compared to Z8–12:OH), exhibited no discernable effect on male behavior. Further studies were then undertaken to show how variations in the ratio of components might affect male behavior (Linn and Roelofs, 1983). The results showed that off ratios did not enhance male response over that observed to the natural blend of components and that, over a wide range of *Z/E* mixtures, males were not able to compensate for the lack of the Z8–12:OH component and exhibit a complete flight to the source.

The CL provides a third, and somewhat different, example from that for RBLR and OFM. Characterization of the functional role of the minor component in this species was not the result of observations using an exaggerated ratio of components, rather they were the result of using a partially identified blend (Bjostad et al., 1980). The major component, Z7–12:OAc, was identified by Berger (1966). Subsequently Bjostad et al., (1980) identified 12:OAc from female gland and airborne collections, and it was shown that females released a 97:3 ratio of Z7–12:OAc to 12:OAc. The initial hypothesis was that 12:OAc was a short-range component, a hypothesis proposed directly from the results reported with RBLR (Baker et al., 1976), and OFM (Cardé et al., 1975a,b). The results of flight tunnel tests confirmed that 12:OAc did not appear to affect upwind flight, rather it enhanced close-range approach and contact with the source, when combined with Z7–12:OAc. It was concluded that the two-component system fit the existing paradigm concerning pheromone components and their functions (Linn and Gaston, 1981a,b).

As with RBLR and OFM, however, our most recent studies indicate that the previously observed effect with 12:OAc represents a response that can be exhibited by a low percentage of the male population to a partial pheromone blend (Linn et al., 1984). The male response to the six-component blend (Z7–12:OAc, 12:OAc, Z5–12:OAc, 11–12:OAc, Z7–14:OAc, and Z9–14:OAc) was in all respects superior to that observed with the two-component mix. These studies also involved subtraction tests with five-, four-, three-, and two-component blends containing Z7–12:OAc. The major component was necessary for any behavior to occur, but it was the only compound that fit this criterion. The subtraction tests showed that all five-component and several four-component blends elicited peak levels of response similar to those with the six-component mix. This suggested that the individual minor components were not acting to initiate specific behaviors, as several substitutions were possible. Rather, the minor components acted as an ensemble to enhance male response to the Z7–12:OAc component and thus effect optimal flight behavior.

Our present view is that individual minor components do not function to trigger specific behaviors in the response sequence, but rather that the full blend

acts as a unit in the male flight response and courtship sequence. It is evident from the above examples that these types of observations may be indicative of a partial, incorrectly characterized, or inappropriate blend of the pheromone and should not be proposed as evidence for the functional importance of individual compounds (Bradshaw et al., 1983), without comparative data for male response to female or gland effluvium/extracts (Linn et al., 1984).

Blend Perception and Active Space of the Pheromone. Data presented in this paper support the hypothesis that blends of components, rather than one predominating component, are acting at long distances from the source and are responsible for initiating and maintaining anemotactic flight (Baker and Roelofs, 1981). This conclusion is based on one of the major results of the present study that the blend of components markedly enhanced male sensitivity (or lowered the threshold for response) to the pheromone. We would conclude, with Baker and Roelofs (1981), that the active space of the pheromone is determined by the appropriate blend of components, not just the major component(s) (Nakamura and Kawasaki, 1977; Nakamura, 1979), and that the dimensions of the active space will be determined by the upper and lower concentration thresholds for the blend (Roelofs, 1978, Elkinton and Cardé, 1984).

The importance of sex pheromones as specific mate recognition signals is one of the formative concepts in research on chemical communication (Cardé and Baker, 1984; Roelofs and Brown, 1982). With the widespread awareness that moth species utilize multicomponent blends (Roelofs, 1980), it has become clear that signal specificity is a function of the composition and ratio of components, and the release rate of the blend (Roelofs and Cardé, 1977; Roelofs, 1978; Baker et al., 1981; Linn and Roelofs, 1983). The importance of a unique blend and ratio of components for the male is that it reduces the time spent in locating a mate. If it is the case, as is often stated, that females are a limiting resource for males, then rapid detection of the chemical signal is of critical importance for the male. A single component or isomer mix could certainly serve this purpose, if it were not for the fact that closely related, cohabitating, species often utilize the same compounds (Roelofs and Brown, 1982; Cardé and Baker, 1984). With a unique blend and ratio of compounds, however, specificity is added to the signal, enhancing the ability of the males to recognize the pheromone of conspecific females, and thus locate a female before conspecific males do, and also to aid the male in discriminating among closely related signals. Rapid detection and recognition of the signal aid the male in locating a female, and also in not wasting time and energy in false trail following (Cardé and Baker, 1984). We would argue that this strongly supports the concept that the blend is the active element in controlling male behavior at all distances from the female.

We recognize that our conclusions concerning the active space of the pheromone are based in large part on flight tunnel observations, but we would propose that they can be tested in the field. Baker and Roelofs (1981) determined

the upper and lower thresholds for the active space of OFM males to the three component blend over a dosage series. We propose to test males in the field, using the procedure developed by Baker and Roelofs, to the single Z8–12 : OAc isomer, the *Z/E* mix, and the three-component blend, over the same dosage series. Our hypothesis would be that the active space for the blend, at any dosage, would be significantly greater than to the single component or partial blend.

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CONTENTS OF DUFOUR GLANDS OF WORKERS OF THREE SPECIES OF *Tetramorium* (Hymenoptera: Formicidae)

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Abstract—The Dufour glands of worker ants of *Tetramorium caespitum*, and the morphologically similar *T. impurum* are filled with similar, but species-specific mixtures of linear hydrocarbons and three homologous sesquiterpene compounds. Glands of workers of *T. caespitum* contain, on average, 70 ng of oil containing C_{13} to C_{17} linear hydrocarbons with *n*-pentadecane (64%) and a mixture of pentadecenes (14%) as major components. *T. impurum* glands are smaller and contain an average of 40 ng of the same mixture but with *n*-pentadecane (49%) and a sesquiterpenoid compound (19%) the major components. Two isomeric pentadecenes, (*Z*)-6-pentadecene and (*Z*)-7-pentadecene are present in both species. *T. semilaeve* workers contain on average only 30 ng of a simple mixture of hydrocarbons with pentadecane contributing more than 90% of the total.

Key Words—Dufour gland, ant, *Tetramorium*, pentadecane, isomeric pentadecenes, sesquiterpene homologues, Hymenoptera, Formicidae.

INTRODUCTION

The Dufour gland is a saclike structure, which, together with the poison gland, is attached to the sting in females and workers of all the Hymenoptera. Its primary function is unknown. Its contents have been examined in a number of ant species and in every case been shown to be a mixture of hydrocarbons, or

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hydrocarbon derivatives, such as long-chain acetates and ketones (Blum and Hermann, 1978; Attygalle and Morgan, 1984a).

We have investigated the contents of a number of myrmicine Dufour glands (cf. Attygalle et al., 1983, and references therein) and in each species we have found a moderately simple, species-specific mixture of hydrocarbons. We describe here the first investigations from the tribe Tetramoriini.

Tetramorium caespitum L. and *T. impurum* Foerster have attracted attention recently because morphologically, they are very similar, in fact almost indistinguishable, except by reference to the male genitalia (Dr. B. Poldi, private communication, quoted in Pasteels et al., 1981). Some work has been carried out on their mandibular gland contents. In addition to the 3-alkanols and 3-alkanones commonly found in myrmicine mandibular glands, 2-undecanone, 2-undecanol, and perillen have been claimed to be identified in the mandibular glands of *T. caespitum* (Longhurst et al., 1980). Pasteels et al., (1980, 1981) have identified very small quantities of chiral 4-methyl-3-hexanol and 4-methyl-3-hexanone in the heads of *T. impurum*.

Whereas *T. caespitum* and the hitherto little known *T. impurum* are found (one or the other) all over Belgium, *T. semilaeve* André comes from the quite different environment of the Mediterranean area. Several points emerged from our studies of these *Tetramorium* species. The two morphologically similar species, *T. caespitum* and *T. impurum* have very similar patterns of hydrocarbons. We have discovered here three new compounds of sesquiterpene type which differ by one or two CH_2 groups which differentiate the species. However, we find this pair of species are easily separated by another chemical test using trail pheromones. For the first time, a mixture of two isomeric alkenes, i.e., pentadecenes which differ only in the position of the double bond, have been found in mymircine Dufour glands. We have compared two techniques by which double-bond positions in such mixtures can be studied, namely methoxymercuration–demercuration and formation of dimethylthioethers. The di(methylthio)ether method is preferred in this work.

METHODS AND MATERIALS

Sources of Insect Materials. Colonies of *T. caespitum* were collected from heathland in Dorset and Surrey in England and various sites in Belgium; *T. impurum* from Belgium and northern France, and *T. semilaeve* from the island of Corsica.

The live colonies were maintained in the laboratory as described previously (Attygalle et al., 1983) in artificial nests formed from conical flasks (250 cm^3) partially filled with plaster of Paris (Wadhams, 1972). Nourishment was supplied in the form of larvae of *Tenebria molitor* and *Musca* and sugar solution.

Identification was made by A. Abbott and J.A. Pontin for the English

samples, by Prof. J.K.A. van Boven for *T. semilaeve*, and R. Cammaerts for *T. impurum*.

Gas Chromatography (GC). The ants were killed by momentary immersion in liquid nitrogen. The Dufour glands were then removed by dissecting the ants in water, under a binocular microscope (magnification ×15). After gently blotting dry, the individual glands were mounted on a small piece of glass and sealed in a section of soft glass capillary tubing (25 × 1.8 mm). This sample was then introduced into the modified injection port of a Pye 104 chromatograph incorporating the solid injector described by Morgan and Wadhams (1972a). Gas chromatography was carried out on a Pye 104 using: (A) 1.5 m × 4 mm ID packed column of 5% OV-101 on Diatomite M (AW-HMDS), with a GC oven temperature of 150°C; and (B) 2.75 m × 4 mm ID packed column of 10% PEGA on Chromosorb W, 100–120 mesh, at 130°C isothermal or at 155°C for 5 min, then programmed at 10°/min to 190°C and then isothermal, in order to look at the higher hydrocarbons.

A Carlo Erba 4160 gas chromatograph with split–splitless injection was employed to analyze hexane extracts of whole gasters on 50 m × 0.24 mm ID WCOT capillary (column C) coated with a mixture of diethylene glycol succinate and polyethylene glycol succinate (DEGS–PEGS). Hydrogen was the carrier at an inlet pressure of 1.2 kg/cm.

For a study of the sesquiterpenoid compounds, a polar capillary column (SP-2340, 30 m × 0.22 mm; column D) was used with helium at 1 ml/min.

Gas Chromatography–Mass Spectrometry (GC-MS). The identification of the major glandular components was confirmed by linked GC-MS, employing a Pye 104 gas chromatograph linked through a glass jet separator to an AEI MS12 mass spectrometer, with a 70-eV electron energy, 5-kV accelerating voltage and 15-kV multiplier voltage. Helium was used in all GC-MS analyses at a flow rate of 20 ml/min. The OV-101 column (A) was employed for its low bleed properties.

Mass spectra of the sesquiterpene compounds were obtained with a fused silica capillary column (CP-19, 38 m × 0.22 mm) linked directly to a Finnigan 3200 E quadrupole mass spectrometer with a 6000 Data System. The carrier gas was helium at 1 ml/min. Ten Dufour glands sealed in a glass capillary were injected by the same solid sampling method (Morgan and Wadhams, 1972a; Morgan et al. 1979a). The column was held at room temperature for 3.5 min and them programmed at 6°C/min to 260°C.

Hydrogenation. For microscale hydrogenation of glandular components, 1% palladium catalyst (Beroza and Sarmiento, 1966) was prepared by evaporating an aqueous solution (150 ml) of palladium chloride (25 mg) and sodium hydroxide (11.2 mg) in contact with 100–120 mesh Chromosorb W (1.5 g) in a rotary evaporator, followed by drying overnight at 150°C. This catalyst was then packed into a glass tube between two silanized glass wool plugs and activated by purging with hydrogen (40 ml/min) for 60 min at 200°C (Attygalle

and Morgan, 1982). The active catalyst was used to prepare a precolumn in the form of a plug of packing (6 cm) between two silanized glass wool plugs on column B. The individual Dufour glands were injected onto the column at 146°C as described previously but using hydrogen as the carrier gas at a flow rate of 40 ml/min.

Ozonolysis of Pentadecenes. The pentadecane and pentadecenes were trapped in a glass capillary (45 × 0.5 mm ID) cooled in liquid nitrogen, from the solid injection and GC separation of the Dufour gland volatiles of three *T. caespitum* workers, employing an all-glass splitter (Baker et al., 1976) attached to the end of column A. The trapping capillary was broken into three pieces and transfered to a solid sample vial (35 × 1.8 mm ID) cooled in ice. Ozonolysis of the trapped alkenes was performed by passing ozone (10 ml/min) from a microozone generator (Beroza and Bierl, 1967) over the capillary fragments using a finely drawn capillary (0.5 mm ID) which extended to the bottom of the vial; after 20 sec, the ozone purge was withdrawn and the vial immediately sealed. The ozonides were cleaved pyrolytically by placing the sealed vial in a solid sampler (Morgan and Wadhams, 1972a) and heating at 200°C for 5 min (Attygalle and Morgan, 1983a). Crushing the vial released the carbonyl products into the carrier gas for analysis on column B.

Methoxymercuration–Demercuration. Fifty workers' gasters were homogenized with *n*-hexane (4 × 50 μl) in a small tissue grinder (prepared from a 5-ml Pyrex test tube and a glass rod; abrasive surfaces on the tip of the rod and base of the test tube were produced by grinding the two together with a slurry of carborundum and water). Following decantation, drying by filtration through anhydrous sodium sulfate (0.1 g, held in a Pasteur pipet plugged with glass wool), evaporation (25 μl), and GC analysis, the extract was treated with methanol (100 μl) and mercuric acetate (1.0 mg, room temperature, 15 hr, dark). After this, sodium borohydride (1 mg) was added to reduce the mercuric complexes to the methyl ethers (RT, 1 hr). Isolation of the latter compounds and unreacted hydrocarbons was performed by partitioning the reaction mixtures between distilled water (1 ml) and *n*-hexane (0.5 ml). Decantation of the hexane followed by evaporation (25 μl) yielded a concentrate which was analyzed by GC and GC-MS on a column of OV-101 silicone.

Formation of Di(methylthio)ethers. The gasters of 25 workers were sealed in a glass tube and chromatographed using our solid injection technique (Morgan and Wadhams, 1972a) and the all-glass splitter (Baker et al., 1976). The pentadecene fraction was collected in a metal U-tube, then transferred to a Reacti-vial (Pierce and Warriner, Chester, U.K.) with hexane (2 × 10 μl). This material was combined with that from a further 25 workers.

Dimethyl disulfide (10 μl) was added to the hexane solution of pentadecenes, followed by a solution of iodine in ether (2 μl, 60 mg/ml). The mixture was left overnight, and aqueous sodium thiosulfate (5% w/w) was added with agitation until the mixture became colorless. The hexane layer was separated

and used for gas chromatography and GC-MS. For this work a 25-m silica capillary column, wall coated with OV-1, was used, temperature programmed from 50 to 280°C at 6°/min. The mass spectrometer was operated in the multiple ion detection mode, monitoring only *m/z* 117, 131, 145, 159, 173, 187, and 304.

RESULTS

T. caespitum. Analysis of single Dufour glands of *T. caespitum* workers revealed detectable amounts of up to 11 components (Figure 1). Components 1, 2, 3, 5, and 7 had retention times on polar (PEGA) and nonpolar (OV-101) chromatography phases corresponding to *n*-tridecane, *n*-tetradecane, *n*-pentadecane, *n*-hexadecane and *n*-heptadecane. Components 4, 6, 8, and 9 were found to correspond in retention times to pentadecene, hexadecene, heptadecene, and heptadecadiene, respectively. Peaks 10 and 11 were recognized as polar substances which did not fit into the hydrocarbon series. On-column mi-

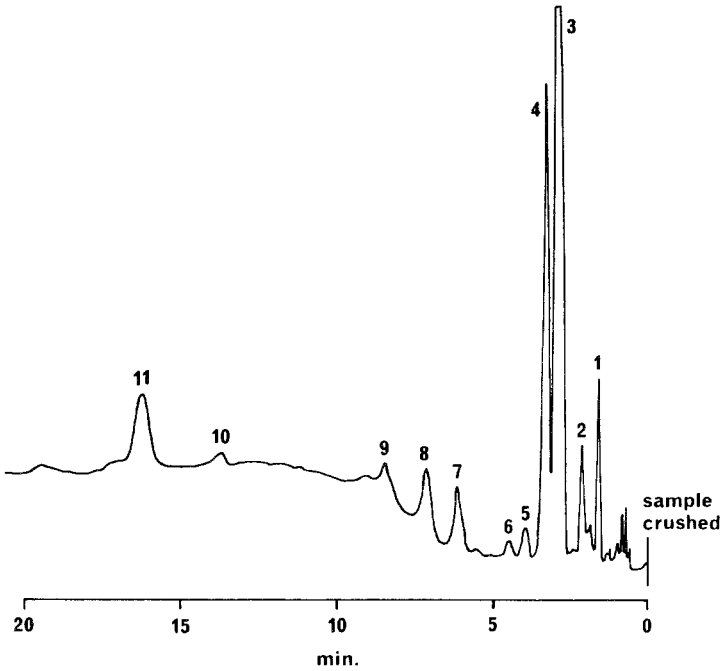

FIG. 1. Typical gas chromatogram from a Dufour gland of a single worker of *T. caespitum* on a 10% PEGA column at 155°C, isothermal for 5 min, then programmed at 10°C min to 190°C and then isothermally. The numbering of the peaks corresponds to the numbers in Table 1.

croscale hydrogenations altered the retention times of components 4, 6, 8, 9, 10, and 11, but did not affect components 1, 2, 3, and 5. Peak 4 disappeared and peak 3 increased in area correspondingly. Peaks 6–11 all were replaced by ones of shorter retention.

Satisfactory mass spectra were obtained for all components. Peaks 10 and 11 gave spectra recognized as belonging to open-chain sesquiterpenoid homologs. Peak 10 (M^+ 236) and Peak 11 (M^+ 250) had very similar spectra but from their behavior on a variety of stationary phases, it was evident they differed by one carbon atom.

To determine the position of unsaturation in the major alkene component, peak 4, material was trapped from several glands, ozonized, and rechromatographed, which revealed four ozonolysis products (hexanal, heptanal, octanal and nonanal), from which it can be deduced that the alkene consisted of 6-pentadecene and 7-pentadecene.

As confirmation of this identification, another sample of pentadecene collected from workers was converted to a mixture of methoxy derivatives via methoxy mercuration–demercuration (Abley et al., 1970; Blomquist et al., 1980), and these products were submitted to combined gas chromatography–mass spectrometry (GC-MS). The methoxy derivatives were not separated by GC, but the mass spectrum showed ions at the following *m/z* values (intensities in parentheses) 171(4), 157(22), 143(35), 129(38), 115(19), 111(8), 97(37), 83(45), 69(100), 55(65). No molecular ion (M^+, 242) was observed. The ions from *m/z* 171 to *m/z* 115 arise from the methoxy derivatives, those at *m/z* 115, 129, 157, and 171 from 6-pentadecene as indicated in Figure 2, while 7-pentadecene gives derivative ions at *m/z* 129, 143, and 157. The absence of other

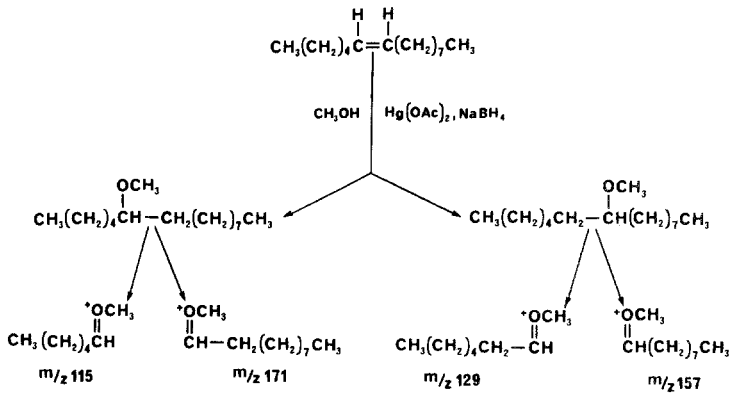

FIG. 2. Scheme showing the formation of two methoxyalkanes from 6-pentadecene by methoxymercuration–demercuration and the production of four intense and characteristic ions by cleavage of the ethers in the mass spectrometer.

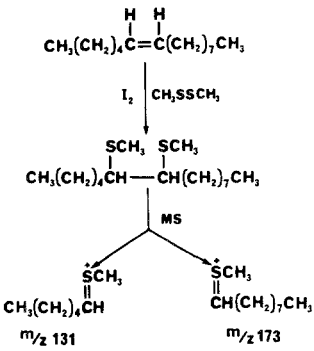

FIG. 3. Scheme showing the formation of the dithioether derivatives of 6-pentadecene and the characteristic ions found in its mass spectrum.

ions means that only these two pentadecenes were present in the original mixture.

Further confirmation was made using the technique of converting the alkenes into their di(methylthio)ethers (Francis and Veland, 1981) and submitting these to GC-MS (Figure 3). The mass spectrometer was operated in the multiple-ion detection mode for greater sensitivity and in order to be able to quantify the proportion of derivatives from the two alkenes. The spectrometer was set to scan ions of *m/z* 117, 131, 145, 159, 173, 187, and 304 (see Figure 4 for an example using *T. impurum*). Only those ions at *m/z* 131, 145, 159, and 173 were detected, so only 6- and 7-pentadecene were found and, from the peak intensities, in the ratio of 1.2 : 1 or approximately equal amounts.

Chromatography on a capillary column, wall-coated with a mixture of DEGS and PEGS, gave baseline resolution of the two pentadecene isomers collected from *T. caespitum* (Figure 5). Coinjection of a synthetic mixture of (*Z*)- and (*E*)-pentadecenes showed clearly that the natural compounds were entirely (*Z*)-pentadecenes; the (*E*)-isomers had retention times almost 2 min shorter. The ratio of (*Z*)-6-pentadecene to (*Z*)-7-pentadecene of 1 : 1.2 found here was different from that obtained from the dithioether experiment; the latter is considered to be more reliable. The quantifications and identifications are summarized in Table 1. Because the amount of material in the Dufour gland varies considerably from one individual worker to another, the percentage composition is calculated for each individual, and then the mean and standard derivation for the set are calculated for each component. It can be seen from Table 1 that the proportions of the major components do not vary much.

T. impurum. Chromatography of the Dufour glands of workers of *T. impurum* showed the same major components in approximately the same proportions as was found for *T. caespitum*. The major difference was that the second sesquiterpenoid with M^+ 250 was now more abundant than the mixture of pen-

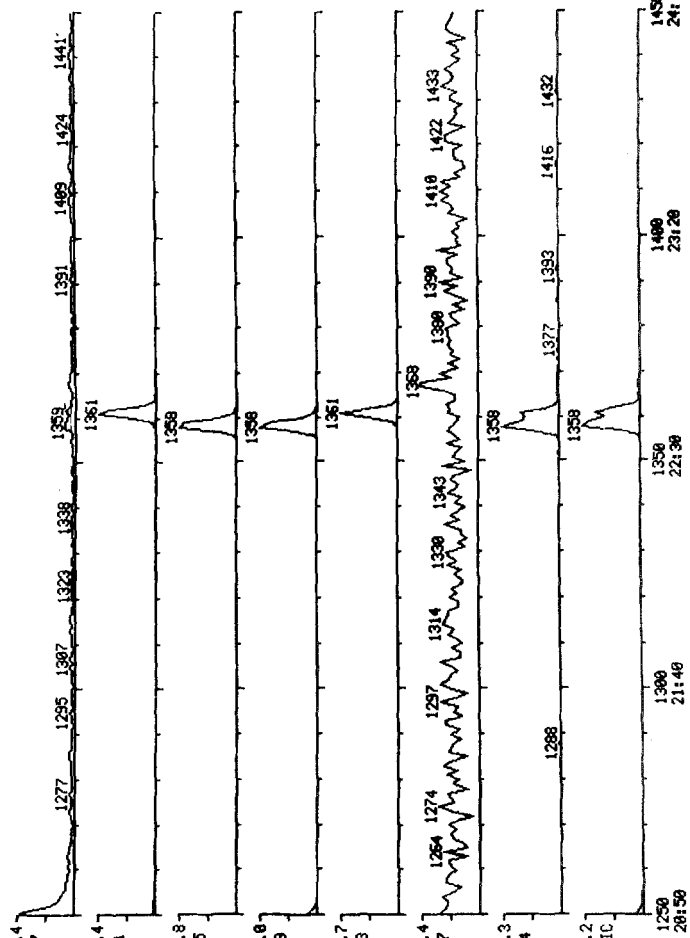

FIG. 4. Results of computerized GC-MS analysis employing multiple ion detection of the dimethyl-thioether derivatives of 6- and 7-pentadecenes from *T. impurum*. The result was similar with *T. caespitium*. The ions at *m/z* 117, 131, 145, 159, 173, 187, and 304 were monitored, their appearances in the mass chromatograms are shown from the top down. Essentially only background noise is seen for *m/z* 117 and 187 (which would arise from 5-pentadecene). The *m/z* 304 mass chromatogram corresponds to the molecular ion. The total ion chromatogram (bottom trace) and that for *m/z* 304 show that the thioether derivatives from 6- and 7-pentadecenes were partially resolved by the capillary column.

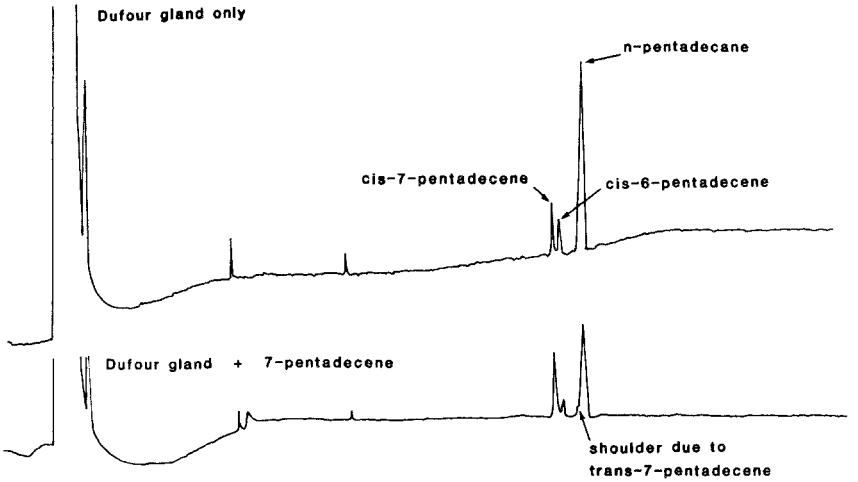

FIG. 5. Capillary gas chromatogram, on column C, of the Dufour gland contents of *T. caespitum* and the same cochromatographed with a mixture of synthetic *cis* or (Z)- and *trans* or (*E*)-7-pentadecenes showing the resolution of the geometric isomers and the absence of *trans* or *E* isomer in the gland.

TABLE 1. QUANTIFICATION OF SUBSTANCES IN DUFOUR GLAND OF *T. caespitum* WORKERS[a]

Peak	Identification[b]	Amount (ng)		Percent	
		M	SD	M	SD
1	tridecane	1.7	0.84	2.4	0.66
2	tetradecane	1.5	0.63	2.2	0.31
3	pentadecane	43.9	13.2	64.0	3.7
4	{(Z)-6-pentadecene, (Z)-7-pentadecene}[c]	9.5	3.2	13.7	1.3
5	hexadecane	t	—	t	—
6	hexadecene	t	—	t	—
7	heptadecane	2.7	1.7	3.7	1.6
8	heptadecene	1.5	1.5	1.9	1.6
9	heptadecadiene	1.0	0.79	1.3	1.1
10	sesquiterpenoid (M^+ 236)	1.2	0.48	1.8	0.93
11	sesquiterpenoid (M^+ 250)	5.8	1.5	8.9	2.6
Mean total			70.4 ± 20.9 ng		

[a] Expressed as the mean (M) with the standard deviation from the mean (SD) for 11 determinations, for both the absolute amount and the percentage of the total; *t* means an unquantifiable trace detected in some samples.

[b] Identification in each case by mass spectrometry, and retention time of authentic compounds for all except the unidentified sesquiterpenoids.

[c] 6-Pentadecene and 7-pentadecene present in a ratio of 1.2:1.

tadecenes, and a third sesquiterpenoid homolog (M^+ 264) was also present in variable amounts (Figure 6). As with *T. caespitum*, the pentadecenes were collected from a GC column and ozonized. The same mixture of *n*-aldehydes was obtained, indicating a mixture of 6- and 7-pentadecenes was present here. Another sample was converted to the dithioethers and submitted to GC-MS with multiple ion detection (Figure 4) at the same time as the sample from *T. caespitum*. The positions of the double bonds were confirmed and the ratio of 6-pentadecene to 7-pentadecene found to be close to 1 : 1 with slightly more 7-pentadecene. Capillary GC showed that both were exclusively the *Z* isomers. There is rather less material in the Dufour glands of *T. impurum*; the quantifications and identifications are summarized in Table 2.

T. semilaeve. The Dufour glands of workers of this species contained still

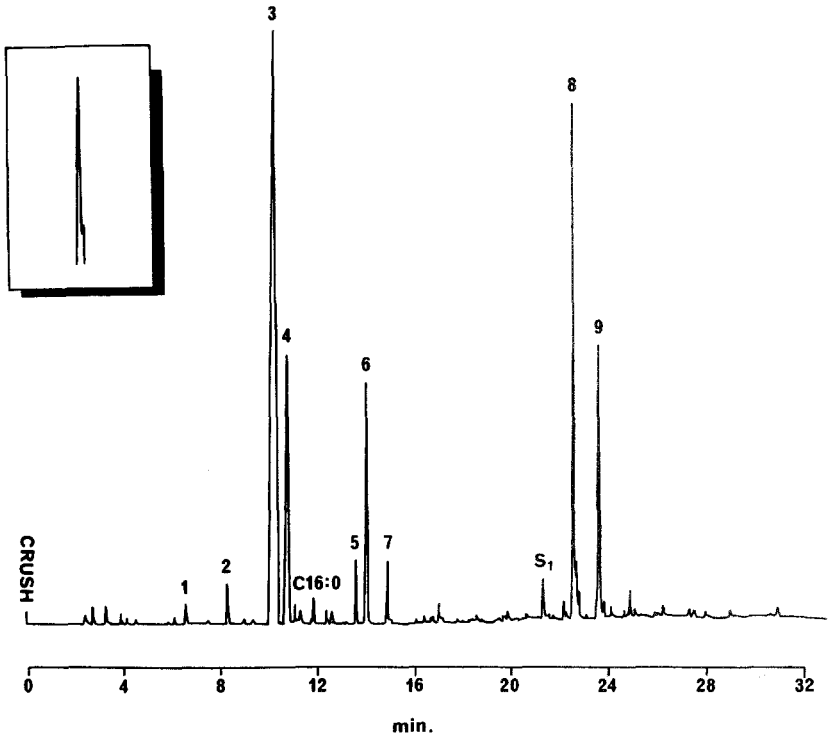

FIG. 6. Capillary gas chromatogram of a single Dufour gland from a worker of *T. impurum* on column D, 3.5 min at room temperature then programmed at 8°C/min to 200°C. Numbers on peaks correspond to those in Table 2. S_1 marks a small amount of the first sesquiterpenoid compound. The hexadecane peak on this sample is also visible. On this phase, the mixed pentadecenes are partially resolved, as shown in the inset using a much faster chart speed.

TABLE 2. QUANTIFICATION OF SUBSTANCES IN DUFOUR GLAND OF *T. impurum* WORKERS[a]

Peak	Identification[b]	Amount (ng) M	Amount (ng) SD	Percent M	Percent SD
1	tridecane	0.4	0.24	0.86	0.40
2	tetradecane	0.6	0.32	1.53	0.37
3	pentadecane	19.5	7.0	48.7	5.8
4	{(Z)-6-pentadecene / (Z)-7-pentadecene}[c]	5.3	2.6	13.0	2.6
5	heptadecane	t	—	—	—
6	heptadecene	3.0	1.2	7.5	1.4
7	heptadecadiene	0.4	—	0.86	—
8	sesquiterpenoid (M^+ 250)	7.1	3.7	18.6	5.2
9	sesquiterpenoid (M^+ 264)	2.8	1.5	9.1	2.7
Mean total		40.7 ± 16.0 ng			

[a] Expressed as the mean (M) with the standard deviation from the mean (SD) for 10 determinations; *t* indicates an unquantifiable amount sometimes detected.
[b] Identification in each case by mass spectrometry, and retention time of authentic compounds for all except the unidentified sesquiterpenoids.
[c] 6-Pentadecene and 7-pentadecene present in a ratio of 1:1.4.

less material, and that almost entirely one component, identified by retention time and cochromatography on different phases as *n*-pentadecane (component 3, Figure 7). Small amounts of three other components were identified by retention times as *n*-tridecane, *n*-tetradecane, and pentadecene. There was insufficient material to investigate whether the pentadecene consisted of positional isomers. The quantifications are shown in Table 3.

DISCUSSION

The Dufour glands of the three species of *Tetramorium* examined here, like those of other myrmicine species, contain characteristic mixtures of hydrocarbons (Attygalle et al., 1983, and references therein), but in addition, two of the species contain three homologs based on a polar sesquiterpene of unknown structure. *T. caespitum* has the largest gland, and *T. semilaeve* the smallest, but all are much smaller than those of *Myrmica rubra* and the seven other *Myrmica* species studied earlier (Attygalle et al., 1983).

The glands of workers of *T. caespitum* contain 70 ± 20 ng of oily hydrocarbons within the range C_{13} to C_{17}. *T. impurum* contains 40 ± 16 ng of a very similar mixture, and *T. semilaeve* is unique in that it has the simplest mixture we have yet encountered. Over 90% of the material in the gland is *n*-penta-

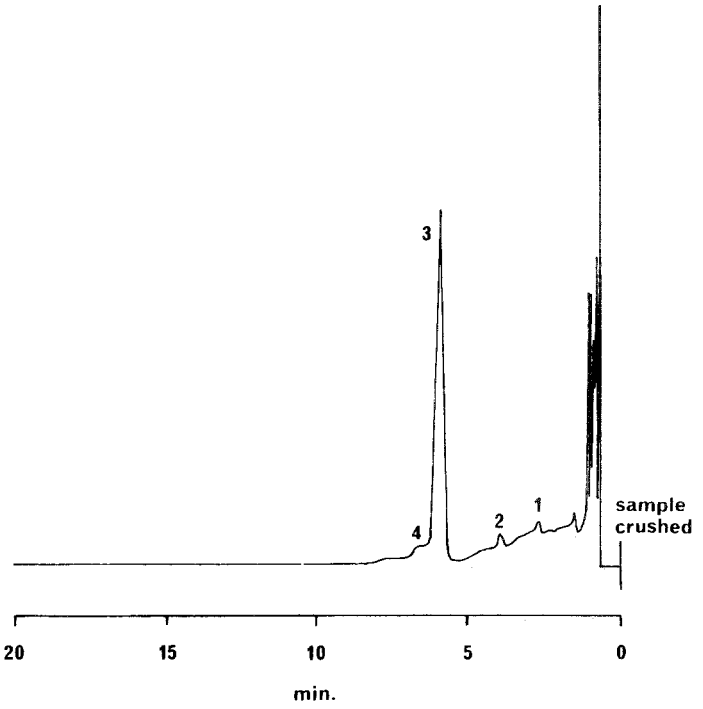

FIG. 7. Gas chromatogram of a single Dufour gland from a *T. semilaeve* worker on a 10% PEG column, isothermally at 142°C. Numbers on the peaks correspond to those in Table 3.

TABLE 3. Quantification of Substances in Dufour Gland of *T. semilaeve* Workers[a]

Peak	Identification[b]	Amount (ng) M		SD
1	*n*-tridecane	1.3		1.6
2	*n*-tetradecane	0.9		0.2
3	*n*-pentadecane	24.5		9.0
4	pentadecene	1.0		0.4
Mean total		27.7	±	11.2

[a]Expressed as mean (M) with standard deviation (SD) for 11 replicates. Percent composition has not been calculated since the amounts of the minor components are so small and variable.
[b]Identification is by retention times only on two columns of different polarity.

decane. The very small amounts of tridecane, tetradecane, and pentadecene observable in some samples are difficult to quantify in relation to the much larger pentadecane peak, making percentage composition rather inaccurate.

The absolute amounts of the different substances vary quite widely from one individual ant to another, as indicated by the large standard deviations (Tables 1–3), but the proportions, as expressed by percentage composition, are remarkably constant. We have examined samples of *T. caespitum* from Dorset, Surrey, and Belgium and *T. impurum* from various sites in Belgium. Although we have not quantified the mean from these various sites, the immediate appearance of the pattern of peaks on the gas chromatograph recorder was the same for the given species regardless of origin.

T. caespitum and *T. impurum* are morphologically very similar, and the first impression of their Dufour gland contents was that they were so alike as to be indistinguishable, but further examination by chromatography at higher temperatures showed they differed clearly in their content of components eluting later. Three peaks were recognized with retentions that behaved anomalously on different phases and did not correspond to hydrocarbons. Satisfactory mass spectra were obtained by capillary GC-MS, which showed that they formed a homologous series of three sesquiterpenoid compounds of molecular mass 236, 250, and 264. All three showed base peaks at *m/z* 69 and with very similar spectra, with the high-mass ions shifted by one CH_2 unit. They have not yet been identified and further work on them is in progress. The substance of mass 250 does not have a mass spectrum close to faranal (Ritter et al., 1977). The series is reminiscent of the three homologous farnesenes (C_{15} to C_{17}) first identified in *Myrmica rubra* (Morgan and Wadhams, 1972b) and later found in other *Myrmica* species (Attygalle et al., 1983) sometimes accompanied by a fourth (C_{18}) (Morgan et al., 1979b). The middle homolog of this new series (M^+ 250) was present in both *T. caespitum* and *T. impurum*. In the latter it was the second largest peak. The lowest homolog was present in quantity only in *T. caespitum*, although it could sometimes be detected in *T. impurum*. The highest homolog was found only in *T. impurum* (Figure 6). The amount and proportions of these sesquiterpenoid homologs vary much more from one sample to another than do the hydrocarbons.

Although pentadecane is the major component in the Dufour gland of all three species, each species has its specific composition, as was found in the eight species of *Myrmica* studied earlier. However, there is a still simpler chemical means of distinguishing these three: through their trail pheromone, present in the poison gland. The trail pheromone of *T. caespitum* consists of a mixture of alkylpyrazines (Attygalle and Morgan, 1983b, 1984b, and unpublished results); that of *T. impurum* is a substance of much higher molecular mass (Attygalle and Morgan, 1984b, and unpublished) and no species follows well the trails of the other two (R. Cammaerts, private communication). Neither do *T. caespitum* and *T. guineense* follow each other's trails (Blum and Ross, 1965).

Determination of Double-Bond Position. 7-Pentadecene has been found in the Dufour gland of a number of ant species, including *Myrmica rubra*, *Anoplolepis custodiens*, *Iridomyrmex humilis*, four species of *Formica*, and four of *Camponotus* (Blum and Hermann, 1978). However, we were surprised to find, on ozonolysis of the *Tetramorium* pentadecene, that it consisted of at least two isomers. We had not encountered alkene isomeric mixtures in our studies of myrmicine and attine ants, although Cavill and Houghton (1973) had reported a mixture of 7- and 8-heptadecenes as minor constituents of the Dufour gland of *Iridomyrmex humilis*, a dolichoderine ant, and the same group found a mixture of 6- and 7-pentadecenes and 7- and 8-heptadecenes in a formicine *Polyrhachis* species (Brophy et al., 1982). We therefore examined the pentadecene from *T. caespitum* and *T. impurum* carefully by ozonolysis, capillary gas chromatography (which separated the pentadecenes as well as separating *E* and *Z* isomers on a high-efficiency column), and by mass spectrometry of two types of derivatives. These two methods have not been compared before, so they are discussed here. The methoxymercuration–demercuration method (Abley et al., 1970) has been used many times for this purpose. The reaction mixture is a heterogeneous system and is difficult to handle once mercury has been displaced. It is not quantitative, yields being typically between 55 and 70%. In contrast, methylthiolation is a homogeneous reaction. All the components are miscible, much smaller quantities of alkene can be used, and the reaction is near quantitative.

By the methoxymercuration–demercuration method, each alkene produces two derivatives (Figure 2), and these are not usually separated by GC; hence a mixed mass spectrum is obtained, and each alkene gives four characteristic peaks. Methylthiolation gives only one product with two intense ions (Figure 3); the spectrum is therefore simpler to interpret. Further, the methoxy derivatives rarely show a molecular ion, so the identification of the derivative is less certain. The dimethyldisulfide derivatives of all the alkenes examined clearly showed a prominent molecular ion (Figure 4). Those of alkenyl acetates showed an M − 60 peak as highest mass.

Finally, the methoxy-derivative method does not distinguish between *E* and *Z* isomers of alkenes whereas the methylthioether method gives stereospecific *trans* addition; the *E* and *Z* isomers yield erythro and threo isomers, respectively, which may give almost identical mass spectra, but they are easily resolved on capillary GC, with the threo isomer eluting first.

The only disadvantage of the methylthiolation method that has emerged is that it does not work well for dienes, and the mass spectra of the derivatives obtained were difficult to interpret. Methoxy derivatives have been used for dienes successfully.

Working with nanogram quantities of Dufour gland alkenes, it was found that dimethylthioethers have several advantages over the mixed methyl ethers produced by the methoxymercuration–demercuration procedure. The absence of 5-pentadecene as more than 0.2% of the total pentadecenes could be dem-

onstrated (Figure 4). The ratio of the two isomers in the two samples showed a difference, with more 6-pentadecene in *T. caespitum* and more 7-pentadecene in *T. impurum*, but it is not known if this is constant for the species. The double bond has the *Z* geometry found for all such examples where it has been investigated.

The Dufour gland contents of eight species of *Myrmica* fall into one of two classes, either dominated by linear hydrocarbons or sesquiterpenes (Attygalle et al., 1983), with C_{17} the major linear hydrocarbons. Among the Attini, *Acromyrmex octospinosus* has homofarnesene (20–100 ng) as almost the only constituent (Evershed and Morgan, 1980), while *Atta cephalotes* and *A. sexdens* belong to the linear hydrocarbon group with C_{17} and C_{19} dominant in *A. cephalotes* and C_{19} and C_{23} [(Z)-9-tricosene] in *A. sexdens* (Evershed and Morgan, 1981). Now *Tetramorium* forms a new group within the myrmicines, with C_{15}-dominated linear hydrocarbons, and a new group of sesquiterpenoids.

On present chemical evidence, supported by behavioral experiments (Attygalle et al., 1983, and earlier references therein), each ant species produces its specific mixture of oily substances in the Dufour gland, which in the *Myrmica* genus, at least, is used as a home-range-marking pheromone (Cammaerts et al., 1977; Attygalle et al., 1983; Morgan, 1984).

It is interesting to note that pure linear hydrocarbons up to *n*-pentadecane (mp 10°C) are liquids at ambient temperature; *n*-hexadecane (mp 18°C) and higher saturated hydrocarbons are solids. Introducing a double bond into the longer carbon chains lowers the melting point considerably (octadecane, mp 28°C; 1-octadecene, mp 17.5°C); a double bond in the middle of the chain is much more efficient at lowering melting point (9-octadecene, mp −30°C), and *Z* double bonds lower melting point more than *E* double bonds. Ants use saturated hydrocarbons up to C_{15} and thereafter use unsaturated hydrocarbons with *Z* double bonds somewhere near the middle of the carbon chain. Some ants use methyl-branched hydrocarbons which have the same effect. Nature thereby ensures that the Dufour gland contents remain liquid.

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IDENTIFICATION OF A VOLATILE ATTRACTANT FOR *Diabrotica*[1] AND *Acalymma*[1] SPP. FROM BLOSSOMS OF *Cucurbita maxima* DUCHESNE

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Abstract—Fractionation of headspace volatiles from *Cucurbita maxima* blossoms by high-performance liquid chromatography resulted in the isolation of a single component which was highly active in an electroantennogram bioassay on *Diabrotica undecimpunctata howardi* antennae. This compound was identified as indole by gas chromatography–mass spectrometry. Field-trapping bioassays were conducted which indicated that indole is a potent attractant of the western corn rootworm, *D. virgifera virgifera*, and the striped cucumber beetle, *Acalymma vittatum*. The southern corn rootworm, *D. u. howardi*, did not respond, despite its strong EAG response. The sex ratio of *D. v. virgifera* found in indole-baited traps varied seasonally. Males were trapped in abundance in late July and later September, 1983, while females were more abundant August and early September. The effectiveness of indole as a *D. v. virgifera* attractant also varied seasonally. A prolonged period of depressed trap catches occurred in early August 1983, during the silking and tasseling period of the corn in the field where trapping was carried out.

Key Words—Attractant, *Diabrotica virgifera virgifera*, *Diabrotica undecimpunctata howardi*, *Acalymma vittatum*, indole, *Cucurbita maxima*, electroantennogram, floral volatile, Chrysomelidae, Cucurbitaceae, Coleoptera.

INTRODUCTION

The chrysomelid genera *Diabrotica* and *Acalymma* contain numerous pest species, including the western corn rootworm, *Diabrotica virgifera virgifera*

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LeConte; the southern corn rootworm, *D. undecimpunctata howardi* Barber; and the striped cucumber beetle, *Acalymma vittatum* (Fabr.). This group is known to show a close association with host plants of the family Cucurbitaceae, particularly with the genus *Cucurbita* (Metcalf, 1979). The adults are most commonly found in the blossoms of *Cucurbita* spp. where they feed on pollen (in staminate flowers) and nectar. Studies have indicated that these beetles show a definite preference for certain species of cultivated *Cucurbita* (Fronk and Slater, 1964; Bach, 1977; Andersen, 1964). In most instances, adults prefer the blossoms of *C. maxima* Duchesne cultivars over those of *C. pepo* L. and *C. moschata* Poir.

No investigations have been made concerning a chemical basis for beetle orientation to the blossoms or foliage of *Cucurbita* spp. However, the effect of plant chemistry of feeding behavior has been intensively studied. A group of tetracyclic triterpenes, the cucurbitacins, are characteristic of the family and serve as potent feeding stimulants for *Diabrotica* and *Acalymma* adults (Chambliss and Jones, 1966; Metcalf et al., 1980, 1982). These relatively large (mol wt $\simeq 500$) and heavily oxygenated compounds are essentially nonvolatile and serve no apparent function in orientation to plants (Branson and Guss, 1983). The purpose of this study was to investigate the role of plant volatiles in the orientation of *Diabrotica* and *Acalymma* adults to the blossoms of *Cucurbita maxima* cultivars.

METHODS AND MATERIALS

Collection of Volatile Material. Plants of two *C. maxima* cultivars, "Blue Hubbard" and "Pink Banana Jumbo" were grown in a field plot at the University of Illinois. Male blossoms were harvested between the hours of 8:00 AM and 10:00 AM and returned to the laboratory on ice. Volatiles were trapped on Porapak-Q (80–100 mesh) by enclosing 20–40 blossoms in a tubular glass chamber (84 × 11 cm diameter) which was closed at both ends with jointed glass adaptors. At one end of the chamber, a short column (2 cm ID) containing approximately 5.0 g Porapak-Q was attached, while at the other, a 35 × 2-cm column containing activated carbon was attached. A vacuum pump coupled to the Porapak-Q column pulled an airflow of 2.0 liters/min through the chamber. Groups of blossoms were aerated for approximately 4 hr. After collection, the Porapak-Q was removed from the column and extracted with anhydrous diethyl ether in a Soxhlet apparatus. The extracts were dried over anhydrous sodium sulfate and concentrated to a volume of 15 ml by vacuum rotary evaporation. Final concentration to 0.5–1.0 ml was performed under a gentle stream of nitrogen.

Analysis of Volatile Mixture. Gas–liquid chromatography (GLC) was carried out using a Varian 2700 series instrument equipped with a hydrogen flame

ionization detector. Columns were packed with 3% OV-17 on Gas Chrom-Q (80–100; 3.7 m × 2 mm ID glass) or 3% SE-30 on Chromosorb-W (100–120; 1.8 m × 2.0 mm ID glass). The carrier gas was nitrogen at 60 ml/min.

For gas chromatography–mass spectrometry (GC-MS), a Hewlett Packard model 5985 GC-MS system was employed. The gas chromatograph was equipped with a 30-m × 0.32-mm ID fused silica capillary column coated with DB-1 (J&W Scientific, Rancho Cordova, Ca.). Helium was used as the carrier gas and electron impact spectra were recorded at a source voltage of 70 eV.

Normal-phase high-pressure liquid chromatography (HPLC) was performed using a single Waters Associates (Milford, Ma.) M-45 pump coupled with a radial-compression-type silica gel column. For preparative separations, a mobile phase of 10% tetrahydrofuran (glass distilled) in hexane (spectrograde) was used. At a flow rate of 2.0 ml/min, 1.0-ml fractions were collected for 6.5 min (13 fractions). The fractions were concentrated for bioassay and analysis under a gentle stream of nitrogen.

Thin-layer chromatography (TLC) was carried out using 0.1-mm silica gel plates on polyethylene terephthalate backing (Eastman Chromatogram, Rochester, N.Y.). The plates contained fluorescent indicator (F254) and were developed in a solvent system of chloroform–benzene–ethanol (83:8.5:1).

Electroantennogram Bioassay. Electroantennogram (EAG) measurements were obtained using a system similar to that described by Visser (1979) with the exception that stimuli were delivered to the antenna in a discrete puff of air. Antennae of *D. u. howardi* females were excised with small scissors after removal of the distal segment. They were then placed between two 0.9% NaCl-filled capillary tubes which covered silver–silver chloride wires. Signals were amplified and recorded by conventional apparatus. Stimuli were delivered to the antenna in 1.0-sec pulses of air (16 ml) traveling through a 1.0-cm ID glass tube. The test stimuli were applied to the inner surfaces of aluminum foil cylinders which fit in the glass delivery tube. A continuous air stream was shunted through the tube for 1.0 sec as a result of opening a solenoid valve by means of a physiological stimulator.

Field Trapping Tests. Traps consisted of white 0.95 liter paper cylindrical containers which were inverted and stapled to the tops of wooden stakes. The external surfaces, except the tops of the traps, were coated with Tack Trap. Test compound was normally applied in 1.0 ml diethyl ether to one surface of a 4.0 × 28.0-cm strip of xerographic transparency. The strip was then wrapped around the center of the sticky surface and securely fastened. Control traps contained an untreated plastic strip. In late season experiments, 1-dr glass vials were used as release sources and were attached to the top of the trap. *D. v. virgifera* sex pheromone, 8-methyl-2-decanol propanoate (5 μg racemic; Guss et al., 1982), was applied in methylene chloride solution to red rubber septa. These were pierced by wires which were attached to the top of the trap. All

traps in comparisons of test volatiles and pheromone contained both a strip and a septum, and each was treated as described above or not treated as appropriate.

The traps were positioned just below canopy level in the center row of a 98 × 18-m plot of hybrid dent corn. In some experiments, each baited trap was paired with an unbaited control. The traps within a control–treatment pair were separated by 10 m and the pairs by 20 m. In other experiments, traps were treated individually. In these cases, all traps were separated by 10 m.

Trapping was also conducted in a small plot containing various *Cucurbita* spp. The traps were placed slightly above canopy level (1.0 m) next to plants of the zucchini cultivar of *C. pepo* or the hybrid *C. pepo* × *C. texana.*

In all field-trapping experiments, traps were baited between 5:30 and 7:00 PM and the insects were removed the following morning between 10:00 and 11:00 AM. Males were distinguished from females by the presence of the supraanal plate (Mendoza and Peters, 1968).

Release Rates of Trap Sources. Indole was applied to the plastic strips used as trap baits by evenly pipetting the desired dosage onto the strip in 1.0 ml of diethyl ether. Strips containing each dosage level were aerated seperately, immediately after preparation in a small glass chamber similar to that described above. The chamber was coupled to columns of activated carbon and Porapak-Q as described above, and each strip was aerated for a period of 2 hr. The Porapak-Q was then extracted with diethyl ether, and the extract was concentrated as described above. Quantifications were made by measurement of GLC peak areas using the 3.7-m OV-17 column run at an isothermal temperature of 160°C.

RESULTS AND DISCUSSION

Analysis and Bioassay of Floral Volatile Mixture. Initial GLC analysis indicated that the volatile mixture released by *C. maxima* "Blue Hubbard" male blossoms is quite complex. Over 40 components were resolved on 3% OV-17 (Figure 1). A fractionation scheme was devised using HPLC and employing the EAG technique for bioassay. Analysis of the fractions by GLC revealed that the entire mixture seen in Figure 1 was eluted within 6.5 min.

Figure 2 displays the EAG responses to the fractions expressed as the ratio of the microvolt response value for each treatment fraction divided by the response to a control air puff. Two peaks of activity are apparent. The first, and most active, was eluted between 2.0 and 2.5 min (fraction 5) and the second peak between 4.5 and 5.0 min (fraction 10).

GLC analysis (OV-17) of the large early peak of activity (fraction 5) indicated that only one sample component was present. A larger quantity of this component was obtained by preparative HPLC of a head space volatile sample collected from the male blossoms of the "Pink Banana Jumbo" cultivar of *C.*

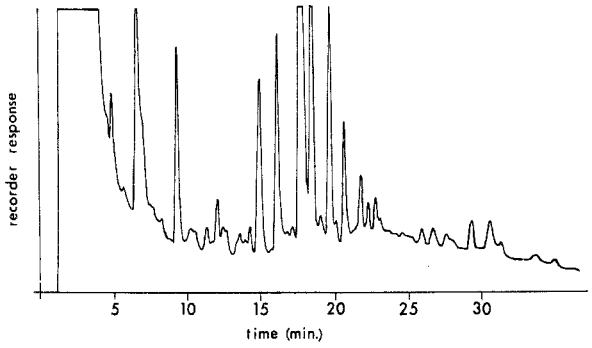

FIG. 1. Gas chromatogram of *C. maxima* cv. "Blue Hubbard" floral volatiles. Column: 3% OV-17 on Gas Chrom Q. Temperature program: 110°C for 5 min, then 4°C/min to 180°C, with a 15-min hold at 180°C.

maxima. Gas chromatography on a capillary column (DB-1) leading to the MS source verified that only a single compound was present. A mass spectrum was obtained which was consistent with the structure of indole, showing a strong molecular ion at *m/e* 117 and major fragment ions at *m/e* 90 and *m/e* 89.

The isolated compound was found to cochromatograph with authentic indole (Aldrich 99% pure) on both the 3% OV-17 (160°C isothermal) and 3% SE-30 (70°C isothermal) columns. Comparison by silica gel thin-layer chromatography resulted in identical R_f values (0.51) for both the active compound and indole as determined by quenching of fluorescence under shortwave UV light. Also, both reacted with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde) to form a pink spot on the developed plate. Ehrlich's reagent is commonly used for the detection of indoles (Durkee and Sirois, 1964) and was found in this case to have a limit of detection of 0.1 μg. The presence of indole in the "Blue Hubbard" floral volatile mixture used for fractionation and bioassay was confirmed by GC-MS and TLC of the unfractionated mixture.

Components responsible for the second peak of activity were not detected by GLC. This activity is most likely due to a single or group of low-boiling polar compounds whose presence was masked by the large solvent peak.

Field Bioassay of Indole. Indole was tested using sticky traps in a corn field containing populations of both *D. v. virgifera* and *D. u. howardi*. *D. v. virgifera* were attracted to baited traps in a dosage-dependent manner (Table 1), while *D. u. howardi* were not attracted to any dosage, despite the strong responses seen in the EAG assay. When traps were observed during the morning hours, *D. v. virgifera* could be seen approaching the source from the downward direction at trap height. Individuals apparently orienting to the bait were seen as far as 10 m downwind from the trap. After reaching the immediate vicinity of the trap, individuals would land directly on the downwind surface or, in some cases, circle the trap and land on the upwind side.

FIG. 2. Electroantennogram responses of female *D. undecimpunctata howardi* to HPLC fractions of *C. maxima* cv. "Blue Hubbard" headspace volatiles. The t/c ratio indicates the response to a treatment fraction (in microvolts) divided by the response to a control air puff.

TABLE 1. MEAN CATCHES OF *Diabrotica virgifera virgifera* AND *Diabrotica undecimpunctata howardi* ADULTS IN TRAPS BAITED WITH VARIOUS AMOUNTS OF INDOLE IN CORN FIELDS, AUGUST 1983 (SIX REPLICATES AT EACH DOSAGE)

	Mean No. beetles/trap ± SE[a]			
	D. v. virgifera		*D. u. howardi*	
Indole (mg)	Male	Female	Male	Female
0	0.0	0.0 a	1.8 ± 0.5	0.2 ± 0.2
1	0.5 ± 0.3	2.7 ± 1.4 b	1.8 ± 0.3	0.0
5	1.5 ± 0.7	8.3 ± 2.9 c	2.2 ± 0.9	0.0
10	2.3 ± 0.8	14.8 ± 6.3 c	1.2 ± 0.3	0.2 ± 0.2

[a]Means followed by a different letter indicate that the total trap catches (male + female) are significantly different at the 5% level as indicated by ANOVA and Student-Newman-Keuls (SNK) multiple-comparison test.

The measured release rates of the plastic sources were quite high during the first 2 hr after application of indole (Figure 3). Andersen (1984) has reported indole release rates of ca. 367 ng/hr for cut blossoms of the "Pink Banana Jumbo" cultivar and ca. 117 ng/hr for "Blue Hubbard." This low release rate (relative to that of the traps) suggests that either floral indole is effective only at short range, or that behavioral cues other than indole contribute to the large numbers of insects found in these blossoms. This is especially true of *D. u. howardi* which shows the same strong preference for *C. maxima* blossoms that *D. v. virgifera* and *A. vittatum* demonstrate, but does not respond to indole as a trap bait. It is noteworthy, however, that the behavioral observations discussed above were made on the morning following baiting. At this time, the release rate of the 1.0 mg trap would be greatly reduced, since approximately 30% of the applied indole is volatilized in the first 2 hr after application (Figure 3). It is possible though, that the complete volatile mixture emitted by *C. maxima* blossoms would be more effective than any single component, including indole. Work is currently in progress to determine the importance of additional floral volatile components in beetle flower selection behavior.

As indicated in Table 1, *D. v. virgifera* catches during this period (August 29–September 2, 1983) consisted almost exclusively of females. In reality, the trap sex ratios were found to vary seasonally. Early in the *D. v. virgifera* emergence season (July 18–20) males and females were caught in nearly equal numbers (Table 2). This may possibly be explained by the phenology of the species and by beetle movement patterns. Males of *D. v. virgifera* are protandrous (Cates, 1969), and the early trapping dates correspond roughly to the period of male predominance in the field (Cates, 1969). The period of female predomi-

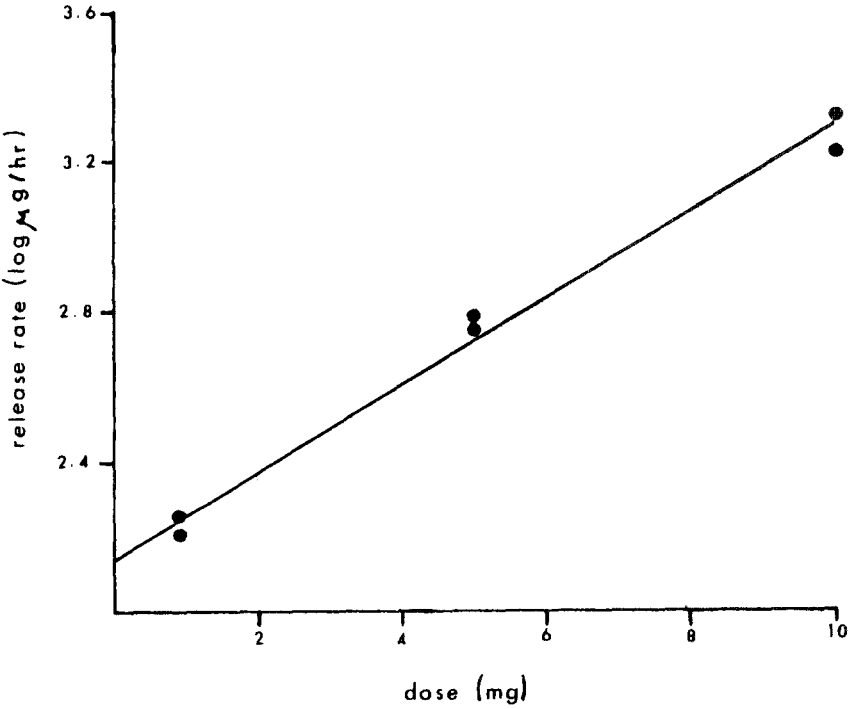

FIG. 3. Indole release rates of plastic strip substrates the first 2 hr after preparation.

TABLE 2. NUMBERS OF *Diabrotica virgifera virgifera* MALES AND FEMALES CAUGHT IN INDOLE-BAITED AND UNBAITED STICKY TRAPS DURING THREE TRAPPING PERIODS

	Indole trap		Control trap	
Trapping period	Male	Female	Male	Female
July 18–20[a]	34	30	11	4
August 29–September 2[b]	24	155	0	0
September 27–29[c]	125	118	9	2

[a] 5 mg indole applied to plastic strips.
[b] 1–10 mg indole applied to plastic strips.
[c] 100 mg indole released from a glass vial.

nance in sticky traps likewise corresponded roughly to the peak of female abundance in the field (Cates, 1969). Also, it has been recently reported that catches in unbaited sticky traps at ground level are biased toward males (Hein and Tollefson, 1984), suggesting a stratification of male and female flight patterns in

the field. If females do fly at higher levels in the field than males, the expected trap sex ratios at canopy level would be skewed in the direction of females. The high numbers of males on July trapping dates would, as mentioned above, be due to male predominance during this period.

The presence of males in the test field during the period of low female response was determined by sex pheromone trapping. In September 1983 (directly following the experiments described in Table 1), *D. v. virgifera* field responses to indole and 8-methyl-2-decanol propanoate, the sex pheromone of the species (Guss et al., 1982), were compared. As expected, almost exclusively males responded to pheromone, while nearly all females were found in indole-baited traps (Table 3). This suggests that the lack of male response is not due to an absence of males in the test field.

In late September 1983, the numbers of males caught in baited traps increased, resulting in trap sex ratios near unity (Table 2). These data were obtained using large dosages of indole (100 mg) released from glass vials instead of the plastic strips normally baited with 1–10 mg. The high release rates of these sources may have been the cause for the increase in male response. However, these experiments were performed after the first seasonal frost, and the corn plants had dried almost completely. The paucity of available food during this period may have resulted in enhanced male response to indole-baited traps. To positively determine the extent of sexual differences in response, comparisons of trap sex ratios will have to be made with those obtained by an independent field sampling method.

In addition to variations in trap sex ratio, the effectiveness of indole as a *D. v. virgifera* lure varied seasonally. Trapping experiments pairing a 5-mg indole-baited trap with an unbaited control trap were performed from July 18 to August 15, 1983. On the first two days of trapping, the mean catches were high, but also quite variable (Figure 4). Between July 20 and August 10, how-

TABLE 3. MEAN TRAP CATCHES OF *Diabrotica virgifera virgifera* WITH 10 mg INDOLE, 5 μg SEX PHEROMONE (8-methyl-2-decyl propanoate) OR THE TWO IN COMBINATION (EIGHT REPLICATES OF EACH TREATMENT)

Bait	Mean No. beetles/trap + SE[a]		
	Males	Females	Total
Control	0.0 . a	0.2 ± 0.2 a	0.2 ± 0.2 a
Indole	0.4 ± 0.3 a	15.7 ± 6.1 b	16.1 ± 6.3 b
Pheromone	11.9 ± 2.4 b	0.1 ± 0.1 a	12.0 ± 2.4 b
Indole + pheromone	10.2 ± 3.6 b	9.6 ± 2.7 b	19.9 ± 6.0 b

[a] Means within a column followed by different letters are significantly different at the 5% level as indicated by ANOVA and SNK comparisons of means.

ever, indole trap catches were consistently low and were not significantly different than control catches (Figure 4). On August 1 and 2, the traps were moved to a second corn field which contained an extremely high *D. v. virgifera* population. Over 50 individuals were counted on some corn plants. In this field on August 2, the indole dosage was increased to 20 mg/trap. Despite these manipulations, the trap catches remained low. After August 10, the catches again increased and continued at high levels for the remainder of the trapping study (Figure 4).

The period of minimum *D. v. virgifera* trap catch corresponded to the time of both the normal maximum emergence in Illinois (Musick et al., 1980) and the silking-tasseling period of the corn. Adults were observed to be feeding on pollen in the vicinity of the tassel and in lesser numbers on the silks. It is possible that during this period of food abundance, *D. v. virgifera* adults show a reduced sensitivity to indole, a food-derived attractant. The response to indole may be related to the degree of satiation of the insect. Replete individuals may perceive the lure but be less apt to make oriented movements toward its source.

Trapping was carried out in a cucurbit field which contained large numbers of *D. v. virgifera* and *D. u. howardi* adults. Also present was a third beetle species, *Acalymma vittatum.* Although related to members of the *Diabrotica*, *A. vittatum* is a cucurbit specialist in the larval stage, and is not normally found in corn fields (Houser and Balduf, 1925). This species was significantly attracted to indole-baited traps (Table 4, paired *t*-test, $P < 0.01$), and the sex ratios (male/female) over the final four days of trapping (August 5–8) were 1.52 for baited traps, and 1.60 for unbaited. These data indicate that male and female *A. vittatum* are attracted equally well to baited and unbaited traps.

The abundance of *D. v. virgifera* and *D. u. howardi* resulted in high control trap catches in cucurbit fields (Table 4). However, numbers of *D. v. virgifera* caught in indole-baited traps (5 mg) were nearly always larger than those found in controls ($P < 0.10$, paired *t*-test). Again, *D. u. howardi* showed no significant response to indole baited traps, with statistically equal numbers of individuals being caught in treatment and control traps (Table 4).

CONCLUSIONS

In past studies, cucurbitacins have been shown to be classic examples of "sign stimuli," as designated by Fraenkel (1959), for *Diabrotica* and *Acalymma* adults. Their narrow taxonomic distribution and their potent feeding stimulant activity make them important factors in the selection of cucurbit hosts by beetles (Metcalf et al., 1982). However, this study indicates that host volatiles are also of importance. A single compound which occurs in the floral essence of the beetle host plant *C. maxima* is strongly attractive to adult *D. v. virgifera* and *A. vittatum.* The relatively broad distribution of indole in floral

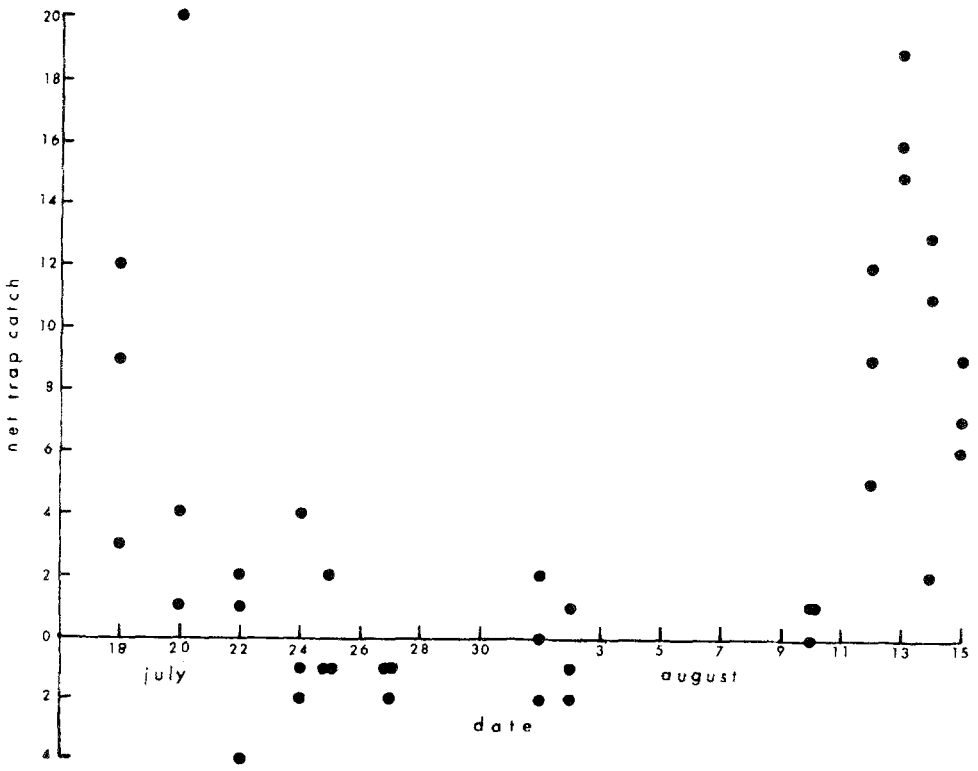

FIG. 4. Catches of *D. virgifera virgifera* in traps baited with 5 mg indole in July and August 1983. Net trap catch indicates the number of *D. v. virgifera* found in baited traps minus the number found in the corresponding unbaited trap. Each spot represents the net catch of one control-treatment pair.

TABLE 4. TRAP CATCHES OF *Acalymma vittatum, Diabrotica virgifera virgifera, Diabrotica undecimpunctata howardi* IN CUCURBIT FIELD USING 5 mg INDOLE LURES (THREE TRAPS OF EACH TREATMENT ON EACH DATE)

Trapping date	*A. vittatum*		*D. v. virgifera*		*D. u. howardi*	
	Indole	Control	Indole	Control	Indole	Control
August 4	30	16	44	22	6	8
August 5	20	7	25	15	9	17
August 6	21	4	7	10	7	9
August 7	16	12	36	29	8	9
August 8	18	5	22	17	17	7
Total	105	44	134	93	47	50

odors (Stowe, 1959) suggests that it does not function as a "sign stimulus" in the *Diabrotica/Acalymma–Cucurbita* association. It may, however, act in concert with other components of the floral volatile mixture to form a unique attractive blend (Visser, 1979).

The cucurbitacins and indole appear to play roles in host selection which are consistent with models described by Kogan (1977). Indole would serve in the "host-finding" phase of host selection by eliciting orientation to the host plant from a distance. Cucurbitacins are minimally volatile and would only act in the "host acceptance" stage by stimulating feeding and arresting locomotion (Metcalf, 1979). Variability in both of these factors could influence the overall preference for a particular *Cucurbita* species or cultivar. The influence of these factors on beetle field distribution will be addressed in a separate report.

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SOLDIER DEFENSE SECRETIONS OF THE GENUS *Hospitalitermes* IN PENINSULAR MALAYSIA

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Abstract—Soldier defense secretions of the genus *Hospitalitermes* (Isoptera, Termitidae, Nasutitermitinae) were chemically analyzed by GC-MS and were found to contain volatile monoterpenes and polyoxygenated diterpenes. Interspecific and intraspecific chemical variations for *H. umbrinus*, *H. hospitalis*, *H. flaviventris*, and *H. bicolor* are described. Interspecific variations in monoterpene and diterpene structures and compositions were evident. A remarkable example of large intraspecific variation from a single species was observed in *H. umbrinus*, indicating that colonies of this species can be separable into two chemically distinct groups. The diterpenes found in one of these groups have unusually high molecular weights.

Key Words—Termites, Isoptera, Termitidae, defense secretions, interspecific variations, *Hospitalitermes* species, diterpenes.

INTRODUCTION

The genus *Hospitalitermes* belongs to the subfamily Nasutitermitinae, in which the soldier castes are characterized by having conical, nozzle-shaped projections on the head from which they eject defensive secretions produced by the enlarged frontal glands located within the head capsules. The genus is restricted to the Old World Tropics with over 22 species having been recorded (Tho, 1982). The soldiers are all monomorphic and are characterized by having long antennal segments, long legs with the hind leg much longer than the body length, and highly pigmented body colors.

Behaviorally, all species recorded have free-ranging habits, in that they march out in long columns without any covered trails. These columns often

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extend more than 50 m from the nest site, and the termites are often observed climbing up to the top of the forest canopy. Food comprises scrapings off the surfaces of leaves, twigs, and the bark of trees (a mixture of dead plant material and lichen). The food material is collected by the worker caste and is rolled into a small ball that is then carried all the way back along the trail to the nest. The soldiers protect their foraging columns by positioning themselves along the fringes of the columns. Defense is effected by shooting chemical secretions at their enemies. Our observations so far have indicated that these termites, while on the march, are seldom attacked by predators or enemies, which would therefore attest to the effectiveness of the soldiers and hence the defense secretions.

The biochemistry and chemistry of the soldier defense secretions of *Hospitalitermes* are therefore of great interest and have been reported for two species, *Hospitalitermes monoceros* (Maschwitz et al., 1972; Prestwich, 1984) from Ceylon and *Hospitalitermes umbrinus* (Chuah et al., 1983) from peninsular Malaysia. In peninsular Malaysia, there are six species of *Hospitalitermes* (Tho, 1982), and in this paper we report our studies of the chemistry of the defense secretions of four species, which includes *H. umbrinus*.

METHODS AND MATERIALS

Termite Material. Soldier termite materials used for the present studies were all collected from within closed canopy dipterocarp forests from the following localities in Peninsular Malaysia: (1) Pasoh forest reserve [2°58′N, 101°55′E; 100 m above sea level (ASL)]—a lowland dipterocarp forest which was the site for intensive ecological studies under the auspices of the International Biological Programme; (2) Fraser's Hill forest reserve (3°43′N, 101°44′E; 1200 m ASL)—an upper hill dipterocarp forest bordering to the oak montane forest; (3) Taman Negara (4°20′N, 102°25′E; 1000 m ASL)—the national park of peninsular Malaysia in the state of Pahang; (4A) Gombak forest reserve (3°20′N, 101°46′E; 300 m ASL); (4B) Klang Gate (3°15′N, 101°46′N; 300 m ASL); (4C) Genting Sempah (3°14′N, 101°46′E; 500 m ASL); (4D) Templer Park (3°22′N, 101°40′E; 200 m ASL)—4A–4D are hill dipterocarp forests in the state of Selangor; (5) Pulau Langkawi (6°22′N, 99°50′E; 200 m ASL)—an island in the state of Kedah; (6) Bukit Buak forest reserve (4°40′N, 103°20′E; 500 m ASL)—a hill dipterocarp forest in the state of Trengganu, and (7) Penang Hill (5°25′N, 100°12′E; 1200 m ASL)—an upper hill dipterocarp forest, most of which has been disturbed.

Analytical Methods. Gas chromatography (GC) was performed on a Pye 104 instrument fitted with a Gow-Mac flame ionization detector. Gas chromatography–mass spectrometry (GC-MS) was performed on a Pye 104 gas chromatograph interfaced to a double-beam Kratos MS30 mass spectrometer by a membrane separator. Mass spectra were obtained at 70 eV with the source and

interface temperatures at 150°C and 230°C, respectively, and data were accumulated by a Kratos DS55 data system. Three glass columns (5-ft × 3-mm 3% OV-17, 5-ft × 3-mm 3% OV-1, and 3-ft × 3-mm 1% SE-30 on 100–120 Gas Chrom Q) were used to analyze the diterpenes. A 10-ft × $\frac{1}{8}$-in. stainless-steel column packed with 10% Carbowax 20 M on 120–140 Gas Chrom Q was also used to analyze monoterpenes.

The monoterpenes (I–IX) have been characterized by GC-MS and coinjection with standards and the results are summarized in Table 1. Similarly, diterpenes (X–XX, Figure 1) were characterized by the NMR and MS data (Vrkoc et al., 1978a,b; Prestwich, 1979; Dupont et al., 1981) and by coinjection with compounds identified (Chuah et al., 1983; Goh et al., 1982, 1984). The results are summarized in Table 2.

The whole gas chromatographic profile of the total secretion was usually obtained by on-column injection at 90°C and then programmed at 8°C/min to 270°C on the 5-ft glass column, or the column was kept isothermal at 90°C for 5 min before temperature programming. The analysis for the diterpenes of *H. umbrinus* chemoform A was done on the 3-ft column. The gas chromatographic profiles are shown in Figures 2 and 3. *H. umbrinus* chemoform A provides two major new diterpenes which have molecular weights of 650 and 664 as shown by MS probe samples. The R_f values of these two compounds on silica gel TLC in EtOAc–Hexane (1 : 20) are 0.37 and 0.38, respectively. These are quite complex molecules and are tentatively assigned as epoxytrinervitenepentaol pentapropionate and epoxytrinervitenepentaol acetate tetrapropionate esters, respectively, based on the mass spectra and [^{1}H]NMR spectra which showed strong propionate CH_2 resonances at δ2.27 and 2.35. A pair of AB doublets (5.8 Hz) centered at δ2.75 and 3.14 were assigned as due to the presence of an epoxy group. Retention indices of all diterpenes were made relative to *n*-alkane standards and R_f values on silica gel TLC plates are summarized in Table 3.

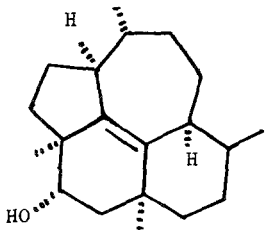
The chemical identities of the major diterpenes were confirmed after liquid chromatographic isolation. Preparative scale separations of the individual terpenoid compounds from the defense secretions (ca. 100 mg or more) of the various species of soldier termites were performed by flash chromatography. Column lengths of 0.5–0.8 m and 1–2 cm diameter were slurry packed with 40–63 μm silica gel (Merck 9385) in dichloromethane, and to separate most of the terpenoids, a gradient elution of 2–10% ethyl acetate in dichloromethane was used. Further purification, if necessary, of the isolated terpenoids was aided by a Waters 440 HPLC instrument fitted with a 25-cm × $\frac{1}{4}$-in. Ultrasphere silica gel column. Elution of the diterpenoids was by a gradient of 2–10% ethyl acetate in hexane. Isolated samples were checked by GC and TLC on 5 × 10-cm 0.25-mm silica gel 60 plates. Proton and carbon-13 NMR spectra were recorded on a Jeol JMN-FX100 spectrometer.

The isolated diterpenoids were identified by their [^{1}H]NMR and mass spectral data as well as by comparison of their retention times with those previously

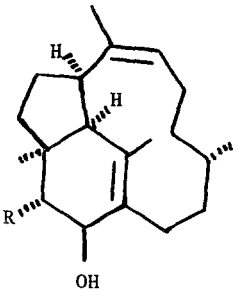
TABLE 1. INTERSPECIFIC VARIATION IN MONOTERPENE HYDROCARBON (%) IN *Hospitalitermes*

Compound	*H. umbrinus A*[a]	*H. umbrinus B*[b]	*H. hospitalis*[c]	*H. flaviventris*[d]	*H. bicolor*
α-Pinene (I)	60–73	65–85	38–48	9–33	61
Camphene (II)	0–3	0–1	0–4	0–8	
β-Pinene (III)	22–30	12–27	15–18	35–53	36
Myrcene (IV)				10–47	
Δ^3-Carene (V)	0–2	0–3	10–18	2–21	
α-Terpinene (VI)	0–1	0–1	3–8		
Limonene (VII)	2–8	2–6	10–22	0–2	3
γ-Terpinene (VIII)			2–3		
Terpinolene (IX)	0–2	0–2	1–2		t[e]

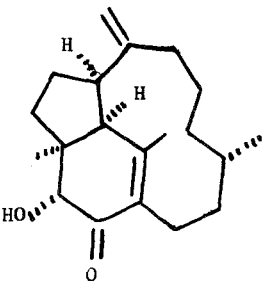
[a]Range for 18 colonies.
[b]Range for 10 colonies.
[c]Range for 7 colonies.
[d]Range for 5 colonies.
[e]t trace = (less than 0.5%).



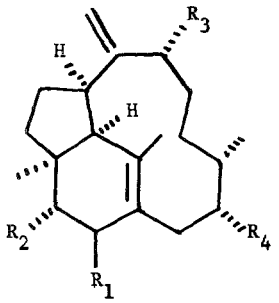
X



XII R=H
XVI R=OH



XIII



XI R_1=H, R_2=H, R_3=OH, R_4=H

XIV R_1=OAc, R_2=OH, R_3=H, R_4=H

XV R_1=OH, R_2=OH, R_3=H, R_4=H

XVII R_1=OH, R_2=OH, R_3=OAc, R_4=H

XVIII R_1=OAc, R_2=OAc, R_3=OH, R_4=H

XIX R_1=OAc, R_2=OAc, R_3=H, R_4=O

XX R_1=OAc, R_2=OAc, R_3=OAc, R_4=H

FIG. 1. Diterpene structures.

identified (Chuah et al., 1983; Vrkoc et al., 1978a,b; Prestwich, 1979; Dupont et al., 1981).

RESULTS AND DISCUSSION

Defensive secretions of the soldiers of *Hospitalitermes* comprise mainly mono- and diterpenoids. The results obtained are summarized in Tables 1 and 2 and Figures 2 and 3. The monoterpenes (I–IX) were all hydrocarbons and were usually dominated by α-pinene as was found for *H. umbrinus*, *H. hospitalis*, and *H. bicolor*. However, a dominant amount of myrcene was found in the secretion of *H. flaviventris*. In general, monoterpenes alone may not be useful for chemotaxonomic diagnosis (Goh et al., 1982). However, when coupled with the diterpene components, which exhibit greater diverse structural

TABLE 2. INTERSPECIFIC VARIATION IN DITERPENE COMPOSITION (%) IN *Hospitalitermes*

Compound	*H. umbrinus A*[a]	*H. umbrinus B*[b]	*H. hospitalis*[c]	*H. flaviventris*[d]	*H. bicolor*
Ripperten-3 α-ol (X)		0–3	1–4	3–5	1
Trinervita-1(15),8(19)-dien-9α-ol (XI)		2–4	1–3	0–2	
Trinervita-1(15),8(9)-dien-2β-ol (XII)		0–3	1–6	4–8	5
2-Oxotrinervita-1(15),8(19)-dien-3α-ol (XIII)		4–9	5–10	4–9	1
Trinervita-1(15),8(19)-2β,3α-diol 2-O-acetate (XIV)	1–5	16–30	4–8	4–10	2
Trinervita-1(15),8(19)-dien-2β,3α-diol (XV)	4–8	37–48	45–55	45–50	11
Trinervita-1(15),8(9)-dien-2β,3α-diol (XVI)		0–1	0–3	0–1	37
Trinervita-1(15),8(19)-dien-2β,3α,9α-triol 9-*O*-acetate (XVII)		12–30	8–18	14–16	2

Trinervita-1(15),8(19)-dien-2β,3α,9α-triol 2,3-*O*-diacetate (XVIII)		0–2	4–12	3–6	34
13-Oxotrinervita-1(15),8(19)-dien-2β,3α-diol 2,3-*O*-diacetate (XIX)		0–3	2–3	1–2	6
Trinervita-1(15),8(19)-dien-2β,3α,9α-triol 2,3,9-*O*-triacetate (XX)		1–2		5–7	
Diterpene UI[e]	22–25				
Diterpene UII[e]	62–70				
Unknown	1–5	0–3	3–5	2–4	

[a] Range for 18 colonies.
[b] Range for 10 colonies.
[c] Range for 7 colonies.
[d] Range for 5 colonies.
[e] Only small amounts of diterpene diol derivatives; mixed acetate–propionate esters of pentahydroxyditerpenes (UI and UII) are also present.

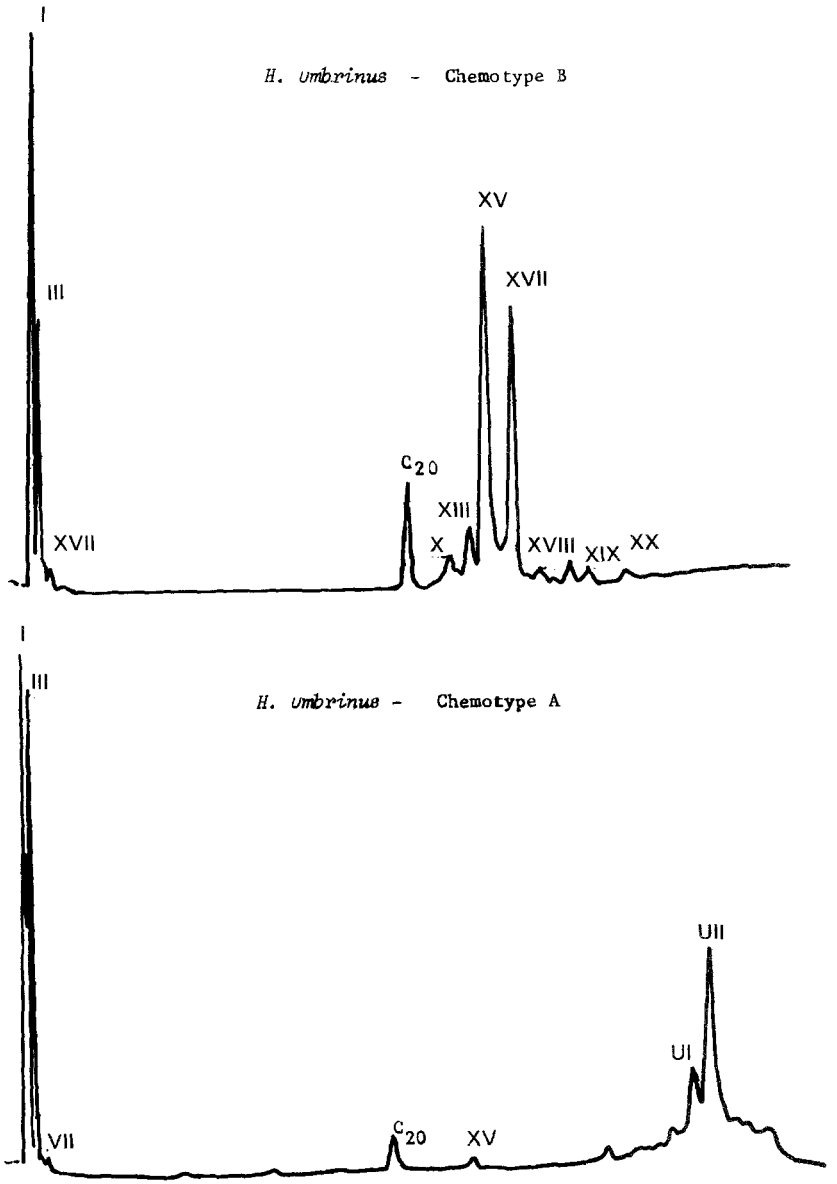


FIG. 2. Gas chromatograms of *Hospitalitermes* soldier secretions.

types and various degrees of oxygenation, the overall chemical composition of the defense secretions does provide some diagnostic patterns depicting intergeneric and interspecific differences. In some cases, monoterpenes can be used as species discriminators in chemosystematics (Prestwich, 1983).

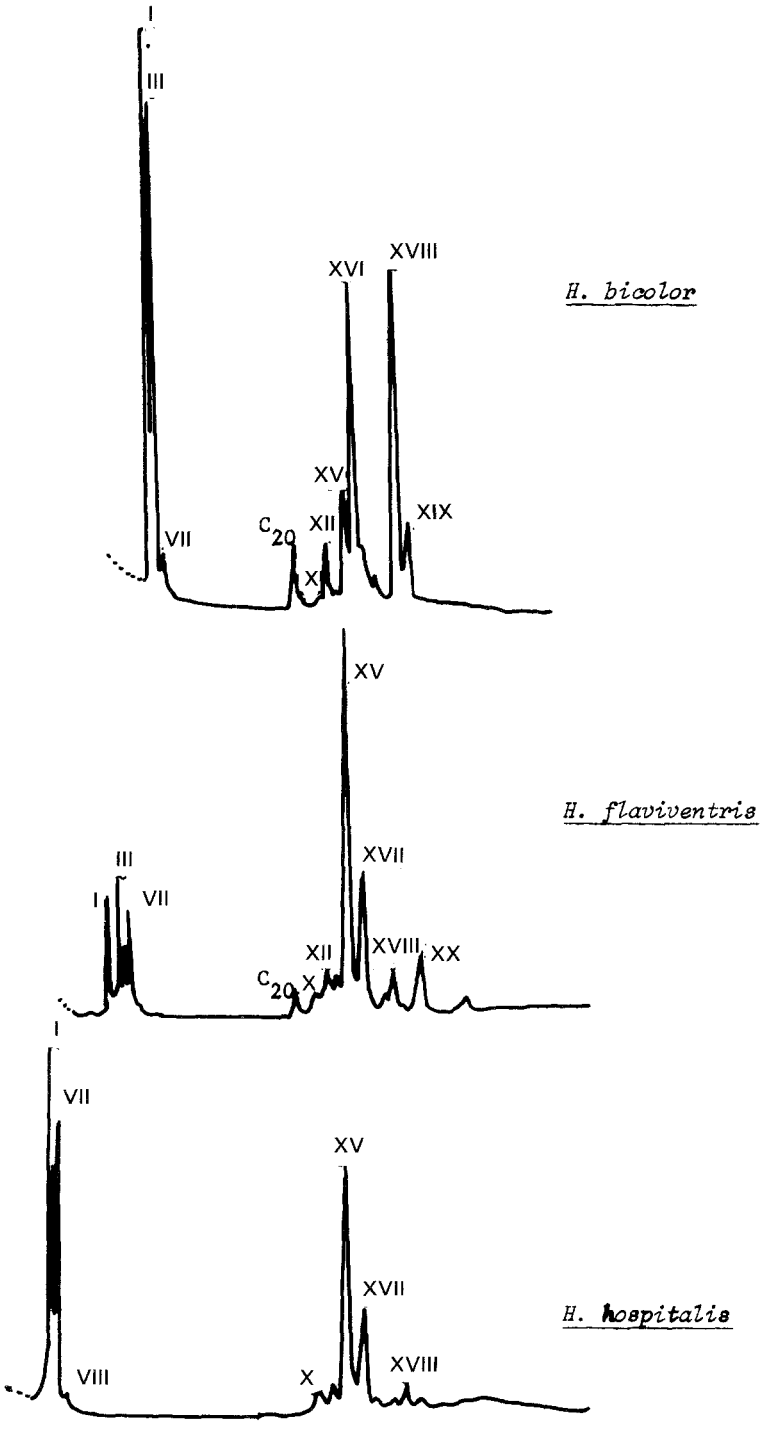


FIG. 3. Gas chromatograms of *Hospitalitermes* soldiers secretions.

TABLE 3. CHROMATOGRAPHIC PROPERTIES OF DITERPENES FROM *Hospitalitermes*

	Mol. wt.	R_f (color)[a]	OV-1[b]	OV-17[b]
Ripperten-3α-ol	288	0.48 (sky blue)	21.8	24.1
Trinervita-1(15),8(19)-dien-9α-ol	288	0.58 (wistaria violet)	22.0	24.3
Trinervita-1(15),8(9)-dien-2β-ol	288	0.54 (violet)	22.3	24.7
2-Oxotrinervita-1(15),8(19)-dien-3α-ol	302	0.61 (lilac)	22.8	25.5
Trinervita-1(15),8(19)-2β,3α-diol 2-*O*-acetate	346	0.36 (pink)	23.8[c]	26.2[c]
Trinervita-1(15),8(19)-dien-2β,3α-diol	304	0.23 (pink)	23.5	26.2
Trinervita-1(15),8(9)-dien-2β,3α-diol	304	0.16 (greyish-blue)	24.0	26.7
Trinervita-1(15),8(19)-dien-2β,3α,9α-triol 9-*O*-acetate	362	0.14 (deep purple)	24.5	27.4
Trinervita-1(15),8(19)-dien-2β,3α,9α-triol 2,3-*O*-diacetate	404	0.42 (greenish blue)	27.1	28.8
13-Oxotrinervita-1(15),8(19)-dien-2β,3α-diol 2,3-*O*-diacetate	402	0.56 (violet)	27.1	29.0
Trinervita-1(15),8(19)-dien-2β,3α,9α-triol 2,3,9-*O*-triacetate	446	0.52 (orange)	29.0	31.0
Diterpene UI[e]	650	0.37 (blue)	33.0[c,d]	
Diterpene UII[e]	664	0.38 (blue)	33.5[c,d]	

[a] 5% Ethyl acetate–dichloromethane (v/v), TLC on silica gel G (stained by vanillin).
[b] Relative retention times; 6-ft × 3-mm 3% OV-1 on 100–120 Gas Chrom Q, 5-ft × 3-mm 3% OV-17 on 100–120 Gas chrom Q.
[c] Compound decomposed in the GC column.
[d] 3-ft × 3-mm 1% SE-30 on 100–120 Gas Chrom Q.
[e] See footnote *e*, Table 2.

The diterpenes of *Hospitalitermes* are mainly dominated by trinervitenes (X–XX) with trinervitenediol (XV) being the most commonly encountered. The only other skeletal type detected in minor quantities is the methyl-shifted rippertenol (X). Interspecific variation is exhibited mainly by the degree of oxygenation of the diterpenes. Thus *H. bicolor* showed a distinct diterpene composition that had a preponderance of the 13-keto triacetate (XIX).

The chemistry of *H. umbrinus* has already been reported previously (Chuah et al., 1983). However, we have now found consistent intraspecific variation for individual colonies. Based on the diterpene components, colonies can be separable into two distinct groups. These are referred to as chemotype A and B for purposes of discussion here. Thus chemotype B colonies are characterized by having large amounts and varieties of the common trinervitenediols (XV), while chemotype A soldier secretions were found to be dominated by two high-molecular-weight diterpenes (UI and UII) as illustrated by the gas chromatograms in Figure 2 and effectively demonstrated in Table 2. Our preliminary studies on the structures of these new diterpenes have shown that they possess unusually high molecular weights. One of these was determined to have a molecular weight of 664 while the other was recorded as 650. These are tentatively assigned as epoxytrinervitenepentaol pentapropionate and epoxytrinervitenepentaol acetate tetrapropionate esters, respectively.

Although the defense secretions are chemically distinct, our examination of the morphological characteristics of the termites from colonies of these two chemotypes has not revealed that they could be taxonomically separated. However, some preliminary ethological observations seem to indicate that soldiers of chemotype B are relatively more aggressive in response to disturbances. Our chemical data are based on collections from various localities in peninsular Malaysia which includes allopatric and sympatric colonies. We therefore infer, from the differences observed, that there are two chemotypes within the species.

On the assumption that such differences could be the result of temporal variation, we have checked and screened soldier termite secretions of several colonies over a period of four years during which we found no major variations. This does imply that the chemical compositions are specific for each colony. In this respect, we are faced with the question of the existence of two "chemically distingishable" species. Current termite taxonomy is based largely on the morphological features of the termite castes. It would be rather difficult to justify the existence of two species based on observed differences in the chemistry of soldier defense secretions. However, speciation in nature need not necessarily be manifested morphologically. Behavioral and physiological changes could precede morphological change. Yet, short of an in-depth study, it can only be concluded here that our present studies have revealed that within the species *H. umbrinus* there are two chemically distiguishable groups of termite colonies.

Phylogenetically, *Hospitalitermes* together with *Trinervitermes* (Prestwich and Collins, 1981) are considered the more advanced genera in the family Na-

sutitermitinae. In these genera, intraspecific variation of the chemical defense secretions has generally been observed to be not common, unlike the case of the primitive *Longipeditermes longipes* (Goh et al., 1984), which demonstrated extreme intraspecific chemical variability in their soldier defense secretions in both allopatric and sympatric colonies. Should the two chemotypes currently observed for *H. umbrinus* prove to be two distinct species, then the present studies for the genus *Hospitalitermes* would provide additional evidence that the more advanced genera exhibit little intravariability. Interspecific differences are discernible, even more so when based on a matrix of the qualitative and quantitative composition of the various monoterpenes and diterpenes.

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Lespedeza PHENOLICS AND *Penstemon* ALKALOIDS: Effects on Digestion Efficiencies and Growth of Voles

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Abstract—*Lespedeza cuneata* contains high levels of phenolics and is a common food plant of the meadow vole (*Microtus pennsylvanicus*); *Penstemon digitalis* contains substantial quantities of alkaloids and is a common food plant of meadow voles and prairie voles (*M. ochrogaster*). We investigated the palatability of these plants and the effects of their secondary compounds on the digestion efficiencies and growth of both species of voles. Voles ate very little of either plant when alternative food was present. Phenolics and alkaloids were extracted from the plants, incorporated into separate artificial diets, and fed to weanling voles for three weeks. *Lespedeza* phenolics reduced the growth of meadow voles but not prairie voles throughout the feeding trial. These compounds disrupted digestion, reducing protein digestibility by more than half. *Penstemon* alkaloids lowered only the initial growth rates of prairie voles and had no significant effect on meadow voles. Prairie voles tended to increase food consumption rates on both *Lespedeza* and *Penstemon* diets. This response offset some of the decrease in digested protein intake in the first case, and offset the increased metabolic cost of processing the diet in the latter case. Our results indicate that although *Lespedeza* phenolics and *Penstemon* alkaloids do influence consumption and digestibility of artificial diets, they do not greatly reduce the performance (i.e., growth or survival) of voles when consumed at levels generally observed for wild voles. However, these compounds probably do place an upper limit on the amount of fresh *Lespedeza* and *Penstemon* that can be consumed and contribute to the generalist feeding strategy of voles.

Key Words—Alkaloids, phenolics, feeding trials, digestibility, growth, *Lespedeza cuneata*, *Penstemon digitalis*, *Microtus pennsylvanicus*, *Microtus ochrogaster*.

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INTRODUCTION

Diet selection by herbivores is largely determined by the phytochemical composition of the available forage and the nutrient requirements/detoxication capacities of the animals. Both plant nutrients and secondary compounds influence the foraging ecology of mammalian herbivores (e.g., Swift, 1948; Swank, 1956; Cooper-Driver et al., 1977; Belovsky, 1978, 1981; Milton, 1979; Bryant and Kuropat, 1980; McKey et al., 1981; Radwan et al., 1982; Fraser et al., 1984; Calvert, 1985), but most studies have simply reported correlations between food habits or palatability and types and amounts of these plant constituents. Only recently have researchers begun to address a more pertinent question regarding plant defenses, viz., do the specific secondary compounds encountered by mammalian herbivores actually inhibit their growth, survival, or reproduction? Also, do differing food habits of closely related species reflect different abilities to detoxify or otherwise circumvent plant chemical defenses (e.g., Jung and Batzli, 1981)?

The meadow vole (*Microtus pennsylvanicus*) and prairie vole (*M. ochrogaster*) are primarily herbivorous, and commonly occur in herbaceous habitats in east-central Illinois. Our previous work (Lindroth and Batzli, 1984a) showed that in a tallgrass prairie habitat, meadow voles consumed substantial quantities of the forbs *Lespedeza cuneata* and *Penstemon digitalis*. In summer, these species comprised 28 and 16% of meadow vole diets, respectively, but preference indices showed that they were neither significantly preferred nor avoided. Cole and Batzli (1979) listed *Penstemon*, but not *Lespedeza*, among the forbs commonly eaten by prairie voles in a similar tallgrass prairie habitat.

Although they are common food plants in our study sites, *Lespedeza* and *Penstemon* contain relatively high levels of secondary plant compounds. As a forage crop, *Lespedeza* is known for its high concentrations of tannins (Donnelly and Anthony, 1969, 1970), and *Lespedeza* plants in our prairie site contain much higher (2–4×) levels of protein-binding phenolics than other common food items (Lindroth and Batzli, 1984a). *Penstemon* contains appreciable amounts of alkaloids, which are not detectable in other food plants (Lindroth and Batzli, 1986). Moreover, in laboratory feeding trials in which prairie voles and meadow voles are provided with a mixture of fresh *Lespedeza* and *Penstemon*, animals lose weight and die within several days (Lindroth, personal observation).

This paper reports results from experiments conducted to evaluate the effects of *Lespedeza* phenolics and *Penstemon* alkaloids on prairie voles and meadow voles. We were interested first in ascertaining whether secondary compounds from major food plants reduce the performance of voles. Second, to help explain the difference in utilization of *Lespedeza* by the two vole species, we tested the hypothesis that prairie voles are more susceptible (i.e., exhibit slower growth or lower digestion efficiencies) to *Lespedeza* phenolics than are meadow voles.

METHODS AND MATERIALS

Palatability Trials. We conducted palatability trials to assess the relative preferences of each vole species for *Lespedeza* and *Penstemon*. *Lespedeza* and *Penstemon* leaves were collected in mid-summer, divided into subsamples, weighed, and fed fresh to six adults of each species. We used the same individuals to test both plant species; the *Penstemon* trial was conducted 24 hr after the *Lespedeza* trial. Trials lasted 15 hr, during which time the voles were housed individually and had access to the test plant ($\simeq$ 5 g), water, and a standard palatable diet (Purina Rabbit Chow, No. 5321). Thus the voles were not forced to eat any of the test plant. To account for evaporative water loss, we also weighed out five additional subsamples of fresh leaves at the onset of each trial. At the conclusion of each trial plant material was collected and all samples were oven-dried (100°C, 24 hr) and weighed. Palatability of each plant species to each vole was calculated as leaf dry weight ingested (dry weight offered minus dry weight not eaten).

Artificial Diets. When using whole plant tissues in bioassays, investigation of the effects of any one plant compound is complicated by the impossibility of controlling the concentrations of a plethora of other plant compounds. Thus we conducted our experiments with an adaptation of an artificial diet developed earlier for use with microtine rodents (Lindroth et al., 1984). Although they do not mimic many of the physical and chemical characteristics of fresh plants, artificial diets are particularly useful for investigations of the action of particular plant compounds or interactions among compounds. All diets described in this study contained 10% protein, consisting of casein supplemented with methionine.

Lespedeza was collected in September 1983 and air-dried leaves were separated, ground, and stored (−20°C) until extracted. The level of protein-binding phenolics in this plant material was similar to that observed in *Lespedeza* in several preceding autumns (Lindroth, 1984; Lindroth et al., 1986). The concentration of condensed tannins was 34% dry weight in quebracho equivalents [vanillin HCl assay, Broadhurst and Jones (1978)], although we doubt that the true level of condensed tannins was quite so high. Ground *Lespedeza* leaves (125-g lots) were extracted sequentially in 2, 1, and 1 liter volumes of 80% ethanol over a period of two days. We then combined the filtrates, concentrated the extract (rotary evaporator, 38°C), mixed it with the dry artificial diet, and dried the diet (28°C) in a forced-air oven for two days. To make the corresponding control diet, a quantity of 80% ethanol equivalent to the final extract volume was added to another batch of artificial diet and dried.

When fed the artificial diet with phenolics equivalent to an 80% *Lespedeza* diet, weanling prairie voles ate only small amounts, lost weight, and died within several days. Because we were more interested in the toxic or digestibility-reducing effects rather than feeding-deterrent effects of *Lespedeza* phenolics, we lowered the phenolic levels to a point where palatability of the diet was not

strongly affected. The artificial diet used for the experiments presented here was approximately equivalent to a natural summer diet consisting of 50% *Lespedeza*.

Leaves of *Penstemon* rosettes were collected in May 1984, freeze-dried, ground, and stored (−20°C) until extracted. The level of alkaloids in *Penstemon* leaves was similar to that observed in preceding spring seasons (Lindroth, 1984; Lindroth et al., 1986), comprising about 0.01% of plant dry weight (Lindroth et al., 1986). We extracted ground *Penstemon* leaves (80-g lots for 30 min) twice in 800 ml of 0.1 N H_2SO_4. We combined the filtrates and basified the extract to pH 10 with a concentrated NaOH–water solution. We washed the basified extract four times with 200 ml $CHCl_3$, then concentrated the combined organic phases to about 300 ml (rotary evaporator, 38°C). This solution was washed three times with 30, 30, and 20 ml of 0.1 N H_2SO_4; the washes were combined and briefly evaporated (rotary evaporator, 38°C) to remove residual chloroform. The resulting solution and corresponding control (equal volume of 0.1 N H_2SO_4) were mixed with dry artificial diet and freeze-dried because alkaloids decomposed when dried in a forced-air oven at 28°C. The alkaloid diet had a concentration of alkaloids equivalent to a natural diet consisting of about 50% *Penstemon* in spring or 100% *Penstemon* in summer (alkaloid concentrations decrease by about half from spring to summer).

Feeding Trials. We conducted the *Lespedeza* diet experiment in autumn 1983, and the *Penstemon* feeding experiment the following spring. For each study, prairie voles were obtained from a colony maintained at the University of Illinois; colony founders came from nearby oldfields. Because we have been unable to breed meadow voles under laboratory conditions, we live-trapped pregnant females in a nearby tallgrass prairie, brought them into the laboratory, and used the ensuing young for the feeding trials. We used the growth of weanling voles as a bioassay of diet quality. Young were weaned at 18 days old and fed a transition diet of rabbit pellets mixed with a control or experimental artificial diet. We divided litters between control and experimental diets to eliminate confounding genotypic or maternal effects. When 20 days old, young were paired with nonsiblings and weighed. We maintained the voles in plastic shoebox cages with hardwood chip bedding and cotton nesting material, at room temperature (22–25°C) on a 15:9 light–dark cycle. Artificial diets and demineralized water were provided ad libitum. We weighed the animals at three- to four-day intervals for three weeks.

Many plant phenolics and alkaloids assimilated by mammals are metabolized by conjugation with glucuronic acid and are then excreted in the urine. We found previously that uronic acid excretion may be used as an index of phenolic detoxication metabolism (Lindroth and Batzli, 1983). To check for evidence of biochemical detoxification of *Lespedeza* phenolics or *Penstemon* alkaloids, we collected 24-hr urine samples from our animals when 42 days old and analyzed these as described in Lindroth and Batzli (1983).

Digestibility Trials. We conducted digestibility trials in order to determine the general mode of action of the plant compounds. Voles were maintained in the same metabolic cages as used for urine collections and fed an artificial diet and water ad libitum for four days. At the end of the trials we dried and weighed feces and excess food and calculated digestibilities as the difference between intake and output. We analyzed samples of food and feces for total organic nitrogen by acid digestion and determination of ammonia by the Nessler method (Association of Official Analytical Chemists, 1975). We did not conduct digestibility trials for all of the voles in the *Penstemon* feeding trial because some of those animals were required for other purposes.

At the conclusion of the digestibility trials, voles were sacrificed, necropsied, and the gastrointestinal tract removed. The carcasses were freeze-dried and extracted with petroleum ether (30–60°C bp) to determine fat content.

RESULTS

Palatability. Fresh *Lespedeza* and *Penstemon* leaves were poorly palatable to both species of voles; ≤ 0.15 g dry weight were consumed (Table 1). Calculations from data in Batzli and Cole (1979) indicate that prairie voles would consume about 3.5 g dry weight of alfalfa in a similar trial. In each trial, the variability in consumption was quite high, with one to several animals showing no consumption. A two-way analysis of variance revealed no significant effects due to vole species ($F_{1,20} = 1.43$, $P = 0.25$), plant species ($F_{1,20} = 2.43$, $P = 0.13$), or interaction ($F_{1,20} = 0.50$, $P = 0.49$).

Growth. The initial (5 day) growth rate of prairie voles on the *Lespedeza* diet was 0.22 ± 0.11 g/day, significantly less than the 0.81 ± 0.05 g/day of control animals ($t_{18} = 4.90$, $P < 0.001$). However, by the end of the trial, voles on the two diets had attained similar body weights (Figure 1). Initial growth rates of meadow voles were also different, 0.30 ± 0.06 and 0.64 ± 0.08 g/day, respectively, for voles fed the *Lespedeza* or control diets ($t_{18} =$

TABLE 1. MEAN CONSUMPTION OF FRESH *Lespedeza* AND *Penstemon* LEAVES BY ADULT VOLES IN PALATABILITY TRIALS[a]

	Consumption (g dry wt ± SE)	
	Lespedeza	*Penstemon*
Prairie voles	0.13 ± 0.05	0.02 ± 0.02
Meadow voles	0.15 ± 0.06	0.11 ± 0.05

[a] (ANOVA showed no significant differences among means at $P < 0.10$). Sample sizes were six for each treatment.

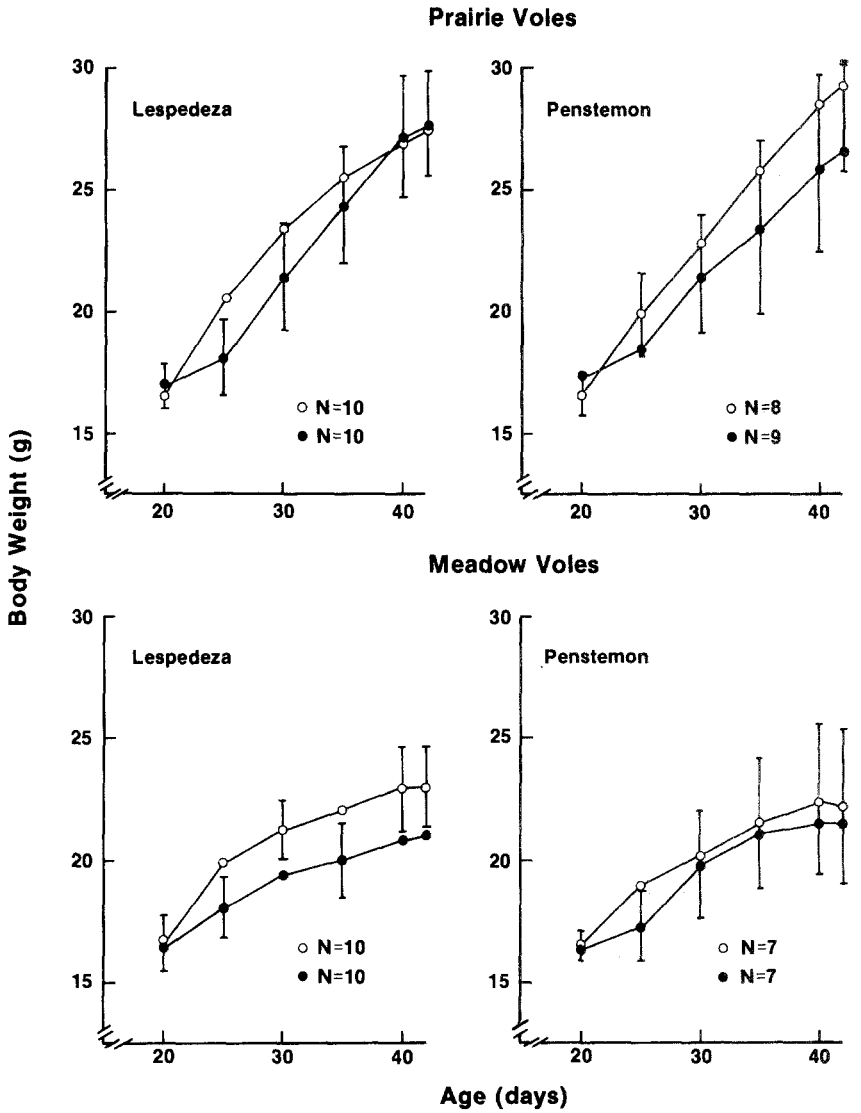

FIG. 1. Growth of weanling prairie voles and meadow voles fed control (○) or experimental (●) diets. Vertical lines indicate the largest 95% confidence intervals.

3.39, $P < 0.001$). At the conclusion of the trial, meadow voles fed *Lespedeza* weighed significantly less than control animals. Moreover, meadow voles as a group grew less than did prairie voles. A two-way analysis of variance of final weights showed that the main effect of vole species was significant, but the effects of diet and species–diet interaction were not (Table 2).

TABLE 2. *P* VALUES FROM TWO-WAY ANOVAS FOR RESULTS OF EXPERIMENTS ON PRAIRIE VOLES AND MEADOW VOLES FED DIETS WITH *Lespedeza* OR *Penstemon* EXTRACTS

Analysis	Source of variation		
	Species	Diet	Interaction
Lespedeza trials			
Final body weight	<0.001	0.636	0.408
Body fat content	0.537	0.181	0.514
Ingested dry matter	0.327	0.012	0.862
Dry matter digestibility	0.860	<0.001	0.838
Digested dry matter	0.347	0.581	0.770
Apparent protein digestibility	0.031	<0.001	0.131
Protein digested	0.969	<0.001	0.835
Uronic acid excretion	0.088	<0.001	0.197
Penstemon trials			
Final body weight	<0.001	0.206	0.539
Body fat content	0.001	0.051	0.231
Ingested dry matter	<0.001	0.227	0.075
Dry matter digestibility	0.782	0.849	0.293
Digested dry matter	<0.001	0.216	0.041
Apparent protein digestibility	0.015	0.377	0.191
Protein digested	0.213	0.204	0.750
Uronic acid excretion	0.003	0.542	0.231

For prairie voles in the *Penstemon* feeding trial, initial growth rates were again significantly different, 0.22 ± 0.15 and 0.68 ± 0.15 g/day, respectively, for experimental and control animals ($t_{15} = 2.15$, $P < 0.05$). Body weights of voles fed the *Penstemon* diet exhibited considerable variability, so we observed no significant differences in weights of animals in the two treatments (Figure 1). Initial growth rates for meadow voles fed the *Penstemon* or control diet showed a similar trend, 0.17 ± 0.13 and 0.49 ± 0.10 g/day, respectively, but were not significantly different ($t_{12} = 1.87$, $P > 0.05$). Voles on the two diets showed similar body weights for most of the feeding trial (Figure 1). Two-way analysis of variance of final weights again showed that only the effect of vole species was significant (Table 2).

In the *Lespedeza* feeding trial, body fat content did not differ significantly between vole species or between treatment and control animals (Figure 2, Table 2). In the *Penstemon* trial, we also found no significant differences between treatment and control animals within each vole species. However, in a two-way analysis of variance, we found that the main effect of species explained a significant amount of the variation and the main effect of diet explained a marginally significant amount of the variation (Table 2). Thus prairie voles contained

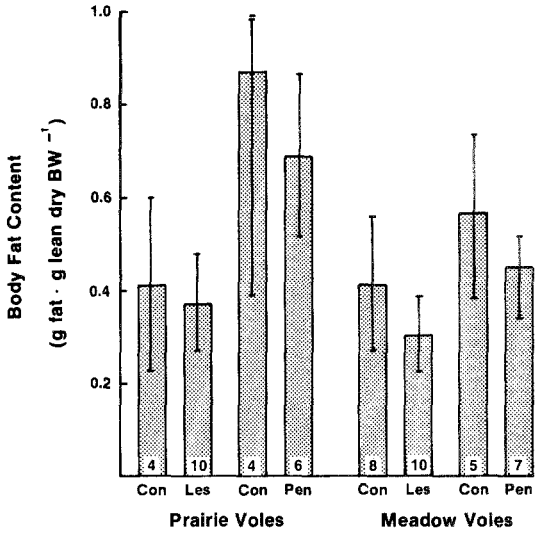

FIG. 2. Body fat content of prairie voles and meadow voles. Vertical lines show 95% confidence intervals; numbers represent sample sizes.

more body fat than meadow voles, and control animals tended to have more body fat than animals fed the *Penstemon* diet. Finally, fat content of all animals in the *Penstemon* trial tended to be greater than that of animals in the *Lespedeza* trial. Reasons for the differences between control animals in the two trials remain unclear, but could be due to differences in the control diets (ethanol vs. sulfuric acid added) or due to differences among animals used in the two trials. The latter possibility emphasizes the need to run control and experimental animals simultaneously in all feeding trials.

Consumption and Digestion of Diets. Voles fed diets containing *Lespedeza* phenolics tended to have higher consumption rates than did voles fed the corresponding control diet (Table 3). Overall digestibility of the *Lespedeza* diet was about 7% less than that of the control diet for both species of voles, but because intake rates were slightly higher in the former case, amounts of digested dry matter were similar for the two diets. Voles fed the *Lespedeza* diet digested only about 44% of the protein digested by voles fed the control diet. Increased consumption offset this difference only slightly; total protein digested by voles on the *Lespedeza* diet was about 51% of that digested by voles on the control diet. Two-way analyses of variance (Table 2) indicated that in general, prairie voles digested protein better than did meadow voles, and that for both species together, *Lespedeza* phenolics significantly increased food consumption and decreased total digestibility, protein digestibility, and amount of digested protein.

Prairie voles fed the *Penstemon* diet consumed more of the diet than did voles fed the control diet, but meadow voles did not show such a response

TABLE 3. MEAN (±1 SE) CONSUMPTION AND DIGESTIBILITY OF *Lespedeza, Penstemon,* AND CONTROL DIETS BY SUBADULT VOLES*

Species and diet	Ingested dry matter (mg/g $BW^{0.5}$/day)	Dry matter digestibility (%)	Digested dry matter (mg/g $BW^{0.5}$/day)	Apparent protein digestibility (%)	Protein digested (mg/g $BW^{0.5}$/day)
Lespedeza digestibility trials					
Prairie vole					
Control (10)	$905 \pm 43^{a**}$	58.3 ± 0.9^{g}	528 ± 26^{i}	77.5 ± 1.2^{o}	67.2 ± 3.2^{s}
Lespedeza (10)	$1076 \pm 44^{a,b**}$	51.3 ± 0.9^{f}	555 ± 29^{i}	33.3 ± 2.1^{m}	34.0 ± 3.0^{r}
Meadow vole					
Control (10)	$974 \pm 47^{a,b}$	58.3 ± 1.2^{g}	567 ± 28^{i}	71.3 ± 1.3^{n}	66.6 ± 3.5^{s}
Lespedeza (10)	1124 ± 92^{b}	51.7 ± 0.8^{f}	576 ± 42^{i}	32.2 ± 1.8^{m}	34.8 ± 2.5^{r}
Penstemon digestibility trials					
Prairie vole					
Control (4)	851 ± 27^{c}	57.6 ± 0.5^{h}	490 ± 13^{j}	77.2 ± 1.9^{q}	62.9 ± 3.1^{t}
Penstemon (6)	982 ± 32^{d}	58.5 ± 0.9^{h}	573 ± 18^{k}	75.8 ± 1.4^{q}	71.2 ± 2.0^{t}
Meadow vole					
Control (6)	1125 ± 44^{e}	58.1 ± 0.5^{h}	654 ± 24^{l}	65.4 ± 3.8^{p}	71.0 ± 6.6^{t}
Penstemon (7)	$1099 \pm 45^{d,e}$	57.5 ± 0.5^{h}	632 ± 27^{l}	71.9 ± 2.9^{p}	76.0 ± 5.1^{t}

[a] Values within each digestibility trial–performance category that bear different superscripts are significantly different (LSD test, $P < 0.05$). Sample sizes are shown in parentheses.

[b] Ingestion rates for prairie voles in the *Lespedeza* trial are significantly different at $P < 0.055$.

(Table 3). Both species of voles digested similar amounts of the two foods. Prairie voles on the *Penstemon* diet digested more dry matter than did control animals, reflecting their increased consumption rate; meadow voles showed similar rates for this parameter. Neither apparent protein digestibility nor amount of protein digested differed between voles fed the *Penstemon* or control diets. Two-way analyses of variance (Table 2) verified that meadow voles ate and digested more of both the control and *Penstemon* diets than did prairie voles. Meadow voles digested protein less well than did prairie voles, but because of their higher intake rates, the two species digested similar amounts of protein. The main effect of diet was not significant for any of the consumption/digestibility parameters for both species together. However, the interactive effect was significant or nearly so for ingested and digested dry matter and suggests that those two vole species respond differently to the presence of *Penstemon* alkaloids in their food.

Uronic Acid Excretion. Prairie voles and meadow voles on the *Lespedeza* diet excreted 2.0 and 2.3 times, respectively, the amount of uronic acid excreted by control animals (Figure 3). Uronic acid excretion did not differ, however, between voles fed *Penstemon* or control diets. In this trial, meadow voles excreted less uronic acid than did prairie voles (Table 2).

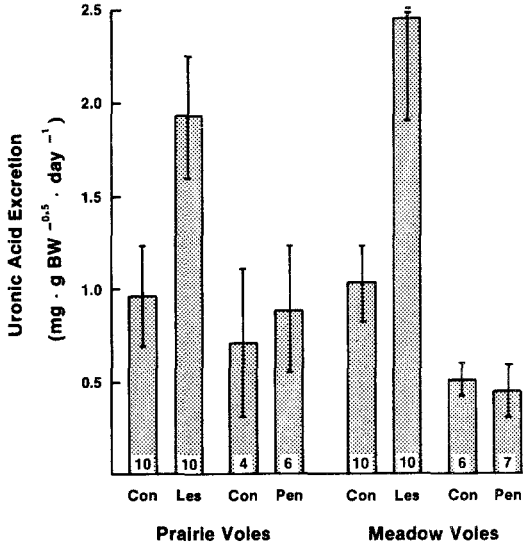

FIG. 3. Uronic acid excretion by prairie voles and meadow voles. Excretion rates are adjusted for weight-dependent average daily metabolic rates [$BW^{-0.5}$, Grodzinski and Wunder (1975)]. Vertical lines indicate 95% confidence intervals; numbers represent sample sizes.

DISCUSSION

That phenolics and alkaloids influence the palatability of plants to mammalian herbivores is well known (e.g., Kendall and Sherwood, 1975; Oates et al., 1977; Bryant and Kuropat, 1980; Lindroth and Batzli, 1984b). Thus it is likely that the phenolics in *Lespedeza* and the alkaloids in *Penstemon* made fresh leaves of these plants unpalatable to voles compared with a highly palatable alternative (rabbit pellets). Results from a previous study (Batzli and Cole, 1979) indicate that in an experiment like ours, prairie voles would consume 20–30 times more alfalfa than they did *Lespedeza* or *Penstemon* in our palatability trials. The reason that voles consume *Lespedeza* and *Penstemon* in the field but not in the laboratory is probably because more palatable, alternative foods are less available in the prairie.

Lespedeza cuneata contains condensed tannins (Bell et al., 1965), compounds that are too large for animals to assimilate and that are purported to reduce the digestibility of dietary protein by binding to the protein itself and/or to proteolytic digestive enzymes (Swain, 1979). Studies with cattle have shown that the dry matter digestibility of *Lespedeza* is inversely correlated with its tannin content (Donnelly and Anthony, 1969, 1970). Booth and Bell (1968), however, found that *Lespedeza* tannins (2% of diet) had no effect on growth or reproductive performance of rats.

Results from our study show that voles required about five days to develop at least partial acclimation to a *Lespedeza* diet. During this time growth rates were slow, most likely because of the physiological and/or deterrent effects of the tannins. After this initial phase, the growth rates of experimental animals more closely matched those of control animals. Cecal and ruminal microbes metabolize a variety of plant secondary compounds, and a change in the diet of an herbivore often induces a shift in the activity and composition of its microbial community (Allison, 1978). Voles have enlarged ceca, and although little is known about the microbial metabolism of tannins, it is possible that the shift in growth rates of voles fed *Lespedeza* diets occurred concomitantly with a change in their cecal microbial community.

Clearly, the most pronounced effect of *Lespedeza* tannins on voles was to greatly reduce the digestibility of protein. Voles fed *Lespedeza* diets digested only about half the amount of protein digested by control animals. Our previous research (Lindroth and Batzli, 1984b) showed that prairie voles require between 5 and 8% dietary protein for normal growth, and this may explain why the growth of voles in this study was not substantially affected. The protein level of our artificial diet was 10% (similar to that of *Lespedeza* in summer), so a 50% reduction in digested protein is near the lower limit of protein that will provide normal growth for voles. Meadow voles may have slightly higher requirements (as suggested by the work of Shenk et al., 1970), and this may

explain why the relative performance of meadow voles fed *Lespedeza* was poorer than that of prairie voles. These results may also explain the low palatability of fresh *Lespedeza* and the poor survival of voles in the preliminary experiment with a higher concentration of *Lespedeza* phenolics. Given that digested protein decreased by more than 50% on a diet with 50% of the normal concentration of *Lespedeza* phenolics, a pure *Lespedeza* diet would probably provide very little, if any, protein to voles.

This study indicates that *Lespedeza* tannins function primarily by disrupting digestive processes rather than by producing a toxic effect (to be toxic, a chemical must first be assimilated). Although uronic acid excretion doubled in animals fed *Lespedeza*, our previous work with assimilable phenolics showed uronic acid excretion to increase by one to several orders of magnitude (Lindroth and Batzli, 1983). Moreover, Booth and Bell (1968) were unable to detect the presence of phenolic degradation products in the urine of rats fed lower levels of *Lespedeza* tannins. Thus, most of the tannins we fed to voles were probably excreted bound to protein in the feces. The relatively small increase in uronic acid excretion probably reflects the presence of smaller, assimilable phenolics that were extracted and incorporated into the artificial diet along with the tannins.

These results lend support to digestibility reduction as the mode of action of tannins as proposed by Feeny (1976) and Rhoades and Cates (1976). This study is one of only a few that have actually demonstrated such a mode of action by tannins in wild animals. Effects of tannins are not limited to the disruption of digestive processes, however. As our earlier work (Lindroth and Batzli, 1984b) showed, tannins also may function as toxins (tannic acid) or feeding deterrents (quebracho) to voles. If tannin structure determines function (Zucker, 1983) it may be that at least for mammalian herbivores, condensed tannins (if consumed at all) operate as digestibility reducers, whereas hydrolyzable tannins do not. This possibility is consistent with the fact that condensed tannins are more refractory to chemical degradation than are hydrolyzable tannins (Roux et al., 1980).

Our data do not support the hypothesis that *Lespedeza* phenolics are more deleterious to prairie voles than to meadow voles. In fact, we found the opposite to be true. Thus some factor(s) other than differential response to phenolics must explain the difference in utilization of *Lespedeza* by prairie voles and meadow voles in tallgrass prairie.

Lindroth et al. (1986) recently identified the alkaloid in *Penstemon digitalis* as boschniakine, a pyridine monoterpene alkaloid. This alkaloid is similar to those that occur in related genera and species of the Scrophulariaceae (McCoy and Stermitz, 1983; Roby and Stermitz, 1984). Except that it appears to act as an attractant to cats [similar to neonepetalactone (Sakan et al., 1967)], little is known about the physiological effects of boschniakine. Pyridine monoterpene alkaloids in general exhibit a variety of physiological effects, including hypotensive, antiinflammatory, and muscular relaxant actions (Cordell, 1981).

Prairie voles and meadow voles differed in their initial response to the *Penstemon* diets; growth rates of prairie voles were significantly lowered (due to toxicity and/or feeding deterrence) whereas those of meadow voles were not. Following this initial phase, the growth rates of prairie voles increased, suggesting induction of enzymatic detoxication systems such as the mixed function oxidases, hydrolases, or glucuronyltransferases, all of which are involved in the metabolism of various alkaloids (Robinson, 1979). Although the final body weights of prairie voles fed control or *Penstemon* diets did not differ significantly (Figure 1), the fact that voles fed the latter diet had elevated intake rates (15%) indicates an increased metabolic cost in the processing of this diet, i.e., a "toxic" response. Growth rates of meadow voles fed the *Penstemon* diet also increased after the first five days (Figure 1), suggesting adaptation to the diet. However, meadow voles on the two diets did not differ for any of the intake/digestibility parameters (Table 3) and, unlike prairie voles, did not exhibit a metabolic cost in utilization of the *Penstemon* diet. Finally, we observed no evidence that *Penstemon* alkaloids were metabolized by conjugation with glucuronic acid. Levels of ingested alkaloids (0.005%) may simply have been too low for us to detect or the compounds may have been detoxified by an alternative biochemical pathway. Our results agree with those of Kendall and Sherwood (1975), who found that reed canarygrass containing various indole alkaloids was unpalatable to voles. Unlike our results, Kendall and Sherwood (1975) also showed that isolated alkaloids decreased consumption of a test diet. However, in a later study Kendall and Goelz (1981) found that intake of an artificial diet by meadow voles increased with nonlethal levels of gramine.

In general the performance of meadow voles on the various diets was inferior to that of prairie voles. Meadow voles tended to consume more food (Table 3) but grew less (Fig. 1) than prairie voles. Thus their efficiencies of conversion of ingested food were lower than those of prairie voles. The two species showed similar capacities to digest diet dry matter, but meadow voles generally digested a smaller proportion of dietary protein (Table 3). One possible explanation for the poor performance of meadow voles is that the artificial diet used in this study may not have been adequate for them. However, another set of meadow voles were fed rabbit pellets during the course of our experiments, and although their initial growth rates were faster, their final body weights and rates of intake for food and digested dry matter did not differ significantly from voles fed the artificial diet (Lindroth, unpublished data). Another explanation, which agrees with our previous experience with meadow voles, is that meadow voles simply perform (grow and reproduce) poorly under laboratory conditions.

That *Lespedeza* phenolics and *Penstemon* alkaloids tended to increase consumption rates by prairie voles is a surprising outcome for compounds that supposedly protect plants from grazing. In an earlier study (Lindroth and Batzli, 1984b), we observed a similar response for prairie voles fed quercetin or tannic acid. This anomaly can be explained in part by the fact that voles in our studies

had no alternative foods from which to choose, whereas in a field situation a variety of alternative foods (some perhaps less noxious) are available. Second, as has been suggested for insect herbivores (Price et al., 1980), longer periods of foraging will lead to longer exposure to predators, and this could indirectly reduce the impact of voles upon plants.

In conclusion, our results show that some of the secondary compounds abundant in the dominant forbs of a local prairie habitat influence consumption and digestibility of diets, but probably do not greatly reduce the performance (growth or survival) of voles when consumed at the levels we have observed. Although *Lespedeza* and *Penstemon* are not excellent foods in an absolute sense, they probably contain more nutrients or fewer chemical defenses than many alternative food plants. We do not yet know how *Lespedeza* phenolics or *Penstemon* alkaloids may influence vole performance when ingested with other secondary compounds within the same plant (e.g., phenolics in *Penstemon*) or in a mixture of plants (e.g., *Lespedeza* tannins may disrupt digestion of other forage plants as well, all of which have lower levels of protein than *Lespedeza*). These compounds probably place an upper limit on the amount of any one species that can be consumed, thereby enforcing a generalist feeding strategy by voles. Finally, we see once again that mammalian herbivores have nutritional needs and adaptations that differ among closely related species. These differences should be recognized and considered in studies assessing the role of forage quality in the dynamics of herbivore communities.

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FEEDING RESPONSES OF SNOWSHOE HARES (*Lepus americanus*) TO VOLATILE CONSTITUENTS OF RED FOX (*Vulpes vulpes*) URINE

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Abstract—This study investigated the influence of the volatile constituents of red fox (*Vulpes vulpes*) urine in suppressing feeding by snowshoe hares (*Lepus americanus*) on coniferous tree seedlings. Pen and field bioassays indicated that the odor of fox urine and its principal component, 3-methyl-3-butenyl methyl sulfide, had a negative effect on feeding behavior of hares. The other sulfur-containing compounds, 2-phenylethyl methyl sulfide and 3-methylbutyl methyl sulfide, as well as six other constituents, were not effective. Synthetic urine mixtures composed of eight and nine volatile constituents, respectively, did not suppress feeding in pen bioassays. However, the mixture of eight compounds in a field bioassay did result in significantly fewer seedlings being eaten by hares than in the control. 3-Methyl-3-butenyl methyl sulfide may act as an interspecific chemical signal which induces a fear or avoidance response in hares. Additional work is required to determine the optimum concentration and release system for 3-methyl-3-butenyl methyl sulfide to be used as a mammalian semiochemical in crop protection.

Key Words—Red fox urine, snowshoe hare, crop protection, sulfur-containing compounds, kairomones, forestry, volatile constituents, feeding suppression, bioassays, synthetic urine mixture, interspecific communication, *Vulpes vulpes*, *Lepus americanus*, 3-methyl-3-butenyl methyl sulfide.

INTRODUCTION

Chemical communication among mammals may involve the use of scent gland secretions, feces, and urine. A major mode of olfactory communication in the

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Canidae is urination. Volatile chemical constituents have been identified in the urine of the red fox (*Vulpes vulpes*) (Jorgenson et al., 1978; Wilson et al., 1978; Bailey et al., 1980) and the wolf (*Canis lupus*) (Raymer et al., 1984). The red fox has also been reported to communicate chemically via the supracaudal gland (Albone and Flood, 1976) and anal sac (Albone and Perry, 1976).

On a behavioral basis, synthetic urinary constituents have induced marking behavior in wild red foxes (Whitten et al., 1980). The major sulfur compound, 3-methyl-3-butenyl methyl sulfide, along with 2-phenylethyl methyl sulfide, are responsible for the characteristic odor of fox urine (Wilson et al., 1978). The former compound, in addition to other sulfides, has also been reported in wolf urine (Raymer et al., 1984). Bailey et al. (1980) reported the presence of 3-methylbutyl methyl sulfide in red fox urine. Thus, these odorous compounds presumably play an important role in social communication, particularly for a solitary species such as the red fox. However, are these compounds meaningful to other species such as mammalian prey?

Certain mustelid scent compounds have suppressed feeding by snowshoe hares (*Lepus americanus*) (Sullivan and Crump, 1984), presumably because of a predator-induced "fear response" in the prey species. Sullivan et al. (1985a) reported that several predator odor preparations reduced feeding on coniferous tree seedlings by hares. Of the urines tested, red fox produced the most effective short-term suppression of feeding (Sullivan et al., 1985a). Reduction or elimination of seedling destruction by hares is a necessary prerequisite to successful forest renewal in many parts of the boreal forest of North America. Thus, this study reports on the influence of the volatile constituents of red fox urine in suppressing snowshoe hare feeding on coniferous tree seedlings.

METHODS AND MATERIALS

Pen Bioassays. Bioassays were conducted at the Applied Mammal Research Institute, Langley, B.C., Canada. The bioassay enclosure (9.15 × 18.3 m) was composed of three sections or pens set in a natural environment (see Sullivan and Crump, 1984). The entire enclosure was covered by a fiberglass roof to eliminate effects of adverse weather. The control and experimental pens (A and B) were enclosed by polyethylene on the walls to keep odors within the respective treatment sections. These pens had all natural vegetation cleared prior to start of trials. Douglas fir (*Pseudotsuga menziesii*) bark mulch covered the ground of each pen to a depth of 20 cm. Each pen had three experimental units, each composed of 10 styrofoam blocks to anchor coniferous seedlings. One-year-old (nursery stock) lodgepole pine (*Pinus contorta*) seedlings were used in all trials with one seedling per block. Thus, there was a maximum of 30 seedlings in each of the control and treatment pens (A or B) at the start of a

given trial. Lodgepole pine is preferred by snowshoe hares over all other coniferous species (see Sullivan et al., 1985a).

The natural habitat (pen C) was not cleared of native vegetation and provided cover and some grasses and forbs for hares. Thus, this pen served as a potential refuge area for hares. Access of hares to pens A or B from pen C was through 30 × 30-cm openings in each adjoining wall. Hares moved freely among all three pens and new animals were always allowed at least five to seven days to acclimate to this experimental configuration. To exert adequate feeding pressure on the experimental material and keep social interactions at a minimum, two hares were used in each trial. All hares used were from our northern study areas at Prince George, B.C. Hares were replaced at regular intervals to overcome potential variability and habituation among animals. Rabbit pellets, grass hay, and water were available ad libitum throughout these trials.

One pen bioassay (screening trial) was conducted for each fox urine compound or test mixture. Real fox urine was tested in an earlier pen bioassay as reported by Sullivan et al. (1985a).

Field Bioassays. Spring field trials (A and B) of red fox urine odors were conducted at Tabor Mountain, 25 km southeast of Prince George, B.C., during April to June 1984. This study area was located in the subboreal spruce biogeoclimatic zone (Krajina, 1969). It was overgrown with deciduous brush species, mainly willow (*Salix* spp.) and Sitka alder (*Alnus sinuata*), with some aspen (*Populus tremuloides*), black cottonwood (*P. trichocarpa*), and paper birch (*Betula papyrifera*); and had experienced coniferous plantation failure because of feeding damage by snowshoe hares. The hare population cycle peaked in this area in 1980–1981 but the peak was much more of a plateau (Sullivan and Sullivan, in preparation) than that described elsewhere (Keith and Windberg, 1978). Thus, the population of hares on Tabor Mountain continued to inflict severe damage to planted coniferous seedlings during the spring of 1984, thereby providing a suitable study area for testing our fox urine compounds.

Additional trials at Prince George may not have subjected seedlings to heavy feeding pressure as the low point in the 10-year hare cycle approaches, with increased numbers not expected before 1987–1988. Thus, fall field tests were conducted in a 4.5-hectare enclosure at the Pineridge Forest Nursery, 130 km northeast of Edmonton, Alberta. The enclosure was composed of 2-m-high steel mesh and was buried in the ground to a depth of 30 cm. This study area was located in the boreal mixed wood zone (Strong and Leggat, 1981) with jack pine (*P. banksiana*) and aspen being the main tree species. Wild rose (*Rosa* spp.) and Saskatoon berry (*Amelanchier alnifolia*) were the dominant shrub species. Within this area, four (30 × 30 m) blocks were established and separated by at least 100 m. In July 1984, each block was cleared of trees and scarified in a manner similar to a typical logging and site preparation prescription. After planting, these blocks acted as plantations of coniferous tree seedlings in our

field trial. The enclosure was stocked with snowshoe hares captured locally and from Prince George. This stocking allowed us to simulate a peak population (5–8 hares/hectare; Keith and Windberg, 1978) of hares, thereby providing a rigorous test of various predator odor compounds in a "field" situation. Both spring and fall represent periods when coniferous seedlings are particularly vulnerable to snowshoe hare feeding attacks. Snow cover is not adequate to protect seedlings, and alternative summer herbaceous foods are not available.

Fox Urine Compounds. Volatile constituents from red fox urine were prepared: 3-methyl-3-butenyl methyl sulfide according to Wilson et al. (1978); 2-phenylethyl methyl sulfide by alkylation of the corresponding thiol (von Braun et al., 1929), which was obtained from 2-phenylethanol via the Bunte salt method of El-Hewehi and Taeger (1958); and 3-methylbutyl methyl sulfide from 3-methylbutyl bromide using lithium thiomethoxide according to Wilson et al. (1978). These and 4-heptanone, 6-methyl-5-hepten-2-one, benzaldehyde, acetophenone, 2-methylquinoline, and geranylacetone (available commercially) were tested in pen bioassays. A synthetic mixture was prepared according to the composition of compounds (8) reported by Wilson et al. (1978) and Whitten et al. (1980). A second mixture, containing 9 compounds and incorporating 3-methylbutyl methyl sulfide in equal proportion with 3-methyl-3-butenyl methyl sulfide (i.e., 50 mg/liter solution), was also prepared and tested in pen bioassays. No odor fixative was added to either of these test mixtures. Real fox urine, collected and frozen prior to use (Triple D Game Farm, Kalispell, Montana), 3-methyl-3-butenyl methyl sulfide, and two test mixtures were also tested in field bioassays. Purity of synthesized compounds ranged from 45% to 98%.

Except for the real fox urine dispensed in 5-ml plastic vials in spring trial A, all compounds were dispensed in 140-μl capillary tubes (75 mm in length), one tube (or vial) attached by a plastic twist-tie to each seedling. The capillary tubes protected the compounds from adverse weather conditions (in field bioassays) and maintained the respective odor around the seedlings for optimum evaluation purposes. Approximately 30 mg of each compound (neat) or test solution was placed in a given capillary tube using a 1-ml microsyringe with 20-gauge needle. A capillary tube was attached to each control seedling to complete the design of a given experiment.

Spring Field Trial A. Each of two blocks (each 400 m^2 in area and separated by 100 m) was planted with 50 (5 $\times$ 10) two-year-old (nursery stock) lodgepole pine seedlings on April 22, 1984, at Tabor Mountain. One block served as a control and the other had real fox urine dispensed in vials. These vials were not protected from prevailing weather conditions, and so fresh urine was added each time the seedlings were checked for feeding damage by hares (days 5, 8, 12, 19, and 25). This trial was terminated on day 25 (May 17), at which time no control seedlings remained. In all trials, feeding (clipping) on

the terminal shoot was considered as mortality to a given seedling. Coniferous seedlings rarely recover from terminal feeding damage, and even if they do, growth and form of the young tree are usually severely impaired.

Spring Field Trial B. Because of the late spring at Tabor Mountain, another trial was initiated on May 17 when four blocks were established and planted in an identical manner to those described above. Blocks were assigned treatments as follows: control, real urine, synthetic urine mixture (8), and 3-methyl-3-butenyl methyl sulfide. Hare browsing of seedlings was checked on days 5, 12, 17, and finally on day 23 (June 9) when the experiment was terminated. Hares had switched to summer herbaceous foods, and hence were no longer feeding on coniferous material.

Fall Field Trial. Each of the four blocks (each 900 m^2 in area) at the Pineridge Forest Nursery was planted with 100 (10 × 10) one-year-old (nursery stock) lodgepole pine seedlings on October 3, 1984. Blocks were assigned treatments as follows (in accordance with available amounts of compounds): control, real fox urine, split block of 50 (5 × 10) seedlings with synthetic mixture (8) and 50 seedlings with synthetic mixture (9), and the fourth block had 30 (3 × 10) seedlings with 3-methyl-3-butenyl methyl sulfide. Browsing of seedlings was checked initially on day 9 and finally on day 20 (October 23) when the experiment was terminated. Snow had covered the majority of seedlings at this time, and the treatments were not designed to be tested through the winter. Monitoring (live-trapping) of snowshoe hares introduced into the enclosure indicated that at least 13 hares were present at the start of the trial. An additional 13 hares were added on day 9 for a total of 26 hares (5.8/ha) which represented a peak density of this herbivore.

Statistical Analysis. Comparison of the number of seedlings surviving in control and treatment blocks was analyzed by chi-square with significance levels $P < 0.05$ and $P < 0.01$.

RESULTS

Pen Bioassays. Of the sulfur compounds tested, 3-methyl-3-butenyl methyl sulfide was very effective in suppressing feeding by snowshoe hares (Figure 1). Control seedlings were completely eaten at the third day after survival rates of 20% and 16.7% at days 1 and 2, respectively. No treatment seedlings were eaten in the first two days. Upon removal of 3-methyl-3-butenyl methyl sulfide after the fourth day, hares readily consumed the unprotected seedlings. 2-Phenylethyl methyl sulfide suppressed feeding (80% survival) after one day but survival of seedlings declined sharply thereafter. This latter response was typical of a novel odor whereby hares react negatively to a new odor for one or two days before realizing that the volatile substance is apparently not biologically meaningful. 3-Methylbutyl methyl sulfide and the synthetic urine mixtures

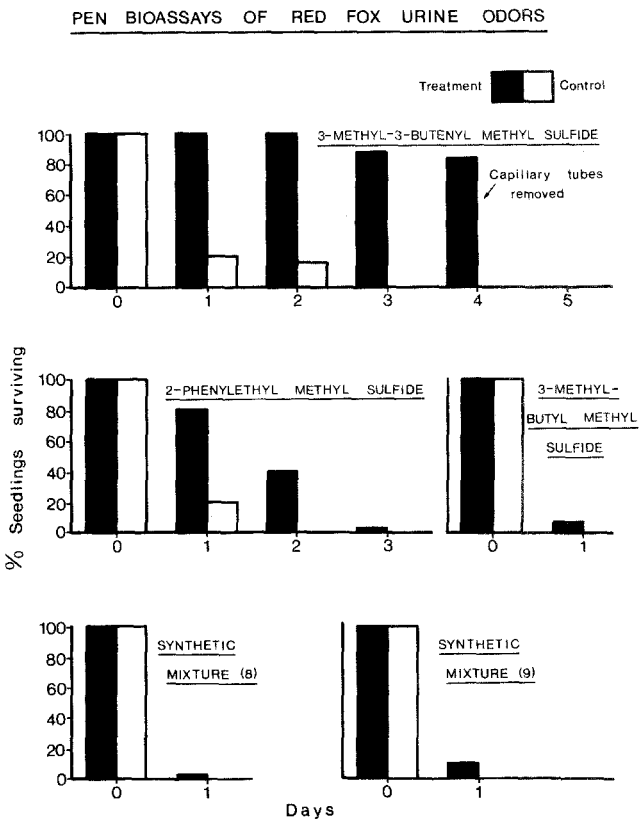

Fig. 1. Effectiveness of volatile constituents of red fox urine in suppressing snowshoe hare feeding on lodgepole pine seedlings in pen bioassays: 3-methyl-3-butenyl methyl sulfide, 2-phenylethyl methyl sulfide, 3-methylbutyl methyl sulfide, and the synthetic urine mixtures (8) and (9).

did not suppress feeding by hares in a pen situation. Individual bioassays of 4-heptanone, 6-methyl-5-hepten-2-one, benzaldehyde, acetophenone, 2-methylquinoline, and geranylacetone indicated that these compounds did not suppress feeding by hares.

Field Bioassays. In the spring trial A, real fox urine (in vials) suppressed feeding by hares compared with the control (Figure 2). This difference was significant ($P < 0.01$) and was maintained throughout the 25-day trial. As indicated in Figure 2, substantial rainfall occurred on days 10, 16, 21, and 22, and this may have contributed to the declining efficacy of the urine odor by dilution in the vials. This was particularly noticeable after day 12, since prior to this check, seedling survival had apparently stabilized at 82%.

To further test real urine and some of its constituents as repellents to feed-

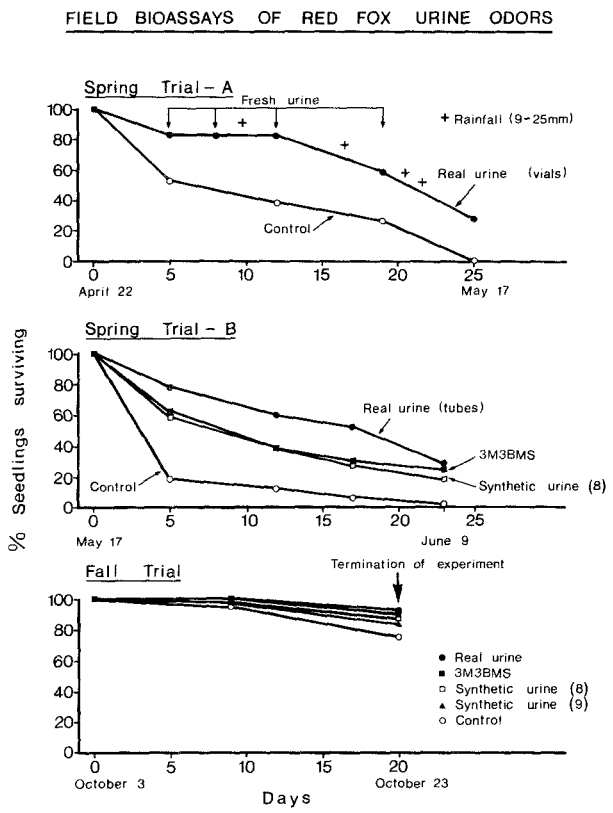

Fig. 2. Field efficacy of volatile constituents of red fox urine in suppressing snowshoe hare feeding on lodgepole pine seedlings: spring trial A, real urine (vials); spring trial B, real urine (capillary tubes), 3M3BMS (3-methyl-3-butenyl methyl sulfide), and synthetic urine mixture (8); fall trial, real urine (capillary tubes), 3M3BMS, and synthetic urine mixtures (8) and (9).

ing by hares, the spring trial B was initiated. Results of this trial are also illustrated in Figure 2. Again, real urine (in capillary tubes) was the most effective odor with significantly ($P < 0.01$) higher survival of seedlings than in the control block throughout the trial. Survival of control seedlings was reduced to 18% at day 5. In addition, real urine was significantly ($P < 0.05$) more effective than 3-methyl-3-butenyl methyl sulfide or the synthetic mixture (8) up to day 17. The difference between real urine and 3-methyl-3-butenyl methyl sulfide at day 5 approached significance ($P = 0.08$). There was little difference between these three odors at day 23. Both 3-methyl-3-butenyl methyl sulfide and the synthetic mixture (8) also significantly ($P < 0.01$) suppressed feeding by hares compared with the control block.

To further assess the negative effect of urine odors on snowshoe hare feeding, and to provide some degree of replication, the fall trial was conducted in habitat typical of coniferous plantations in the boreal forest. As a result, intensity of feeding was reduced compared with the spring trials (see Figure 2). There was little feeding up to day 9 in this trial but, following an introduction of more hares, significant differences among treatments appeared at day 20. Hares consumed more control seedlings (25%) than those in the real fox urine (8%) block ($P < 0.01$). In addition, the differences between the control block and 3-methyl-3-butenyl methyl sulfide ($P = 0.08$) and synthetic mixture (8) ($P = 0.07$) approached statistical significance and may certainly be biologically meaningful results. There were no significant differences among the various treatments.

DISCUSSION

This study has demonstrated that the odor of red fox urine and its principal volatile constituent, 3-methyl-3-butenyl methyl sulfide, will suppress feeding on lodgepole pine seedlings by snowshoe hares. This conclusion corroborates that obtained by Sullivan et al. (1985a) for red fox urine tested on hares in a short-term pen bioassay. Although field results were not as outstanding as those obtained with certain mustelid scent-gland compounds (Sullivan and Crump, 1984), suppression of feeding in the presence of real urine and 3-methyl-3-butenyl methyl sulfide was still highly significant when compared with a control. Pen bioassays clearly demonstrated that 3-methyl-3-butenyl methyl sulfide was the functional compound responsible for altering hare feeding behavior, of the eight volatile constituents identified by Jorgenson et al. (1978) and the additional 3-methylbutyl methyl sulfide reported by Bailey et al. (1980).

3-Methyl-3-butenyl methyl sulfide may be potentially regarded as a kairomone (Brown et al., 1970), since it presumably acts as a scent-marker signal to hares that a fox is, or was recently, in the area. As discussed for snowshoe hares and certain mustelids (Sullivan and Crump, 1984), this interspecific communication is of adaptive advantage to the hare if it avoids the marked area, and hence is less likely to be preyed upon. The red fox is an opportunistic predator whose diet is influenced by food availability. Several dietary studies of the red fox in North America indicate that their major prey is small rodents and lagomorphs (Errington, 1935; Scott, 1943; Scott and Klimstra, 1955; Pils and Martin, 1978). In boreal forest habitats during years of hare abundance, the diet of this canid is dominated by snowshoe hare (Jones and Theberge, 1983). Thus, 3-methyl-3-butenyl methyl sulfide may act as an interspecific signal between predator (fox) and prey (hare).

Vernet-Maury et al. (1984) reported that several volatile compounds, isolated from red fox feces (and urine) induced stress in laboratory rats. Of the

compounds tested, 3-methyl-3-butenyl methyl sulfide and 2-phenylethyl methyl sulfide did not cause stress. The result with the former compound is in marked contrast with our results for the hare–fox interaction. The presence of 3-methyl-3-butenyl methyl sulfide in wolf urine (Raymer et al., 1984) may explain the significant suppression of feeding by hares (Sullivan et al., 1985a) and black-tailed deer (*Odocoileus hemionus columbianus*) (Sullivan et al., 1985b) when tested with the urine odor of this other canid species. In addition, deer avoided food associated with red fox urine (Sullivan et al., 1985b).

It is not clear at this stage why real fox urine was more effective than 3-methyl-3-butenyl methyl sulfide and the synthetic mixture (8) in the second spring trial. If one or more components of real urine were missing from the synthetic mixture, then the conclusive results obtained by Whitten et al. (1980) for induction of marking by wild red foxes would not be expected. The eight compounds identified were mixed together in a test solution very similar to that used by Whitten et al. (1980). We did not include an odor fixative so as to eliminate any artificial smell associated with the urine. However, it may have been useful to include an inert binding compound such as squalene to retard volatility of the compounds, particularly the highly volatile 3-methyl-3-butenyl methyl sulfide and 2-phenylethyl methyl sulfide. The inclusion of 3-methylbutyl methyl sulfide was an attempt to improve the efficacy of synthetic urine by adding another compound. The latter compound had little effect compared with the original synthetic mixture (8). It should be noted that 3-methylbutyl methyl sulfide was tested as a novel odor and had no effect on hare feeding reported by Sullivan et al. (1985a). There was little difference between real urine and the synthetic mixtures as well as 3-methyl-3-butenyl methyl sulfide in the fall field trial.

The original hypothesis of Sullivan et al. (1985a) that the negative but transient response of hares to predator odors was due to evaporative loss of the active repellent compound(s) rather than habituation should be discussed in light of our results. Clearly, the survival of seedlings in both control and treatment blocks in the field declined over time. In the first spring trial (Figure 2), much of this decline might be explained by dilution of the urine by rainfall. However, in the subsequent trials, all treatments were dispensed in capillary tubes and were essentially weatherproof. If the decline in efficacy was due to habituation, then the differences between treatment and control should not have been maintained for periods of 25 days (spring trial A) and 23 days (spring trial B). Hares which habituate to an olfactory stimulus in a treatment area would presumably consume seedlings at a level comparable to the control situation. A sudden increase in consumption of treatment seedlings did not occur in these trials. Therefore, perhaps the concentration and release rates of compounds, particularly 3-methyl-3-butenyl methyl sulfide, is the critical factor in maintaining a predator-induced "fear response" in snowshoe hares. Additional work is required to determine the optimum concentration and release system for 3-methyl-

3-butenyl methyl sulfide to produce a functional avoidance response in snowshoe hares. Such investigation might help explain the poor efficacy of synthetic urine mixtures in pen compared with field bioassays.

The reduced intensity of feeding in the "open" plantation habitat (fall trial) compared with the overgrown habitat at Tabor Mountain was not surprising. In a habitat manipulation study, Sullivan and Moses (1986) reported a dramatically reduced abundance of hares in a scarified habitat, but the amount of feeding damage to planted coniferous trees was still unacceptable. This was the case in our study as well, where 25% of seedlings were destroyed after only 20 days. A scheme of integrated management is urgently required combining habitat alteration and predator odor repellents to reduce or eliminate feeding damage by hares and related herbivores. This study and those of Sullivan and Crump (1984) and Vernet-Maury et al. (1984) have clearly demonstrated the potential of mammalian–predator semiochemicals for use as area repellents to protect forest and agricultural crops.

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IDENTIFICATION OF CANTHARIDIN IN FALSE BLISTER BEETLES (COLEOPTERA, OEDEMERIDAE) FROM FLORIDA

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Abstract—Cantharidin, a potent vesicant and antifeedant, is identified for the first time in two oedemerid beetles from the western hemisphere. Amounts of the substance per beetle were found to range from 2 to 7 μg in *Heliocis repanda* and from 15 to 35 μg in *Oxycopis thoracica*. Females had two to three times more cantharidin than males of the same species. Cantharidin loads of these beetles are sufficient to irritate human skin.

Key Words—Cantharidin, defense, chemical, vesicant, antifeedant, *Heliocis repanda*, *Oxycopis thoracica*, Coleoptera, Oedemeridae, sexual dimorphism.

INTRODUCTION

Beetles of the family Oedemeridae in Florida, Puerto Rico, Japan, and elsewhere occasionally cause severe skin blisters in humans (Herms, 1925; Vaurie, 1951; Fleisher and Fox, 1970; Kurosa, 1977; Arnett, 1984). Gentle manipulation can cause the insects to discharge vesicating droplets from the elytra and pronotum, but most cases of dermatitis arise after these "false blister beetles" are crushed on the body (Fleisher and Fox, 1970; Arnett, 1984). Patch tests using squashed beetles suggest that most oedemerid species can irritate human skin (Kurosa, 1958).

The vesicant in adult oedemerids is thought to be cantharidin (Arnett, 1984), the potent antifeedant widespread in "true blister beetles" (family Meloidae) (Carrel and Eisner, 1974), but evidence for this idea is scant. To our knowledge only one false blister beetle has been rigorously tested. Kurosa and

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Watanabe (1958) identified pure cantharidin in extracts of *Xanthocroa waterhousei* Harold, a Japanese species, by mixture melting point as well as elemental and infrared spectral analyses. Based on gas chromatographic and mass spectral evidence, we here report that cantharidin also is present both in *Oxycopis thoracica* (Fabricius) and in *Heliocis repanda* (Horn) from central Florida.

METHODS AND MATERIALS

Insects. Nine *O. thoracica* adults were collected April 27, 1984, at UV lights at the Archbold Biological Station in Highlands County, Florida. Transported alive to our laboratory, they were placed by sex into two groups (N = 4 males, 5 females), weighed, and frozen.

Sixty-seven *H. repanda* adults caught from April 20 to May 10, 1984, while feeding on the flowers of dwarf chestnut, *Castanea pumila* (L.), outside the Doyle Conner Building in Gainesville, Alachua County, Florida, were preserved together in a vial of alcohol. Beetles were removed from the vial, air-dried for 0.5 hr, sexed (N = 31 males, 36 females), and then a subset (N = 40 beetles total) was placed by sex into eight groups of five individuals each. Each group was weighed and then frozen. To ascertain the amount of cantharidin leached from beetles, two aliquots (1 ml each) of the alcohol (13.2 ml total) used to preserve all 67 beetles were placed separately in the freezer.

Chemical Analysis. After thawing, beetles in each group were cut into several pieces and submitted as a group to acid hydrolysis and extraction (Carrel and Eisner, 1974; Carrel et al., 1985). Samples of alcohol were treated as extracts.

Cantharidin in each extract was determined by capillary gas chromatography with flame ionization detection (Carrel et al., 1985). A fused silica column (27 m × 0.329 mm ID) coated with 0.25-μm DB-5 (J&W Scientific, Rancho Cordova, California) was used in a Varian 3700 gas chromatograph with the injector and detector temperatures maintained at 190° and 320°C, respectively. The column was programmed from 100°C upon injection to 270°C at 20°C/min. Air, hydrogen, and helium flow rates were 300, 30, and 30 ml/min, respectively. A Hewlett-Packard 3390 integrator was used to quantify the chromatograms. Benzophenone (Aldrich Chemical, Milwaukee, Wisconsin) was used as an internal standard to verify the retention time of cantharidin and to permit accurate quantification of the amounts of cantharidin in unknown extracts. Authentic cantharidin from Inland Alkaloid, St. Louis, Missouri, served as a reference standard.

The identification of cantharidin as a component in oedemerid extracts was verified by gas chromatographic–mass spectrometric (GC-MS) analyses using a Carlo Erba 4160 capillary gas chromatograph coupled to a Kratos 25 mass spectrometer. The GC-MS data for extracts and the cantharidin standard were

obtained using a 0.25-μm methylsilicone capillary column (30 m $\times$ 0.25 mm ID) run at 60°C for 2 min, then programmed to 250°C at 15°/min with helium (2.5 ml/min) as the carrier gas. Electron impact (EI) mass spectra were collected at 70-eV ionizing voltage with the separator at 230°C and the source at 200°C. The EI spectra were acquired and processed by a Kratos 55 data system.

RESULTS

Cantharidin was detected in both oedemerid species we examined. Representative chromatograms of the extracts for each species are shown in Figures 1 and 2. GC-MS comparisons with the reference compound confirmed the authenticity of cantharidin in the extracts.

Quantitative measurements, presented in Table 1, reveal two major differences in the cantharidin content of individual oedemerids. First, *O. thoracica* adults each contained much more cantharidin (15–35 μg/beetle) than *H. repanda* adults (2–7 μg/beetle). Second, intraspecific comparisons show that females had two to three times more cantharidin in their bodies than did males. Because both species showed the same modest size dimorphism (males and females each weighed approximately 7 and 10 mg, respectively), the species and sexual differences are maintained when cantharidin amounts are expressed on a body weight basis (see Table 1). Cantharidin (72 μg total) in the alcohol used to preserve 67 *H. repanda* was omitted from our calculations; its inclusion would only slightly change our data for individuals of this species.

DISCUSSION

The results in this paper represent the first definitive demonstration of cantharidin in oedemerids from the western hemisphere. The amounts of cantharidin we detected in oedemerids (0.03–0.3% of beetle weight) are low in comparison to those in meloids (1% of beetle weight on average) (Dixon et al., 1963; Walter and Cole, 1967), but they are sufficient to irritate human skin (Sollman, 1948; J. Carrel, unpublished observations). If cantharidin is widespread in oedemerids, then at least in a biochemical sense there is nothing "false" about these blister beetles, as their common name suggests.

That cantharidin serves as a defensive function in oedemerids, much as it does in meloids (Carrel and Eisner, 1974), seems reasonable to propose. Our data in Table 1 suggest three specific topics for future research on the defensive chemistry of oedemerids. First, is the cantharidin content of individual beetles correlated with an aspect of their behavioral ecology? Our findings show that a nocturnal species, *O. thoracica*, contains more cantharidin than a strictly diurnal species, *H. repanda*. We suspect that beetles moving about freely in the

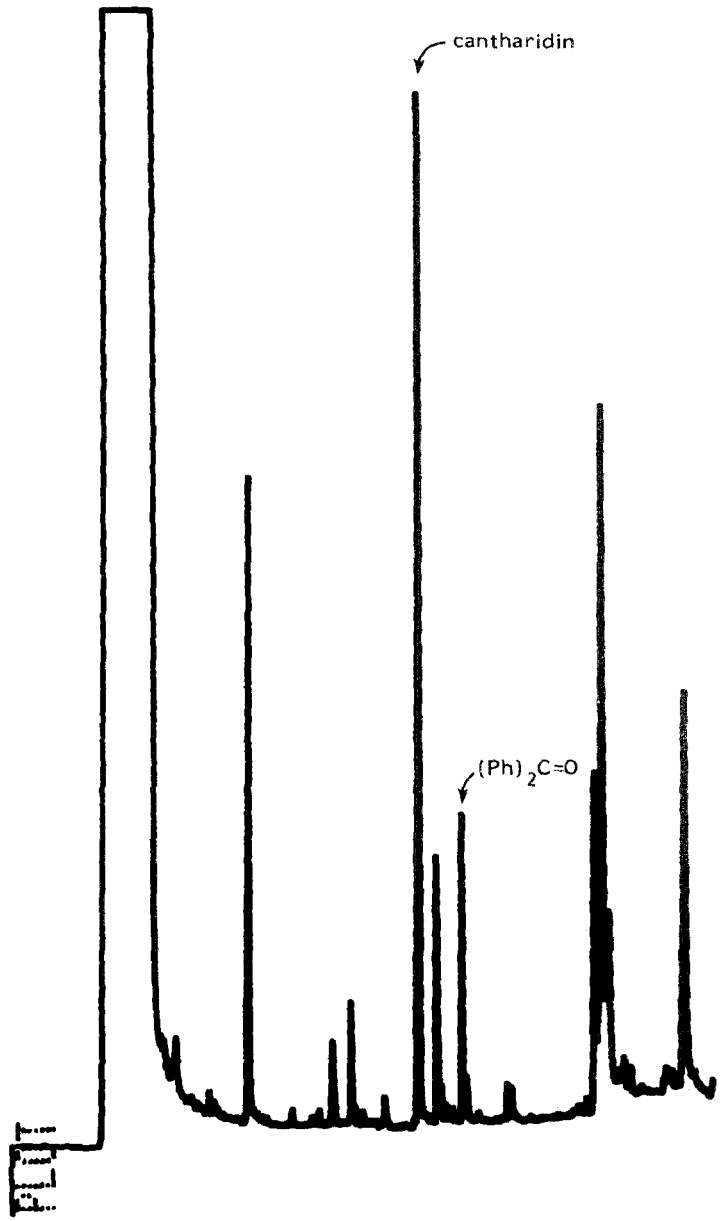

FIG. 1. Gas chromatogram showing the quantitation of cantharidin in an extract of five adult female *Oxycopis thoracica*. Labeled peaks of interest indicate an injection that was found to contain 211 ng cantharidin using 24.4 ng of added benzophenone as the internal standard. Retention times are 5.16 and 5.71 min, respectively.

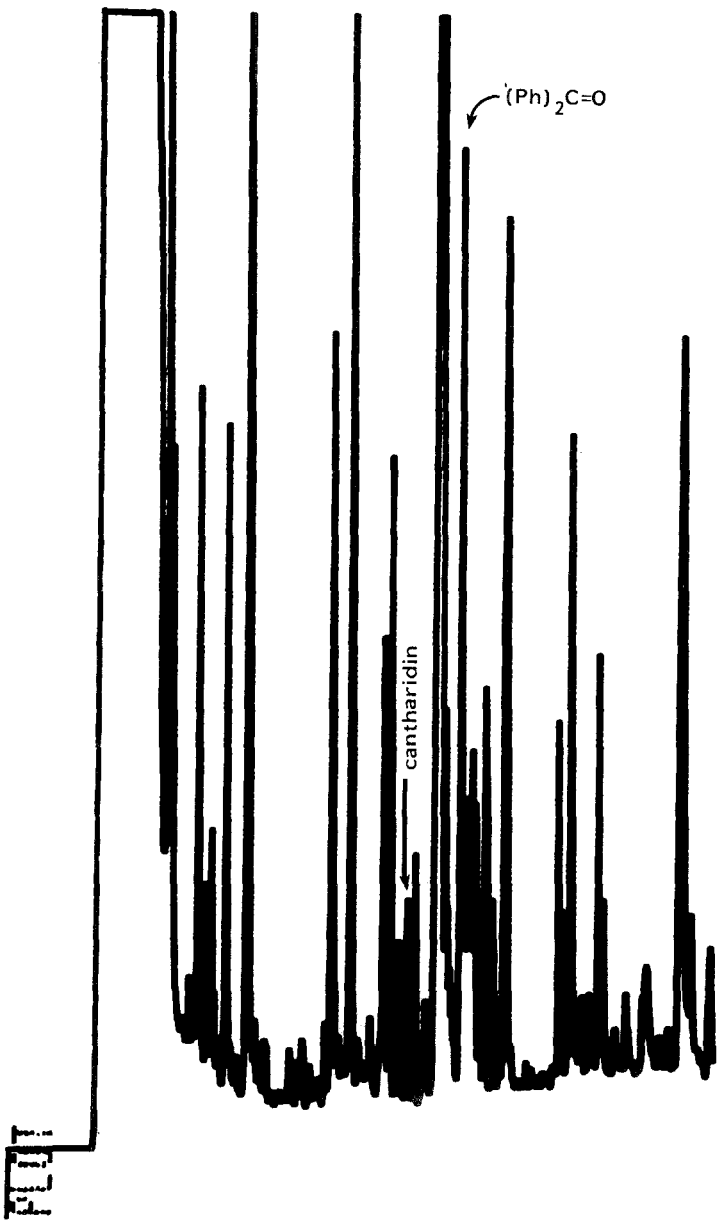

FIG. 2. Gas chromatogram showing the quantitation of cantharidin in an extract of five adult male *Heliocis repanda*. Labeled peaks of interest indicate an injection that was found to contain 34.3 ng cantharidin using 61.1 ng of added benzophenone as the internal standard. Retention times are identical to those in Figure 1.

TABLE 1. CANTHARIDIN IN OEDEMERID BEETLES FROM FLORIDA

Species	Sex	Number of samples analyzed	Beetle body mass (mg)[a]	Cantharidin[a]	
				μg/beetle	μg/mg of beetle
Oxycopis thoracica (Fabricius)	Male	1	6.7	15.5	2.3
	Female	1	10.4	35.2	3.4
Heliocis repanda (Horn)	Male	4	7.3 ± 0.4 [b]	2.1 ± 0.1 [b]	0.29 ± 0.03 [b]
	Female	4	10.3 ± 0.4	7.4 ± 0.8	0.72 ± 0.08

[a] Values are means ($\bar{X} \pm$ SEM) for samples containing four or five beetles.
[b] Adjacent values in a column are significantly different at $P < 0.01$ using Student's t-test.

dark might depend primarily on chemical mechanisms to deter predators, whereas their day-active counterparts might rely on a combination of visual and chemical defenses.

Second, what is the adaptive explanation for sexual differences in the cantharidin content of oedemerids? Perhaps female beetles have more of the substance in order to achieve a greater degree of protection for themselves and for the gametes they harbor than that enjoyed by males. A similar situtation exists in the meloid *Epicauta pennsylvanica* (Carrel, 1971), but the opposite is true for several other meloid species (Sierra et al., 1976).

Third, what is the source of cantharidin in oedemerids? Is it the case, as in meloids (Sierra et al., 1976), that male beetles biosynthesize the substance de novo from farnesyl pyrophosphate and then transfer it during mating to females? Or do they obtain cantharidin through dietary means? We are conducting predation and biosynthetic studies with oedemerids and meloids to answer these questions.

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IDIOSYNCRATIC VARIATION IN CHEMICAL DEFENSES AMONG INDIVIDUAL GENERALIST GRASSHOPPERS

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Abstract—The defensive secretion of the lubber grasshopper, *Romalea microptera*, shows extreme chemical variation among individual adults of the same sex within a single wild population. Certain phenolic compounds were absent in some individuals and present in others. Concentrations of compounds, when present, varied over two to three orders of magnitude. Chemical variation attributable to individuals accounted for 60–88% of the total quantitative variation and was evenly contributed by all individuals in both sexes. Cluster and regression analyses showed no discernible predictable patterns in the defensive secretion variation. The specificity of chemical cues used by predators may explain why these defenses are so idiosyncratic.

Key Words—Lubber grasshopper, *Romalea microptera* (= *guttata*), Orthoptera, Romaleidae, phenolic secretion, inter-individual quantitative and qualitative variation, idiosyncratic patterns.

INTRODUCTION

Arthropod defenses often involve chemical secretions whose taste, odor, or irritancy command rapid predator attention (Blum, 1981). Successful defense is

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assumed to require predator recognition of relatively predictable chemical cues, and subsequent avoidance of prey. These allelochemicals are often viewed as characteristic, species-specific signals of limited variability (Brower and Brower, 1964; Whittaker and Feeny, 1971; Rothschild, 1973). Consequently, studies on chemical variation in defensive secretions have primarily focused on species differences and secondarily on population differences (Tschinkel, 1975; Pasteels et al., 1983; Prestwich, 1983; Pasteels and Gregoire, 1984). Variation between individuals within a population is rarely examined, and the amount of this variation is generally unknown (Blum, 1981). The extent to which chemical variation occurs and the ecological scale at which it takes place have considerable bearing on how such defenses may operate. For example, it will affect how predators may perceive and respond to chemically defended prey and how selection for and adaptation against such predators may operate.

The lubber grasshopper, *Romalea microptera* Beavois (Orthoptera; Romaleidae) (= *R. guttata* Houttyun; Kevan, 1980) is an aposematic, generalist herbivore that is unpalatable to a number of different predators (Whitman et al., 1986). This insect produces a principally phenolic secretion from metathoracic glands. The secretion is produced as a froth generated by respiratory air from the metathoracic spiracles. The pooled, frothy, metathoracic spiracular secretion has been previously examined chemically and has also been shown to be repellent to certain ants (Eisner et al., 1971). In contrast to pooled samples, we analyzed metathoracic secretions from individuals. We wished to know whether or not individuals within a defined population showed any significant quantitative or qualitative variation in these chemical defenses. We also wanted to know whether or not such variation, if any, showed predictable patterns.

METHODS AND MATERIALS

Secretion Collection. Metathoracic glandular secretions were collected from sexually mature males and females in a single wild population occurring within a 10-m-diameter area of early successional habitat in Athens, Georgia. All individuals were sampled within a 15-min period. Secretions were collected from individuals in the field by gently squeezing the thorax while applying a microcapillary (10 μl) to the metathoracic spiracular orifices. Capillaries were sealed in the field and then stored cold ($-10°C$). Quantity and quality of secretion stored in this manner did not change over six months. Fresh live weight (± 0.01 g) of the insect and the volume of secretion collected (± 0.25 μl) were also recorded.

Qualitative Secretion Analysis. Compounds were identified by gas chromatography–mass spectroscopy. Splitless injection (10 psi) capillary GC-MS of undiluted secretion and ether extracts (2 μl) from pooled secretions of male or female insects were carried out on a Finnigan 4021 using a 30-m $\times$ 0.3-mm-

ID DB-5 fused silica bonded phase column (J&W), programmed at 50 °C for 1.5 min, then 100°C to 250°C at 15°C/min. The mass spectrometer was operated in EI mode at 70 eV, 1400 V and 10^{-7} amp/V. Scanning was done at 45–350 amu with 1.5 sec/scan. Spectra were identified using libraries and confirmed with authentic samples. Identified compounds were reconfirmed in samples by retention time and co-chromatography using GC (Varian 6000) with on-column injection, flame ionization detection, and the same conditions as GC-MS, and using HPLC.

Quantitative Secretion Analysis. Secretions diluted to 1% in 18 mohm deionized water were analyzed by gradient reverse-phase HPLC (Varian 5500) using a 15-cm × 4-mm-ID C_{18} tri-methyl end-capped bonded silica column (5 μm) (Varian Micropak HCN-N-Cap 5) with a 4-cm × 4-mm-ID guard column (Varian Universal) packed with the same type of phase (Vydac SC reverse phase), and kept at 35°C. A convex solvent elution gradient of water, methanol, and 1% H_3PO_4 was used. Gradient commenced at 80:15:5 and finished at 20:75:5 composition, respectively. Peaks were detected at 275 nm. Stop-flow spectral scanning was used to confirm absorption maxima of peaks with some samples. Samples (three replicates of 10 μl of a 1 or 2% dilution of the secretion of each individual) were spiked with a fixed amount (56.7, 157, or 201 ng) of 4-hydroxybenzoic acid as an internal standard. Repetitive analyses of pooled secretions (N = 30 females or 20 males), collected at the same time and place as secretions from individuals, showed that retention times were accurate to ±1% (P = 0.95) and peak heights were accurate to ±10% (P = 0.95). Minimum detectable quantities per injection were: hydroquinone, 3.4 ng; *p*-benzoquinone, 23.7 ng; phenol, 3.8 ng; catechol, 4.1 ng; guaiacol, 2.5 ng; 4-methoxybenzaldehyde, 0.5 ng; unknown, 1.2 ng as 4-hydroxybenzoic acid equivalents. Peak heights were measured by an integrator (Varian 402). Data analysis was carried out on an IBM 4371 using CMS-SAS statistical packages.

RESULTS

Components of Pooled Secretions. GC-MS analysis of pooled secretions showed that they were exceedingly complex. Consequently our subsequent analyses focused on the 'phenolic' compounds and an unknown that comprised the major components detected by HPLC/$UV_{275\,nm}$ and GC-FID. Some of the identified compounds were similar, or identical, to those reported previously from pooled secretions (Meinwald et al., 1968; Meinwald and Hendry, 1969; Eisner et al., 1971). For example, we found phenol, hydroquinone, *p*-benzoquinone, guaiacol, and romallenone—although we did not quantify the last component. However, there were some notable qualitative differences in our pooled samples as compared to previous studies. We did not detect *p*-cresol, 2,5-dichlorophenol, or the ketones isophorone and verbenone found by Eisner

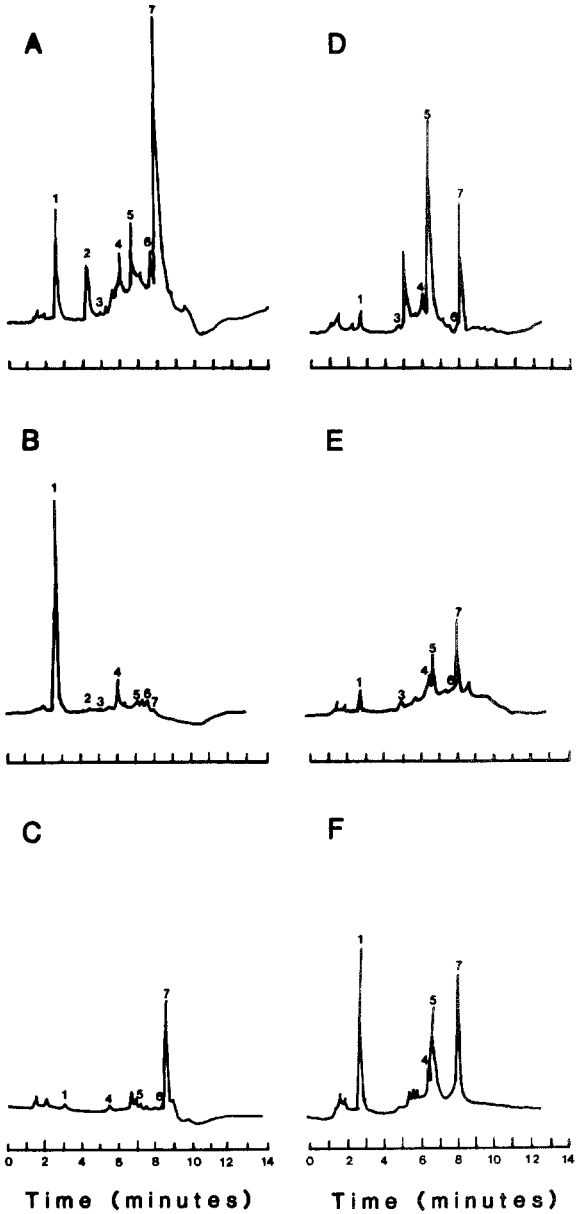

FIG. 1. HPLC traces (0.2 μl) of secretions of *Romalea microptera*: A, pooled females (N = 30); B, C, individual females; D, pooled males (N = 20); E, F, individual males. 1, hydroquinone; 2, catechol; 3, *p*-benzoquinone; 4, phenol; 5, guaiacol; 6, 4-methoxybenzaldehyde; 7, unknown. The internal standard peak has been deleted for clarity. Missing peak numbers denote concentrations below detectable limits.

et al. (1971). Since the halogenated phenol was purported to be a sequestered herbicide degradation product (Eisner et al., 1971; Berger, 1976), its absence in our samples, collected in an unsprayed area, is not surprising. We detected catechol and 4-methoxy benzaldehyde as major components not previously reported, and an unknown major component detected by HPLC/$UV_{275\,nm}$ that we are presently unable to analyze by GC or GC-MS.

Qualitative Variation among Individuals. The compounds identified in pooled secretions were quantified in secretions from 10 female and 11 male individual insects from the same population, using HPLC. Secretions from individuals differed substantially from pooled secretions for both males and females (Figure 1). Secretions from certain individuals lacked detectable amounts of some compounds that were found in other individuals (Table 1). In particular, males were qualitatively more variable than expected (χ^2, $P < 0.05$) due to the sporadic occurrence of catechol (Table 1), so much so that this component is not detectable in pooled male secretions because of its low frequency of occurrence and consequent dilution. Therefore, pooled secretions were not qualitatively representative of the secretion of individuals.

Quantitative Variation among Individuals. Concentrations of compounds showed extreme variation among individuals within a sex (Table 2). A minimum of fivefold (males, guaiacol) and a maximum of nearly 70-fold (males, hydroquinone) variation in concentration occurred within sexes. This variation in concentration increased to 90-fold and 100-fold (phenol and unknown, respectively) across sexes. While absolute variation (s^2) was greater in females, relative variation (i.e., coefficient of variation, $s/\bar{X}$) was as high or higher in males and was not constant among compounds, ranging from 66 to 183%. Analysis of variance showed that all compounds significantly differed in con-

TABLE 1. QUALITATIVE VARIATION IN PHENOLICS AND AN UNKNOWN BETWEEN INDIVIDUAL FEMALE (♀) AND MALE (♂) *Romalea microptera*, EXPRESSED AS FREQUENCY OF OCCURRENCE IN INDIVIDUALS

Compound	Frequency (%)	
	♀ (n = 10)	♂ (n = 11)
Hydroquinone	100	100
Catechol	70	27[a]
p-Benzoquinone	70	73
Phenol	100	100
Guaiacol	100	100
4-Methoxybenzaldehyde	100	73
Unknown	100	100

[a] Significantly lower frequency than expected (χ^2, $P \leq 0.05$)

TABLE 2. QUANTITATIVE VARIATION IN PHENOLICS AND AN UNKNOWN (AS 4-HYDROXYBENZOIC ACID EQUIVALENTS) BETWEEN INDIVIDUAL FEMALE (♀) AND MALE (♂) *Romalea microptera*[a]

Compound	Mean concentration (ng/μl Secretion) (coefficient of variation, %)		Concentration range (ng/μl secretion)		Mean composition (%)	
	♀	♂	♀	♂	♀	♂
Hydroquinone	1631[b] (132[c])	506(98)	104-7240	85-1383	19.1	22.9
Catechol	938 (65[c])	303(105)	122-1720	99-671	11.1	13.7
p-Benzoquinone	1363 (106)	659(134)	434-4506	227-2829	16.0	29.8
Phenol	1336[b] (84[c])	152(116)	68-3269	36-609	15.6	6.9
Guaiacol	777[b] (74[c])	371(67)	115-1700	183-917	9.1	16.8
4-Methoxybenzaldehyde	130[b] (88[c])	26(183)	18-323	4-142	1.5	1.1
Unknown	2364[b] (87[c])	195(66)	74-5709	52-456	27.7	8.8

Variable	Mean (coefficient of variation, %)		Range	
	♀	♂	♀	♂
Number of Compounds	6.4(11)	5.7(14)	5-7	4-7
Fresh weight (±0.01 g)	5.56[b] (17[c])	2.37(11)	2.70-7.49	1.92-3.15
Secretion volume (±0.25 μl)	2.73[b] (26[c])	1.14(28)	1.75-4.00	1.00-2.00

[a] Means and other statistics are based only on individuals containing these compounds (see Table 1). Similar statistics are also shown for some other insect variables.
[b] Mean significantly different between sexes (*t* test, $P \leq 0.05$).
[c] Variance significantly different between sexes (*F* test, $P \leq 0.05$).

centration among individuals within a sex and between sexes (F test, $P < 0.0001$). Variance from partial sum of squares was apportioned 60–88% to individuals, 9–39% to sex, and 1–4% to analytical error between replicates, depending on the compound (Table 3).

The analysis of variance showed that variation among individuals was the major source of variation. However, the ANOVA could not distinguish between variation contributed by one or a few individuals at extreme values of concentrations versus equal contributions to variation by all individuals. Maximum-likelihood estimation was used to determine the best fit of data to, and distribution along, a normal distribution generated by probit analysis from the range of compound concentrations found. The resultant χ^2 values for testing deviation from a normal distribution were not significant ($P = 0.65$–0.99, depending on sex and compound), and all data points were evenly placed along the probit distribution. These results indicate a good fit of data to normality, even contribution to variance by each individual, and the classification of all individuals as members of a single population.

In contrast to the extreme variation in secretion component concentrations among individuals, the variables fresh weight, secretion volume, and the number of components present in the secretion all had small coefficients of variation that were similar among variables and sexes (Table 2). This showed that only the secretion components were showing the extensive quantitative variation.

Although individual variation predominated, sexual differences were apparent and significant. Defensive secretions of females contained more of all components except catechol and *p*-benzoquinone. The lack of significant differences between sexes for these compounds is almost certainly a reflection of the extreme variation and does not imply equality of the means. Relative composition of the secretion was different between sexes; males had higher propor-

TABLE 3. PARTITIONING OF VARIANCE, FROM PARTIAL SUM OF SQUARES, IN CONCENTRATION OF PHENOLICS AND AN UNKNOWN (AS 4-HYDROXYBENZOIC ACID EQUIVALENTS) FOR *R. microptera*

Compound	r^2 (% variance) partitioned to		
	Sex	Individuals	Analytical error
Hydroquinone	13	86	1
Catechol	24	72	4
p-Benzoquinone	9	88	3
Phenol	38	60	2
Guaiacol	19	77	4
4-Methoxybenzaldehyde	26	72	2
Unknown	39	60	1

tions of *p*-benzoquinone and guaiacol, while females had higher proportions of phenol and the unknown (Table 2). Males and females differed significantly in weight and secretion volume but not in the total number of components in the secretion (Table 2).

Patterns of Variation. Patterns of variation were described using regression and ordination. Relatively few significant correlations existed between concentrations of different compounds. Only nine of 42 possible pairwise regression analyses had $P \leq 0.1$, with only phenol vs. *p*-benzoquinone and guaiacol vs. unknown being both significant and common to both sexes (Table 4). The independent variables insect fresh weight, volume of defensive secretion, and number of components in the secretion (i.e., secretion "quality") accounted for little variation in compound concentration, and not with any consistent pattern. Only six of 42 of the possible pairwise regression analyses had $P \leq 0.1$. The strongest relationship was between *p*-benzoquinone and secretion volume in females only (Table 4). Cluster analysis was used to investigate patterns among individuals. When insect fresh weight and secretion volume were used as ordinating variables, two clusters clearly separating the sexes were produced (Figure 2A). However, when compound concentrations were used, the analysis produced diffuse clusters that were not sexually differentiated and were very different from those produced by insect fresh weight and secretion volume (Figure 2B). Thus, patterns in the chemical variation were not very predictable.

TABLE 4. LISTING OF CORRELATION COEFFICIENTS (r) AND PROBABILITIES (P), BY SEX (♀ = FEMALE, ♂ = MALE), FOR PAIRWISE REGRESSIONS BETWEEN CONCENTRATIONS OF DIFFERENT COMPOUNDS AND BETWEEN COMPOUND CONCENTRATIONS AND OTHER VARIABLES FOR *R. microptera* WHEN $P \leq 0.1$

Variable 1	Variable 2	Sex	r	P
Hydroquinone	Catechol	♀	0.68	0.09
p-Benzoquinone	Phenol	♀	0.80	0.03
p-Benzoquinone	Phenol	♂	0.92	0.001
p-Benzoquinone	Secretion volume	♀	0.99	0.001
Phenol	Guaiacol	♂	0.69	0.02
Phenol	Number of compounds	♀	0.62	0.06
Phenol	Unknown	♂	0.65	0.03
Phenol	Secretion volume	♂	0.75	0.008
Guaiacol	4-Methoxybenzaldehyde	♀	0.81	0.005
Guaiacol	Unknown	♀	0.87	0.001
Guaiacol	Unknown	♂	0.65	0.03
4-Methoxybenzaldehyde	Unknown	♀	0.89	0.0005
4-Methoxybenzaldehyde	Number of compounds	♀	0.60	0.06
4-Methoxybenzaldehyde	Fresh weight	♀	0.72	0.02
4-Methoxybenzaldehyde	Secretion volume	♀	0.62	0.06

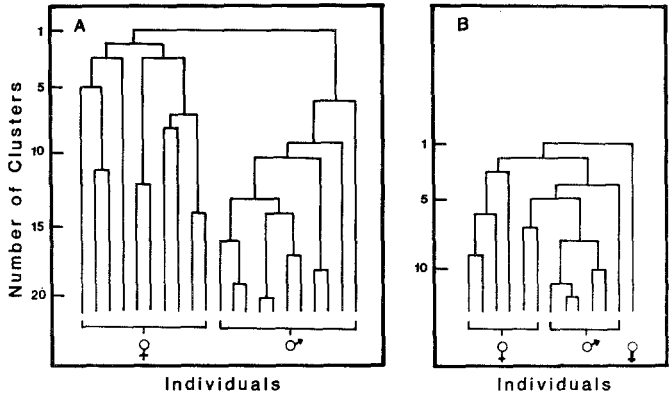

FIG. 2. Cluster analysis dendrograms for relatedness between individual female (♀) and male (♂) *Romalea microptera*: (A) using fresh weight and secretion volume as ordinating variables; (B) using concentrations of hydroquinone, *p*-benzoquinone, phenol, guaiacol, and 4-methoxybenzaldehyde as ordinating variables. Only 13 individuals had all five of these compounds in common.

Concentrations of compounds were not strongly correlated with each other or with obvious attributes such as insect sex, size, and volume of defensive secretion produced.

DISCUSSION

Our data show that lubber grasshoppers show extensive inter-individual qualitative and quantitative chemical variation in glandular defensive secretions. Furthermore, the patterns of chemical variation appear to be unpredictable. What sources or causes of chemical variation might be responsible? Chemical changes in the quantity and quality of arthropod defensive secretions are known to occur and to be influenced by a number of factors (Blum, 1981). Stage, age, sex, season, instar, social caste, physiological condition, population, genotype or colony, defensive history and regeneration, and dietary sequestration have all been previously reported as sources or causes of variation (Wallbank and Waterhouse, 1970; Tschinkel, 1975; Owen, 1978; Blum, 1981; Brower et al., 1982; 1984; Prestwich, 1983; Goh et al., 1984).

We do not believe that these factors explain the bulk of the variation we found for the following reasons. We sampled insects of the same developmental stage. These adults were very likely of similar age because of the relatively synchronous development of this insect (Jones, Whitman and Blum, unpublished data). We took sexual variation into account and found it always to be considerably less than that due to inter-individual variation. We can eliminate season and instar because we looked at one point in time at a single stage. Since

R. microptera is not a social insect, neither social caste nor colony are applicable. The generally poor correlations between insect fresh weight and secretion component quantity imply little direct influence of physiological condition. The maximum-likelihood probit analysis clearly showed that we were sampling a single population. Studies on the distribution of *R. microptera* in the sampled habitat indicate that individuals have a high probability of having a common genotype because egg pods hatch synchronously and larvae form sibling aggregates that do not disperse widely (Jones, Whitman and Blum, unpublished data).

Furthermore, we had no knowledge of the defensive history of the individuals we sampled, and it is conceivable that discharge of secretion prior to sampling could have affected variation. However, there was very little variation in the expected indicators of secretion use, i.e., secretion volume and the number of components in the secretion, and they were generally poorly correlated with secretion component quantity. Although *R. microptera* can sequester compounds from the diet (Eisner et al., 1971; Jones et al., 1986a), the compounds we quantified are produced on all diets and are almost certainly not sequestered (Jones et al., 1986b). While the distribution and abundance of precursors of defensive compounds in the diet accounts for some variation between individuals, it is similar in magnitude to the degree of variation due to sex that we found here (Jones et al., 1986b). We therefore feel justified in eliminating the above factors as significant contributions to inter-individual variation. We consider that the majority of the two to three orders of magnitude of variation in compound concentration, as well as qualitative variation in compound distribution that we found, is attributable to the individual insects themselves. Thus lubber grasshopper defensive secretions are truly idiosyncratic, and a typical species or population defensive secretion composition does not really exist.

Because most analyses of chemical variation in arthropods have been based on pooled samples, the extent to which inter-individual variation occurs is generally unknown (Blum, 1981). We know of a few studies where inter-individual variation was examined. With one exception, these studies report no detectable or obvious variation, or they report variation among individuals that is less than that found between castes, sexes, colonies, populations, or geographic areas. In all these cases, quantitative variation was less than one order of magnitude (Tricot et al., 1972; Doyen, 1973; Tschinkel, 1975; Daloze and Pasteels, 1979; Goh et al., 1984). The exception is a report of variation in cyanide yield from individual millipedes that is of a similar magnitude to the variation we found (Eisner et al., 1967). In their study, however, individuals were collected from an undefined population in a woodland, over a six-month period, and the quantitative contribution of these factors to variation was not determined. The occurrence of such extensive inter-individual chemical variation in *R. microptera* strongly suggests that future studies with other arthropods should avoid the pooling of defensive secretion samples unless there is a priori knowledge that inter-individual variability is not significant. It also shows that, at least for *R.*

microptera, comparison of defenses among populations is not necessarily the most meaningful within-species comparison.

How might such extensive variation arise and be maintained within a population? We suggest two alternative explanations that are based on the signal specificity used by predators to select or reject chemically defended prey within a prey population. Suppose that most of the predator species interacting with *R. microptera* respond to threshold concentrations of a gestalt odor or taste that can be produced from a large number of combinations of compounds and concentrations in a manner analogous to that suggested by Blum (1974) and Tschinkel (1975). In this situation, selection for a specific composition of secretion would not occur, provided the composition is above this threshold. Secretion quality and component quantity might not be tightly regulated and any inherent variation, or variation subsequently arising by other means, would not necessarily be selected against.

Alternatively, suppose that different species within the guild of predators interacting with *R. microptera* respond to different sets of specific chemical cues, in a manner analogous to that proposed by Pasteels et al. (1983); or are subsequently conditioned to different prey cues, as has been shown for a sawfly predator (Pasteels and Gregoire, 1984). In these situations, divergent predator selection pressure would favor divergent composition within prey populations. We know that different predator species vary in their responses to *R. microptera* (Whitman et al., 1986). We also know that the same predator species varies its responses to different *R. microptera* secretions (Jones et al., 1986a). However, the specificity of the chemical cues used by these predators is unknown, and so choosing between these alternate explanations as reasons for the maintenance of idiosyncratic variation must await further study.

The occurrence of such notable inter-individual variation in an insect chemical defense, and its fundamentally idiosyncratic and unpredictable nature, is directly analogous to the recent discovery of extensive chemical variation within and among individual plants (Denno and McClure, 1983; Jones, 1983). These discoveries illustrate the potentially important contribution of heterogeneity to the operation of chemical signals and defense in both animals and plants.

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SEX PHEROMONE OF *Adoxophyes orana*: Additional Components and Variability in Ratio of (Z)-9- and (Z)-11-Tetradecenyl Acetate

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Abstract—Twelve products related to the sex pheromone main components (Z)-9- and (Z)-11-tetradecenyl acetate (Z9–14 : Ac and Z11–14 : Ac, respectively), were identified in female pheromone gland extracts of the laboratory-reared summerfruit tortrix moth, *Adoxophyes orana* F.v R. These are the geometric isomers and the alcohols of the main components, (Z)-9-dodecenyl acetate, (Z)-11-hexadecenyl acetate, and saturated acetates of 12–22 carbons. The ratio of Z9–14 : Ac to Z11–14 : Ac in individuals varied from 3.5 : 1 to 11 : 1 with an average of 6.2; their total added up to 462 ng/female with an average of 182 ng for 2- to 7-day-old individuals. No qualitative or quantitative differences were observed between laboratory and field insects. Z9–14 : Ac, Z11–14 : Ac and the corresponding alcohols were also found in female effluvia. Addition of either of the two alcohols to a blend of the two acetates augmented trap catch in the field. The same was true for (Z)-9,(E)-12-tetradecadienyl acetate which was not detected in gland extracts.

Key Words—Summerfruit tortrix, *Adoxophyes orana*, Lepidoptera, Tortricidae, sex pheromone, (Z)-9-tetradecenyl acetate, (Z)-11-tetradecenyl acetate, (Z)-9-tetradecen-1-ol, (Z)-11-tetradecen-1-ol, individual variation.

INTRODUCTION

Adoxophyes orana (Fischer von Röslerstamm) was one of the first species known to use a sex pheromone of more than one compound (Meijer et al., 1972). The two components, (Z)-9-tetradecenyl acetate (Z9–14 : Ac; for other short forms, see Table 1) and Z11–14 : Ac, were also found in two related species, the smaller

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tea tortrix, *Adoxophyes* sp. (Tamaki et al., 1971a), and the summerfruit tortrix, *A. orana fasciata* (Tamaki et al., 1971b), in Japan.

Meanwhile, reports on multicomponent pheromones have become numerous. Tamaki et al. (1979) later described the *Adoxophyes* sp. pheromone as a four-component blend with another seven related compounds present. From behavioral observations, Den Otter and Klijnstra (1980) suggested that *A. orana* also used additional pheromone components in courtship. In connection with a study on mating disruption, Charmillot (1981) observed that males responded to a broader range of Z9–14:Ac/Z11–14:Ac blends than reported originally. For these reasons, a reinvestigation of the *A. orana* sex pheromone appeared appropriate. A preliminary account of this work has been presented (Guerin et al., 1982).

METHODS AND MATERIALS

Insects. Wild populations of *A. orana* were sampled at opposite ends of Switzerland, at Vich near Lake Geneva and Güttingen near Lake Constance. A laboratory colony derived from a 10-year-old culture (ca. 100 generations) at Changins was reared on the diet used for *Eupoecilia ambiguella* (Rauscher et al., 1984). Sexed pupae and adults (provided with sucrose solution) were kept in a 16:8-hr photoperiod at 24°C, 56% relative humidity with 3000–6000 lux in the photophase, and 18°C, 85% relative humidity, and 1 lux in the scotophase. Sex glands extracts were made from 3- to 7-day-old females during calling which, under this regime, occurred at the end of the scotophase.

Pheromone Gland Extraction. Employing a method similar to that described by Klun et al. (1979), batch extracts of some 5–25 excised female glands were obtained using ca. 1 μl/gland of hexane (Merck "for residue analysis") and 5 μl in the case of single glands. The terminal abdominal segments were forcefully everted with blunt forceps, and the tip incorporating the gland was sliced off with a scalpel blade. Extraction was made in a V-bottomed vial for 10 min; for direct gas chromatographic (GC) analysis, the gland wash was transferred to another vial, concentrated at room temperature, and injected. Extracts were stored at −80°C in microampoules.

Collection of Female Effluvia. Fifty females were placed in a 1-liter glass bottle containing nylon gauze as a resting site 24 hr before collection. Synthetic air (50 ml/min) passed over the insects during calling (3 hr) onto 1 ml of 1-mm glass beads held in a Pasteur pipet. The beads were washed with 0.5 ml hexane and the eluate concentrated at room temperature.

Gas Chromatography (GC). For GC analysis, we employed 15- to 50-m glass capillary columns (0.3–0.4 mm ID) coated with the polar phases Silar 10c or SP-1000 in a Carlo Erba gas chromatograph equipped with a Grob '76-type splitless injector. Typical programs were 60°C for 3 min, 20°/min to 100°C

and 3–5°/min to 240°. The elution sequence is given in Table 1. Silar 10c gave a better separation of *E*9–14 : Ac and *Z*9–14 : Ac; SP-1000 was superior for the pair *Z*9–14 : Ac/*E*11–14 : Ac and eluted the alcohols with less tailing.

Electroantennographic detection (GC-EAD) (Arn et al., 1975) was carried out using male antennae from various moth species of known sensitivity (Guerin et al., 1985).

Gas Chromatography–Mass Spectrometry. GC-MS was carried out on a Finnigan 4000 quadrupole instrument equipped with a 6115 data system operating in the EI mode. Complete mass spectra were obtained from injections of 5–15 female equivalents (FE). A systematic search was made for compounds structurally related to the previously identified pheromone components, using, among others, the key ions *m/z* 61 ($CH_3COOH_2^+$) for acetates and *m/z* 166, 194, and 222 for monoenic acetates and alcohols of 12, 14, and 16 carbons ($M^+ - CH_3COOH$ and $M^+ - H_2O$, respectively). Mass fragmentograms (MF) to confirm retention times were recorded by injecting 10 FE and synthetic standards at levels similar to the gland constituents (1–100 ng).

To determine the location of double bonds of monoenes, dimethyl disulfide (DMDS) derivatives (Buser et al., 1983) were prepared by keeping an extract of some 140 FE in 20 μl hexane plus 60 μl DMDS and 10 μl 6% ethereal iodine solution overnight at 40°C in a flame-sealed ampoule. Approximately 30 μl 5% $Na_2S_2O_3$ solution was added and, after rigorous mixing, the organic phase removed and concentrated. Mass spectra (*m/z* 35–535) of the adducts were obtained from injections of 25 FE.

Field Tests. These were done in 6–10 replicates in apple orchards using rubber caps for dispensing the chemicals and tetra traps with flaps (Arn et al., 1979). Treatment positions within replicates were systematically varied; catch differences were assessed by two-way analysis of variance and Duncan's multiple-range test after log $(x + 1)$ transformation ($P = 0.05$).

Chemicals. Acetates were purified by argentation chromatography (Heath et al., 1977); alcohols were derived from acetates. Isomeric purity was better than 99.97% for monoenes and 98% for dienes.

RESULTS

Chemical Analysis

Identification of Pheromone Gland Components. A listing of the compounds found in gland extracts of laboratory-reared females, along with identification criteria, is given in Table 1. *Z*9–14 : Ac and *Z*11–14 : Ac were main components at the ratio of 85 : 15, as previously established. The corresponding *E* isomers were present in the range of a few percent. Two additional monoenic acetates were identified as *Z*9–12 : Ac and *Z*11–16 : Ac. The alcohols *Z*9–14 : OH

TABLE 1. COMPONENTS IDENTIFIED IN *A. orana* FEMALE SEX PHEROMONE GLANDS[a]

Compound	Short form	Identified by	Amount (ng/FE)
Dodecyl acetate	12:Ac	GC-MS	0.04
(*Z*)-9-Dodecenyl acetate	*Z*9–12:Ac	GC-MS, MF, GC-EAD	0.2
Tetradecyl acetate	14:Ac	GC-MS	0.1
(*E*)-9-Tetradecenyl acetate	*E*9-14:Ac	GC-MS, MF	2
(*Z*)-9-Tetradecenyl acetate	*Z*9-14:Ac	GC-MS, DMDS	155
(*E*)-11-Tetradecenyl acetate	*E*11-14:Ac	GC-MS, DMDS, GC-EAD	4
(*Z*)-11-Tetradecenyl acetate	Z11-14:Ac	GC-MS, DMDS	27
(*Z*)-9-Tetradecen-1-ol	*Z*9-14:OH	GC-MS, DMDS, GC-EAD	4
(*Z*)-11-Tetradecen-1-ol	Z11-14:OH	GC-MS	0.4
Hexadecyl acetate	16:Ac	GC-MS	0.005
(*Z*)-11-Hexadecenyl acetate	Z11-16:Ac	GC-MS, MF, DMDS, GC-EAD	3
Octadecyl acetate	18:Ac	GC-MS	0.005
Eicosyl acetate	20:Ac	GC-MS	0.005
Docosyl acetate	22:Ac	GC-MS	0.005

[a]In order of elution on Silar 10c and SP-1000.

and Z11–14:OH were present in the percent range at the ratio of the corresponding acetates. Smaller amounts of saturated acetates from 12 to 22 carbons were also found. Analysis of extracts from insects collected near Lake Geneva and Lake Constance revealed no qualitative or quantitative difference to the laboratory material.

The DMDS procedure confirmed the structures of the monoenic acetates except for *Z*9–12:Ac and *E*9–14:Ac (concentration too low). A DMDS adduct (M^+, 306; A^+, 117; B^+, 189) also confirmed the presence of *Z*9–14:OH. No evidence for monounsaturated acetates of 10, 13, 15, 17, or 18 carbons or for monounsaturated alcohols of 12 or 16 carbons was found with this method.

The presence of *Z*9–12:Ac, *E*11–14:Ac, *Z*9–14:OH, and Z11–16:Ac in the extract was confirmed with GC-EAD using *E. ambiguella*, *Zeiraphera diniana*, *Maliarpha separatella*, and *Mythimna unipuncta* as detector species, respectively (Figure 1). In each case, the intensity of the EAD versus the FID signal matched that obtained with authentic standards.

As reported by Priesner (1983), *A. orana* male antennae carry receptor cells specialized for the dienes (*Z*)-9,(*E*)-11-tetradecenyl acetate (*Z*9,*E*11–14:Ac) and (*Z*)-9,(*E*)-12-tetradecenyl acetate (*Z*9,*E*12–14:Ac). However, our analyses revealed no trace of these compounds in female extracts. Detection limit was 1 pg/FE in GC-EAD using *Spodoptera littoralis* for *Z*9,*E*11–14:Ac and *Ephestia kuehniella* for *Z*9,*E*12–14:Ac, and 1 ng for both in GC-MS (m/z = 192 for $M^+ - CH_3COOH$). A search for 10-methyldodecyl acetate, a pheromone component of the smaller tea tortrix, *Adoxophyes* sp. (Tamaki et al., 1979), by GC-MS was equally negative at a detection limit of 1 ng/female.

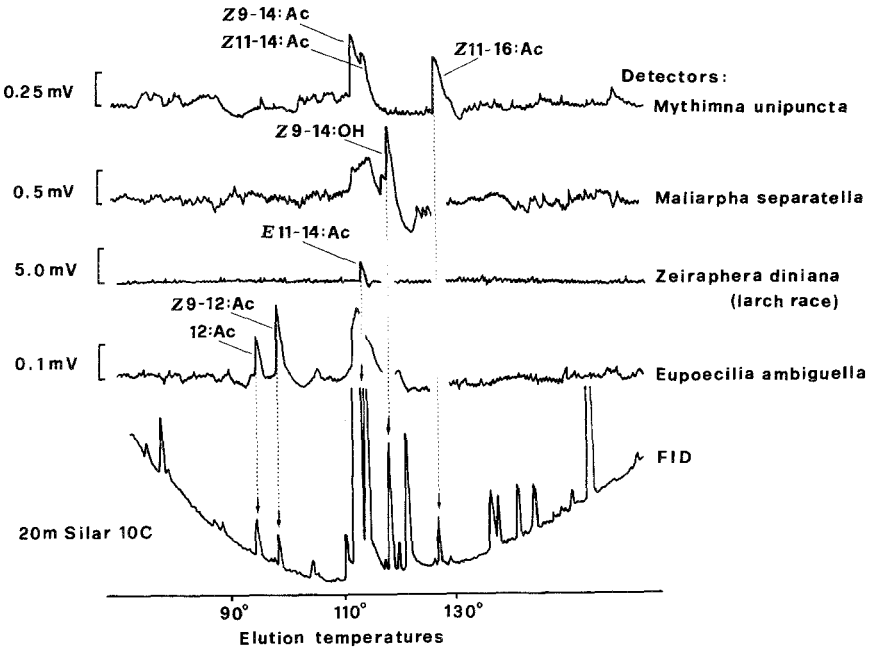

FIG. 1. Combined GC-EAD traces of *Adoxophyes orana* sex gland extracts with detector species selective to Z11-16:Ac, Z9-14:OH, *E*11-14:Ac, and Z9-12:Ac. Silar 10c, 20 m × 0.3 mm glass column, 40°C for 2 min, 10°/min to 60°C, 5°/min to 180°C.

Analysis of Effluvia. GC-MS of a glass-bead wash indicated the presence of Z9-14:Ac, Z11-14:Ac, and the corresponding alcohols in the female effluvia. The overall amount (1.5 ng from 50 females during 3 hr) was very low, probably due to low trapping efficiency. The isomer ratio of 11.5:1 for both alcohols and acetates and the proportion of alcohols (30% of the total blend) in the effluvia differed markedly from the analysis of the female glands but are more in line with the field results.

Analysis of Individuals. Due to the relatively high pheromone content, quantitation of the main components in individual glands was possible by GC with flame ionization detection. Forty laboratory-reared females of less than 2 hr up to 8 days old were analyzed (Figure 2).

The Z9-14:Ac + Z11-14:Ac content was lowest in newly emerged females (9 ± 3.4 ng), increased through day one (103 ± 16 ng), remained high from days 2 to 7 (182 ± 15), and dropped off on day 8 (80 ± 15). The highest amount (460 ng) was found in a 4-day-old. The ratio of Z9-14:Ac to Z11-14:Ac showed considerable variation, with values ranging from 3.5:1 to 11:1 in the 1- to 8-day-old individuals. The mean value for the laboratory stock was 6.2:1, similar to those of the pooled samples of wild material (5.3:1 for Lake Geneva and 5.8:1 for Lake Constance).

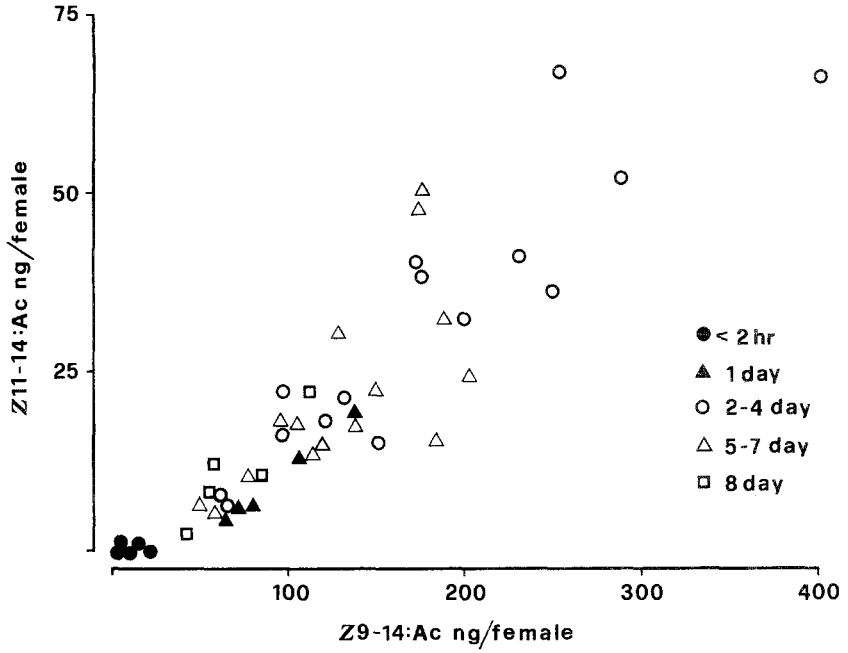

FIG. 2. Content of the main pheromone components in individual *Adoxophyes orana* female sex glands with age.

Field Responses

Attraction to Z9-14:Ac/Z11-14:Ac Blends. While Z9-14:Ac alone was ineffective, blends with Z11-14:Ac at a total cap load of 100 μg were attractive to males over a wide range (Figure 3). At locations where overall catch was high, the maximum was at 5-10% Z11-14:Ac, closer to the ratio found in the female effluvia than in the gland extract. At other locations no distinct attraction maximum was observed; the response curve was equally broad when the total dose was lowered to 10 μg. Increasing the dose of a 85:15 blend by factors of 10 up to 10 mg/cap (the highest dose tested) augmented the catch significantly.

Effects of E *Isomers.* *E*9-14:Ac and *E*11-14:Ac were each tested at various doses added to a blend of 90 μg Z9-14:Ac and 10 μg Z11-14:Ac. At levels of 0.5% and above, *E*9-14:Ac strongly inhibited trap catch, as observed by Minks and Voerman (1973) (Figure 4). Isomeric purity of Z11-14:Ac was less critical: inhibition was only found at levels of 5% *E*11-14:Ac of the binary blend or higher. On the other hand, the positive effect of high doses (10%) of *E*11-14:Ac reported earlier (Guerin et al., 1982) could not be substantiated.

Effects of Corresponding Alcohols. In tests similar to the above, Z9-14:OH and Z11-14:OH both augmented trap catch at levels of 10-50 μg (Figure 4).

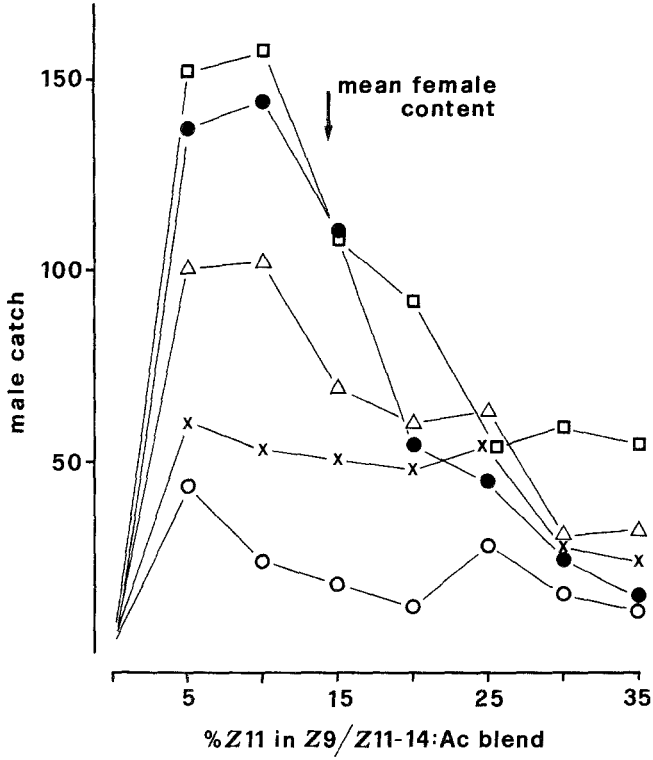

FIG. 3. Field captures of *Adoxophyes orana* males with different blends of Z9–14 : Ac and Z11–14 : Ac at Vich •—•, Güttingen ×—×, Geneva △—△, and Châteauneuf ○—○ (first) and □—□ (second) flight (the latter at half scale).

These effects were obtained in two consecutive years, although not in all experiments. *E*9–14 : OH could be present at up to 10% of Z9–14 : OH without affecting trap catch.

Effects of Other Pheromone Gland Components. No effects on catch were obtained when 12 : Ac, Z9–12 : Ac, 14 : Ac, or Z11–16 : Ac were added at levels between 0.1 and 50 μg to the blend of 85 μg Z9–14 : Ac and 15 μg Z11–14 : Ac. Addition of the full complement of acetates at the proportions found in the gland was also without effect. Traps containing 100 μg Z11–16 : Ac in addition to the binary blend caught an average of ca. 10 males per trap of the noctuid *Oligia latruncula* in June 1981.

Effects of Dienic Acetates. Two compounds for which Priesner (1983) found receptor cells on *A. orana* male antennae were combined with the 90 : 10 blend of Z9–14 : Ac and Z11–14 : Ac at levels ranging from 0.1 to 50 μg. Addition of 2 μg of Z9,*E*12–14 : Ac resulted in a twofold increase in trap catch but

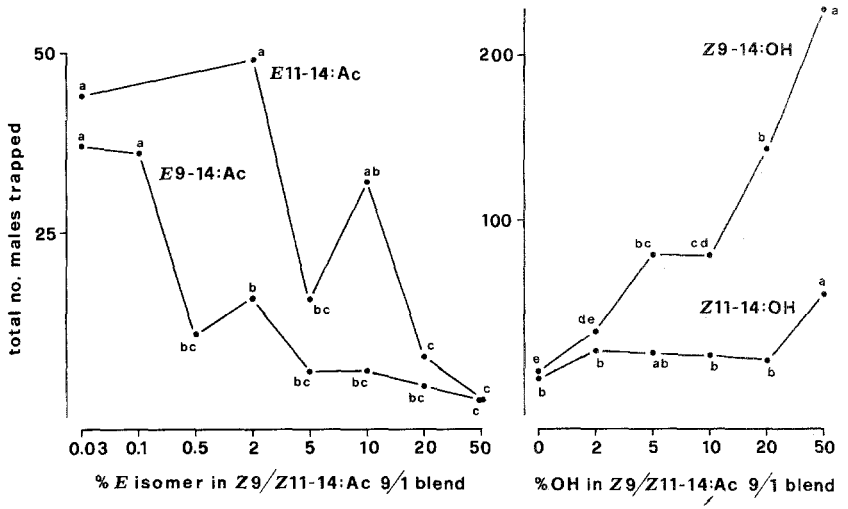

FIG. 4. Field captures of *Adoxophyes orana* males with a blend of 90 μg Z9–14 : Ac and 10 μg Z11–14 : Ac and different proportions of *E* isomers and corresponding alcohols added. Points marked with the same letters within a curve are not significantly different at $P = 0.05$.

was inhibitory at 50 μg. This confirms the results obtained by Voerman (1982). Z9,*E*11–14 : Ac, on the other hand, reduced trap catch to one half at 2 and totally at 50 μg. Neither of the binary mixtures of Z9,*E*12–14 : Ac with Z9–14 : Ac or Z11–14 : Ac was attractive.

DISCUSSION

In addition to confirming the presence of Z9–14 : Ac and Z11–14 : Ac, this study revealed 12 related components in the female pheromone glands of *A. orana*. Three of these, 12 : Ac, 14 : Ac, and *E*11–14 : Ac also occur in *Adoxophyes* sp. (Tamaki et al., 1979). The dodecenyl and hexadecenyl acetates common to both may be the Z-9 and Z-11 isomers identified here, present as possible byproducts of biosynthesis. Several components found by Tamaki et al. (1979) could not be detected in *A. orana*, notably 10-methyldodecyl acetate which is biologically active for *Adoxophyes* sp. At our detection limit, we would have found the compound if present at a similar proportion to the main components as in the smaller tea tortrix. We also identified the alcohols corresponding to the main components, *E*9–14 : Ac, and even-numbered saturated acetates up to 22 carbons.

The ratio of Z9–14 : Ac to Z11–14 : Ac showed considerable variation between individuals. This is in contrast with the strict control of the Z11–14 : Ac/

*E*11–14:Ac ratio in *Argyrotaenia velutinana* (Miller and Roelofs, 1980), possibly because of less closely linked biosynthetic pathways for the positional than the geometric isomers (see Roelofs and Bjostad, 1984).

Similar to the observations by Minks and Voerman (1973), male captures were made over a wide window of isomer ratios, ranging from 5 to 25% of *Z*11–14:Ac in the blend. This compares favorably to the 7–20% found in the female glands. According to Den Otter (1977), the male receptor cells are not tuned to the perception of a particular isomer ratio.

The effect of the additional pheromone gland components on male behavior has not been fully elucidated. *Z*9–14:OH and *Z*11–14:OH added to the main components strongly enhanced trap catch in more than one experiment. An explanation for some lack of consistency might be that these compounds exert an effect not amenable to measurement by trap catch under all conditions. *A. orana* males bear receptor cells sensitive to *Z*9–14:OH (Priesner, 1983).

As reported by Minks and Voerman (1973), attraction is strongly inhibited by a fraction of a percent of *E*9–14:Ac in the blend. Contrary to observations by the same authors, *E*11–14:Ac can be present at up to 20% of *Z*11–14:Ac without loss of activity. This situation is similar to that observed in *Adoxophyes* sp. where *E*11–14:Ac is a synergist (Tamaki et al., 1979).

Based on this study we currently use a *Z*9–14:Ac of high isomeric purity in a 90:10:10:2 blend of *Z*9–14:Ac, *Z*11–14:Ac, *Z*9–14:OH and *Z*11–14:OH.

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CHEMICAL MATE RECOGNITION AND RELEASE OF MALE SEXUAL BEHAVIOR IN POLYBIINE WASP, *Belonogaster petiolata* (DEGEER) (HYMENOPTERA: VESPIDAE)

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Abstract—The results of bioassays using freshly killed gynes of *Belonogaster petiolata* (Degeer) and hexane extracts of their tagmata and abdominal glands provide evidence for the existence of chemical signals in the venom, thorax, and head of gynes that serve as mate recognition cues and releasers of copulatory behavior in conspecific males. Attraction of males to the source of the chemicals occurs over short distances (a few centimeters).

Key Words—Polybiine wasp, *Belonogaster petiolata*, Hymenoptera, Vespidae, chemical signals, mate recognition, copulatory behavior, venom.

INTRODUCTION

Chemical communication and the production of pheromones in social wasps is probably the least explored aspect of the communicative and social biology of this group of insects. The majority of studies have been directed at determining and describing the source and functions of pheromones, rather than their chemical nature and composition. Only three pheromones, a queen substance (Ikan et al., 1969), a thermoregulatory pheromone (Veith and Koeniger, 1978) and an alarm pheromone (Veith et al., 1984) have been chemically characterized from the Vespinae, and one of the active components of the ant-repellent allomone of the sixth sternal gland of *Polistes fuscatus* has been identified (Post et al., 1984b). In addition, two compounds have been identified from the seventh sternal gland of *Vespa crabro* (Wheeler et al., 1982), although the function of this gland is still unknown.

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There are indications that the queens of certain vespine species produce substances that attract males and release copulatory behavior. Sandeman (1938) reported that freshly killed queens of *Dolichovespula sylvestris* were attractive to males and produced attempted copulation. Thomas (1960) noted that males of *Vespula germanica* which had recently mated queens, became attractive to other males, indicating that some of the queen's sex attractant may have been transferred to the former males. This behavior has also been observed among males of *V. maculifrons* under laboratory conditions (Ross, 1983). Initial detection of queens by vespine males appears to be visually achieved (Batra, 1980; Post, 1980; MacDonald et al., 1974; Akre et al., 1982; Ross, 1983) and, with the possible exception of *V. atropilosa* (MacDonald et al., 1974), males contact and mount females on a substrate. It has been suggested that mate recognition and release of copulatory behavior at this stage is mediated by short-range olfactory or contact pheromones in *Vespa crabro germana* (Batra, 1980), *Vespula atropilosa* (MacDonald et al., 1974), *V. consobrina* (Akre et al., 1982), and *V. maculifrons* (Ross, 1983). In particular, conspecific males were attracted to the queen's thorax in *V. crabro* and to her gaster in *V. maculifrons* (Batra, 1980; Ross, 1983, respectively).

Post and Jeanne (1983a, 1984a) reported the presence of a sex pheromone in the venom of *Polistes fuscatus* and *P. exclamans* that attracts males over short distances and releases copulatory behavior, although they were not able to establish how the sex pheromone is released by the females. The same authors found that this attractant is not species specific and that its action in these species is supplemented by a chemical cue on the cuticle (of at least the thorax and gaster) of females that is species specific (Post and Jeanne, 1984a). Long-range attraction of male *P. fuscatus* to conspecifics in their territories is visually mediated (Post and Jeanne, 1984b).

Observations (M.G. Keeping, unpublished data) on the mating behavior of *B. petiolata* in the field and laboratory have produced indications that the females of this species secrete a pheromone which attracts males, facilitates mate recognition, and releases copulatory behavior. Males and gynes emerge during late summer (February and March), when adult population size reaches its peak. During March and April, males fly to different nests, where they land and attempt to mate with resident gynes; there is no territoriality between males such as that observed in various species of *Polistes* (Kasuya, 1981; Lin, 1972; Post and Jeanne, 1983b; Turillazzi and Cervo, 1982).

Copulation in the field and laboratory is initiated when a male approaches a female and the pair undergoes either mutual antennation (male and female antennate each other's heads and antennae) or unilateral antennation (male antennates female's thorax and abdomen). The male then mounts the female and immediately begins vibrating his antennae against hers, with the latter generally becoming hooked in the curled section at the end of the male's antennae. The male's head is tucked back and down so that his mouthparts contact the female's

thorax; at this stage he curves his abdomen down to one side of the females's abdomen and attempts to establish genital contact. Genital extrusion only occurrs once contact has been established—this seldom lasts longer than about 5 sec—but is achieved several times during copulation. The male generally vibrates his wings while chasing or attempting to mount a female.

Since large numbers of adults are present on the nests during the mating period and since there is no specialized visual signaling by females (the sexes are also similar in their gross morphology and coloration), it appeared unlikely that visual cues provide the only means whereby males ultimately distinguish between the sexes. Agitated females also produce a strong, acrid odor which persists for some time in containers in which females have been kept; the same odor is evident from crushed venom glands and sacs, and it seems reasonable to expect that this odor may function in alarm or allomonal contexts or in mate attraction. An alarm response to venom has been demonstrated for three vespine species (Maschwitz, 1964, 1984), three species of *Polistes* (Jeanne, 1982; Post et al., 1984a), and *Polybia occidentalis* (Jeanne, 1981); it remains to be fully investigated in *Belonogaster*.

The present study was carried out to investigate whether males of *B. petiolata* are attracted to and recognize conspecific females on the basis of chemical cues, and if so, what body parts or glands in the female may be responsible for producing the odors involved.

METHODS AND MATERIALS

Colonies of *B. petiolata* were collected from Sterkfontein, Transvaal, during late March 1985, when males were still actively mating. Males and females were placed into separate cages soon after capture, and the males were used in the bioassays after a week of isolation. Trials were performed by placing 15 males in a Perspex box (14 × 10 × 10 cm) together with a test object for 10 min; interactions were recorded in terms of antennation of the test object and homosexual copulatory attempts. Our justification for using the latter interaction in quantifying the response of males to the test object is the same as that of Post and Jeanne (1983a), namely that homosexual copulatory attempts involved an identical sequence of behaviors to heterosexual ones (see Introduction) and they allowed us to eliminate the visual cue of the female in determining the efficacy of various female body parts in the chemical release of copulatory behavior. Antennation was interpreted in terms of the attractiveness of the test object to males.

Bioassays Combined with Visual Cue. Initially males were tested against gynes which were freshly killed, by freezing at −20°C for 15 min, and mounted on a wall of the test box. Females were handled gently before freezing to prevent agitation and release of venom. Controls consisted of presenting a dead female (dried at 60°C for 36 hr) to the males in an identical manner. In these

trials, the number of mountings (with the usual sequence of behaviors) of test and control females by males and how many of these resulted in genital linkage were scored in addition to the interactions mentioned above.

Bioassays in Absence of Visual Cue. Hexane extracts of body parts and glands of females were prepared by placing them in solvent at −4°C for 36 hr in the following quantities: 10 heads in 500 μl; 10 thoraxes in 1000 μl; 10 venom sacs and glands in 100 μl; 10 Dufour's glands in 100 μl; 10 sternal glands (sixth abdominal sternite) in 100 μl; and abdominal sternites 2–5 in 1000 μl. For each bioassay, an extract of one of the above was syringed onto a 2.7-cm-diameter disk of filter paper in appropriate quantities to obtain one body part or gland equivalent to test against the males. The solvent was allowed to evaporate, and the disk was pinned in position against the side of the test box. Controls consisted of presenting filter paper disks onto which pure hexane was syringed in a quantity equal to that of the extract used in the corresponding test; again the solvent was allowed to evaporate before presentation.

Controls and tests were paired, because male activity varied with the time of day. The same males were always used for the test and control of a particular trial, so that individual differences in male behavior were constant between the two. The experiments were carried out between 10 AM and 4 PM, since this coincided with the period of greatest male activity. Lighting was provided in the form of overhead neon tubes, together with a 60-W incandescent lamp which also maintained temperatures between 24.5 and 26°C. Consecutive trials were separated by at least 10 min, during which time the males were transferred back to a general holding cage and the test box was thoroughly cleaned. For each trial, males were taken at random from the holding cage and allowed to settle down in the test box at the correct temperature, before commencing with the bioassay.

RESULTS

Bioassays Combined with Visual Cue. Male activity in the presence of the dried (control) female was low; no homosexual copulatory attempts were observed and antennations of the dried female were infrequent (Table 1). When a freshly killed gyne was placed into the test box, there was a noticeable rise in male activity and aggression; there was also a significant increase in the number of homosexual copulatory attempts and antennations of the freshly killed female (Table 1). A significantly higher number of mountings was observed for freshly killed females than for dried females (freshly killed female = 119; dried female = 4; $P = 0{,}005$; $T = 0$; $N = 10$; Wilcoxon matched-pairs signed-ranks test); 41 of these mountings resulted in genital linkage with freshly killed females, but no genital linkage was attempted with dried females; thus the lack of genital contact with the latter was not due to the inability of males to grasp the hardened, inflexible genitalia of a dried female.

TABLE 1. RESULTS OF BIOASSAYS USING FRESHLY KILLED *B. petiolata* GYNES AND HEXANE EXTRACTS OF TAGMATA, ABDOMINAL GLANDS, AND VENOM.[a]

Test object	Antennation of test object		Homosexual copulatory attempts		Number of trials
	Control	Test	Control	Test	
Whole female	88	425[b]	0	27[c]	10
Hexane extract of:					
Head	28	140[b]	5	45[b]	10
Thorax	46	180[b]	9	41[b]	10
Venom gland and sac	32	211[b]	25	111[b]	10
Dufour's gland	43	57	72	74	10
Sixth sternal gland	35	35	41	33	10
Sternites 2–5	40	57[b]	12	28	10

[a] Male response is measured in terms of antennation of the test object and homosexual copulatory attempts by 15 males in the test box during a 10-min trial period. Comparison of tests and controls carried out using Wilcoxon's matched-pairs signed-ranks test.
[b] Significant at $P = 0.005$.
[c] Significant at $P = 0.01$.

Bioassays in Absence of Visual Cue. Hexane extracts of head, thorax, and venom elicited significantly higher numbers of antennations of the filter paper disk and homosexual copulatory attempts than the controls (Table 1). Venom extract proved to be particularly efficacious in releasing copulatory behavior (Table 1); the number of male responses was highest at the start of a trial and declined as time progressed. Extract of sternites 2–5 was attractive to males but was not capable of releasing a significant amount of copulatory behavior (Table 1).

In both sets of bioassays (with and without a visual cue), males responded with attempted copulation when in close proximity to the test object (a few centimeters), indicating that the chemical signals produced by females are close-range attractants.

DISCUSSION

The significantly higher number of antennations and mountings (together with genital linkage), directed at freshly killed gynes compared to dried females, provides evidence for the production by gynes of *B. petiolata* of a pheromone which attracts conspecific males and releases copulatory behavior. In this instance the visual cue was identical for both control and test females, and any significant increase in the release of copulatory behavior in the presence of the freshly killed female can only be accounted for in terms of chemical cues

present on one or more body parts of the latter. The odor produced by freshly killed gynes was also capable of releasing homosexual copulatory behavior, which further diminishes the role of close-range visual cues in mate recognition by males. Males which had recently mounted a freshly killed gyne were generally the subject of subsequent homosexual copulatory attempts, indicating that the substances involved are transferable from females to males. This same phenomenon has been observed during normal mating in *Vespula germanica* (Thomas, 1960) and *V. maculifrons* (Ross, 1983).

The odor contained in venom is clearly involved in the release of male sexual behavior in *B. petiolata*, but its action is probably augmented by the substances produced or harbored in or on the head and thorax (Table 1). Post and Jeanne (1984a) found that males of *P. fuscatus*, in addition to responding strongly to the venom of their species and to a smaller degree to that of *P. exclamans* and *Vespula maculifrons*, also responded to whole thorax and gaster or extracts derived from these; in the latter case, the responses of the males were species specific, which suggested that a chemical cue was present at least on the cuticle of the thorax and gaster and this allowed males to recognize and copulate with conspecific females. The transferability of odors from females to males of *B. petiolata* indicates that the pheromone in this species may also have a cuticular component, and as suggested for *Polistes* by Post and Jeanne (1984a, b), venom may be spread over the surface of other body parts (e.g., the head and thorax) during grooming, thereby increasing the attractiveness of the latter to males. This is supported by the finding that extract of sternites 2–5, which do not have cuticular glands (M.G. Keeping, personal observation), was attractive to *B. petiolata* males and probably contained traces of venom.

Since the pheromone produced by gynes of *B. petiolata* is only effective over short distances, it is likely that long-range attraction of males occurs on the basis of visual cues and may entail a response chain such as that described for *P. fuscatus* by Post and Jeanne (1984b). However, the absence of territoriality in *B. petiolata*, and consequently the lack of a need for males to differentiate between conspecifics and other wasp-sized objects and ultimately between free-flying male and female conspecifics (as occurs in *P. fuscatus*), suggests that the visual response chain would be relatively simple, with its major component being the sight of a populated nest. These possibilities remain to be tested. Recognition of females by males on the nest would subsequently be achieved using chemical cues.

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SELECTIVE OLFACTORY CHOICES OF THE HONEYBEE AMONG SUNFLOWER AROMAS: A Study by Combined Olfactory Conditioning and Chemical Analysis

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Abstract—A bioassay based on an olfactory conditioning method simulating the foraging situation in laboratory conditions was coupled with chemical analysis of volatile sunflower blends. Behavioural data obtained from foragers' responses to volatile fraction point out that honeybees need to use only a limited fraction acting as a "simplified aromatic pattern" of the plant, among hundreds of compounds constituting the whole aroma. This active fraction included 27 polar compounds among which 14 were identified. Extension of such data to crop pollination and plant improvement is discussed.

Key Words—Honeybee, sunflower aroma, foraging behavior, olfactory conditioning, active volatile fraction.

INTRODUCTION

The sunflower crop has become very important in these last years, and the sunflower is now the second oil plant in the world (Bonjean, 1983). This fact is linked to a better knowledge in the field of plant improvement, and, more particularly, to the discovery of cytoplasmic male sterility (Leclercq, 1969), which allowed the creation of hybrid varieties.

At the beginning, *Helianthus* genus was allogamous and submitted to entomophilous fecundation (Putt, 1940; Free and Simpson, 1964); foraging insects, particularly honeybees, are needed for hybrid seed production (Radford

and Rhodes, 1978; Parker, 1981a, b) and lead to a benefit in oil seed production: increase in seed yield, oil rate, and seed germination ability (Benedek and Manninger, 1972; Furgala et al., 1979; Radford et al., 1979; Parker, 1981c).

Sunflower hybrid seed production is strictly dependent on pollen transport from a male fertile line to a male sterile line. However, sometimes low seed production was due to a selective foraging behavior of honeybees; field observations thus pointed out a constancy of foragers to some sunflower lines, which led to a lack of entomophilous cross-pollination (Shein et al., 1978; Parker, 1981a, b); Freund and Furgala, 1982). Such a behavior, defined in honeybees, as a "floral constancy" (Grant, 1950; Free, 1963), is based on a learning process in which chemical cues are mostly implied (Masson, 1982): bees, by landing on a plant, memorize plant aroma during food uptake (nectar, pollen) and then are likely to use olfactory parameters as cues for selective orientation.

The aim of this work was thus to determine how far foragers are able to use volatiles as cues for discriminating different sunflower lines. Chemical analysis carried by Etievant et al. (1984) and based on both gas chromotography and mass spectrometry applied to extracts coming from solvent or headspace extractions, showed qualitative and quantitative variations in aroma composition of different sunflower genotypes. A recent study based on a compared analysis of chromatographic profiles of volatile constituents emitted by living heads of different sunflower hybrid parent lines (Marianne, Mirasol, and Primasol varieties), confirmed previous data and identified the specific constituents of each genotype (Pham-Delegue et al., in preparation).

The existence of such genotypic differences among sunflower aroma compositions being proved, the ability of bees to use these differences as cues for selective orientation towards one given genotype has to be studied. A plant aroma is a highly complex chemical blend, and one can ask if all constituents of the blend are equally identified, or if only a few of them are particularly significant and sufficient to lead to a food-oriented behavior. In order to answer these questions, the role and nature of the chemical implied in pollinator attractiveness were studied by a combined chemical and behavioral analysis.

The chemical data have been described elsewhere (Etievant et al., 1984); we will refer to that work while developping, in this paper, the behavioral aspects of the study. Behavioral data were carried out from a bioassay previously set up and based on an olfactory conditioning method (Pham-Delegue, 1983; Pham-Delegue and Masson, 1985).

METHODS AND MATERIALS

Plant Material. In order to provide general information on sunflower aroma constituents, a bulk sample was composed of flower heads from the cultivars H_9P_2, US 894, Marianne, and Mirasol and their inbred lines; flower heads were

frozen at −25°C immediately after their collection and analyzed during the following six months.

Choosing the Extraction Method. Methods for extraction and concentration of sunflower volatile constituents, as well as for analyzing the composition of resulting extracts, were described in detail by Etievant et al. (1984).

The kind of extract used for this behavioral study first had to be chosen, because the analysis of the former authors clearly pointed out that dichloromethane and headspace extraction methods were both valid. In a first step we tried to condition honeybees in a same way to both kinds of extracts. This conditioning was obtained in both cases but six times more material was needed for the headspace extract (three flower heads compared to half a head for the dichloromethane extract); these data confirm the conclusions drawn by Etievant et al. (1984) relative to the different concentration of each kind of extract. Therefore, taking account of bioassay duration, we chose the extract obtained by dichloromethane extraction of sunflower heads.

Splitting of Bulk Sample Obtained by Dichloromethane. The solvent extract was separated into polar and apolar fractions by adsorption chromatography (Etievant et al., 1984). The dilution factor of these two fractions had been carefully adjusted to that of the global extract before their use in behavioral tests. The results of our tests allowed us to divide the polar fraction into three subfractions (A, B, C) using a gas chromatograph set up with a $\frac{1}{8}$-in. glass capillary column packed with chromosorb W (80–100 mesh, acid-washed, DMCS-treated) soaked with a 10% SE 52 phase. Operating conditions were as follows: N_2 was used as the carrier gas (2.5 ml/min); the oven was programmed from 60 to 180°C at 2°C/min; the amount injected was about 40 μl.

The chromatogram was arbitrarily separated in three parts and substances eluted from each of these parts were successively trapped into three glass capillary tubes filled with 10 mg of previously conditioned Porapak Q.

The content of each trap was then eluted by 5 ml of Freon 11 and the resulting fractions A, B, and C were concentrated to the same volume before their use in bioassays. The constituents of the different extracts were separated using a Girdel 300 gas chromatograph fitted with a split/splitless glass injector and a flame ionization detector. Column characteristics were: length 37 m; inside diameter 0.4 mm; SE 52 phase; film thickness 0.6 mm. Temperature was programmed from 40 to 180°C at 3°C/min. Helium was used as a carrier gas (17.1 ml/min). Peak areas were calculated by using a Spectra-Physics Minigrator.

Identification of polar and apolar fraction constituents was detailed by Etievant et al., (1984). The B fraction included compounds eluted from acetic acid ethyl ester to 2-hexanal (*trans*). The B fraction included compounds eluted from eucalyptol to myrtenal, and C fraction, compounds eluted after verbenone, on to the end of the chromatogram of the polar fraction.

The scheme of the different events needed for global extract preparation

from flower heads and for separation of different fractions is reported in Figure 1.

Bioassay. Foraging behavior of honeybees was observed in a flight room (12 m^3) with laboratory controlled conditions (temperature: 25 ± 1°C; humidity: 55%; photoperiod: 12 hr/12 hr) (Figure 2). Foragers come from a honeybee colony of 3000 workers clustered around a fertile 1-year-old queen and its brood. They are presented to a conditioning device set up by Pham-Delegue (1983) and based on the principle of the artificial flower feeder described by Waller (1972) (Figure 2). Honey bees visiting the device undergo an associative conditioning, in which a sugary solution (50% sucrose) is the unconditioned stim-

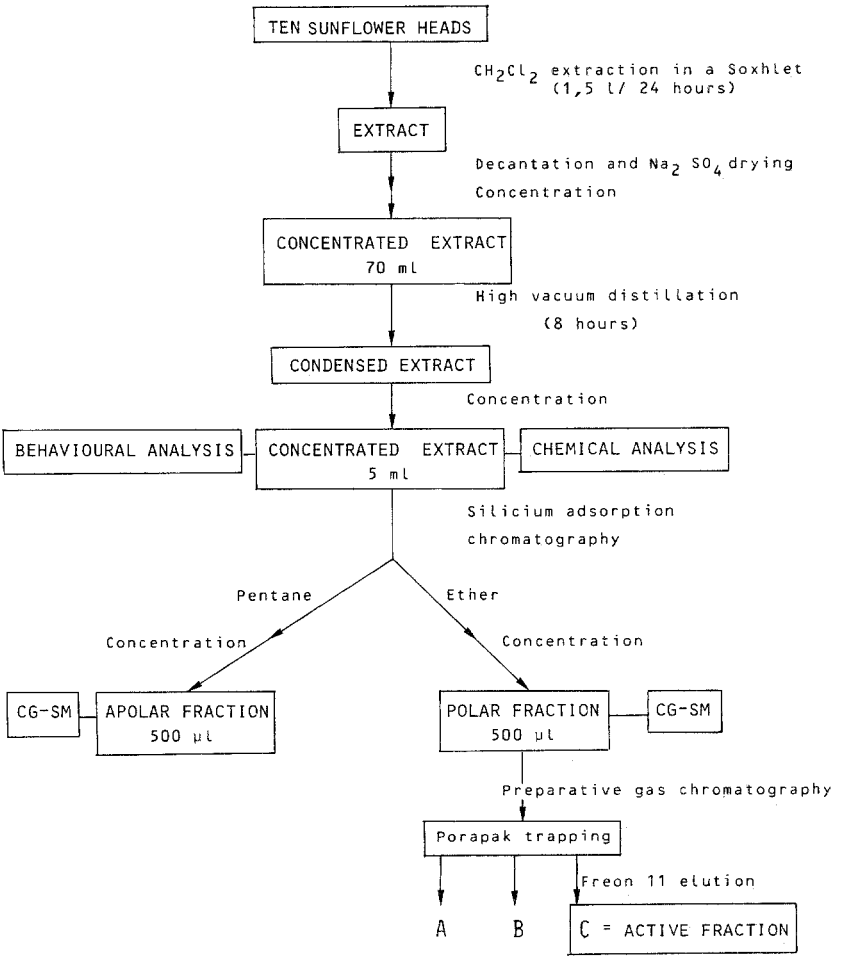

FIG. 1. Chemical processes leading to the isolation of an active fraction.

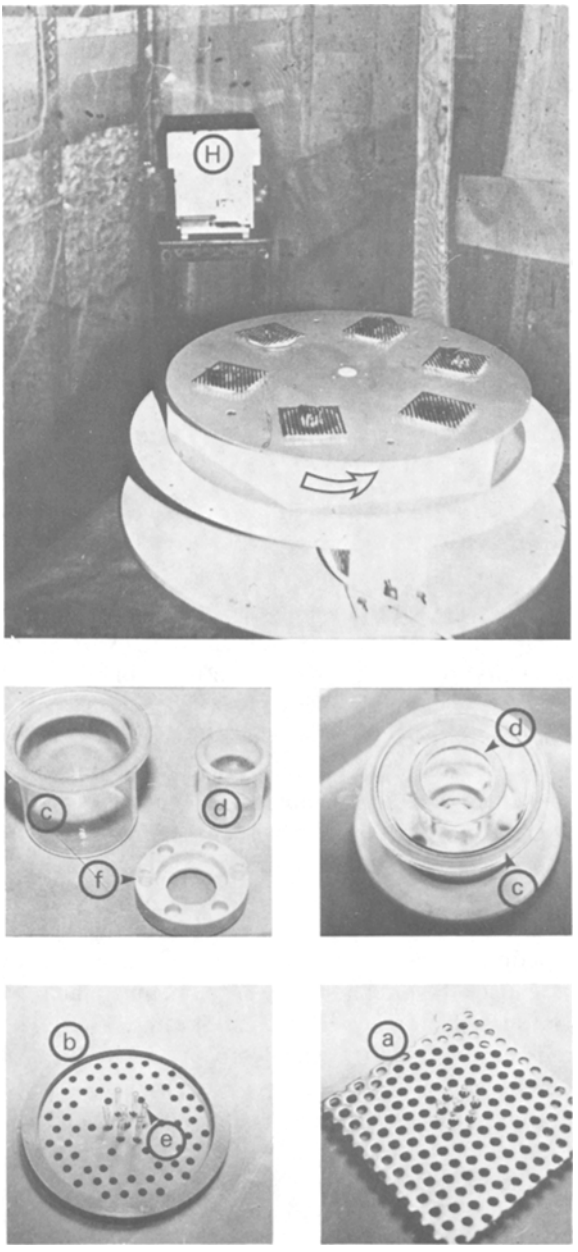

FIG. 2. Artificial flower feeder device used for the test: foragers flying freely from the hive (H) in a flight room are presented to the device set up on a rotating table ($\frac{1}{3}$ rpm). Conditioning period: association of the conditioning scent with sucrose solution in six artificial flower feeders (cf. Figure 6); a, perforated metal screen; b, plastic perforated lid; c, 80-ml glass beaker; d, 15-ml glass beaker filled with the sucrose solution; e, glass pipet, ID: = 1 mm; f, scent diffusor.
Testing period: three unscented sites (paraffin oil) alternating with three scented sites. No sugary reinforcement.

ulus, and a scent diffusing close to the food source is the conditioned stimulus. The conditioning period lasted 2 hr; conditioning efficiency was then tested, without any food reward.

The choice was given between the conditioning scent and an unscented control, or between the conditioning scent and different scents. The testing period was divided into four phases of 5 min, each phase corresponding to 20 successive responses (or visits) at least. Each testing phase followed by a 10 min reward period where the conditioning stimulus was associated with the sugary solution again. Such a protocol allowed a high visit frequency during tests and a high reproducibility of bee responses.

A statistical analysis of the distribution of visits among the different stimulus allowed to define how far foragers are able to discriminate (pooled χ^2 applied to percent visit distribution).

RESULTS

Volatile extracts obtained from splitting were diluted in Freon 11 and kept in sealed glass tubes in a deep freezer. Thus, before any experiment with such extracts, it is necessary to check if Freon 11, a very volatile substance (boiling temperature = 23°C), would induce any disturbance in bee choice.

Control of Solvent Effects. Foragers were trained to visit an unscented food source (paraffin oil as unscented control). Then bees were given a choice between paraffin oil added with a large amount of Freon 11 (1 ml Freon 11 in 1 ml paraffin oil) and pure paraffin oil. In such experimental conditions, visit distribution was perfectly random (61 visits to paraffin oil and 63 visits to paraffin oil added with Freon 11).

In order to control possible interaction between Freon 11 and scents, another control experiment was set up with a pure odorant, geraniol. This compound, which is part of the aroma spectra of numerous plants, was the major constituent of Nasonov pheromone (Boch and Shearer, 1962) which acted as a recruiting pheromone on slightly scented food sources (Frisch, 1923; Free, 1962). In this control experiment honeybees were trained to a 1% geraniol solution (paraffin oil as solvent) and were then given a choice between the conditioning solution and the same solution with added Freon 11 (volume per volume). No significant discriminatory effect linked to Freon 11 was apparent (56 visits to geraniol scented sites and 64 visits to sites diffusing geraniol added with Freon 11). Thus, one could conclude that Freon 11, which was not significantly identified, will not disturb aroma recognition.

Existence of Active Fractions. The global extract kept in Freon 11 was diluted in paraffin oil to limit evaporation speed. In order to determine the dilution ratio sufficient for an efficient conditioning (i.e., a significant recognition of the extract), several dilutions were made. A dilution of the initial extract (5

ml) at a ratio of 100 μl/ml of paraffin oil corresponding to half a flower head extraction, allowed a highly significant conditioning (66 visits to scented sites vs. 29 visits to unscented controls, during the testing period) (Table 1).

The polar and apolar fractions obtained from the global extract were then respectively tested against the global extract being considered as the conditioning stimulus. It appears that the apolar fraction is significantly discriminated from the global aroma, while the polar fraction is confused with the conditioning aroma (Table 1; Figure 3-A). Thus the polar fraction (Figure 5) may be considered by honeybees as very similar to the global aroma (Figure 4).

Because of the low quantities of global extract being kept, from this phase of experimentation experiments were carried out with the polar fraction as a conditioning stimulus. Conditioning efficiency of this polar fraction was first checked (80 visits to sites diffusing the polar extract vs. 31 visits to unscented controls, during the testing period) (Table 1). Then the three fractions (A, B, C) obtained from preparation chromatography (Figure 5) were successively presented versus the conditioning polar fraction. Behavioral responses indicated that only fraction C is statistically confused with the polar fraction (Table 1,

TABLE 1. TOTAL NUMBER OF VISITS[a] TO SUNFLOWER EXTRACTS AND ITS FRACTIONS DURING CHOICE EXPERIMENTS AFTER CONDITIONING

	Global extract			Polar fraction of the global extract		
	Conditioning scent (N_1)	Testing scent (N_2)	N^b	Conditioning scent (N_1)	Testing scent (N_2)	N^b
Paraffin oil	66	29	8	80	31	5
Polar fraction of the global extract	61	51	14		—	
Apolar fraction of the global extract	77	48	11		—	
Fraction A of the polar fraction		—		56	36	4
Fraction B of the polar fraction		—		67	37	9
Fraction C of the polar fraction		—		54	41	8

[a] Number of visits recorded along four successive tests, each test including at least 20 landings ($N_1 + N_2 > 80$).
[b] Mean number of different foragers performing the tests.

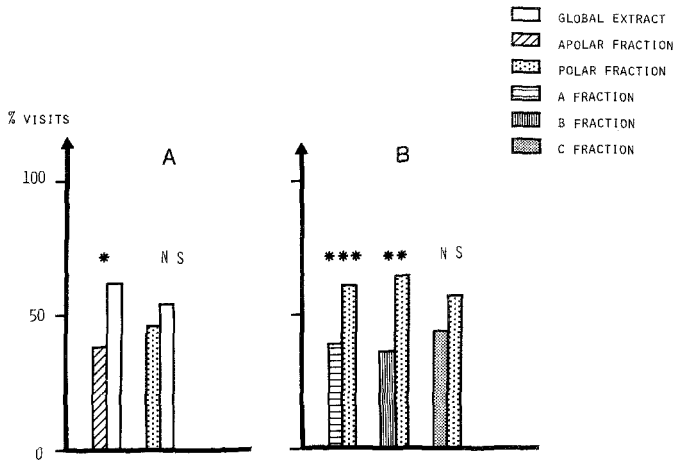

FIG. 3. Foragers behavioral responses (as a percent of visits) tested after a conditioning to: (A) the global sunflower extract; (B) the polar fraction previously defined as active. Values were calculated from four test experiments of at least 20 visits each.

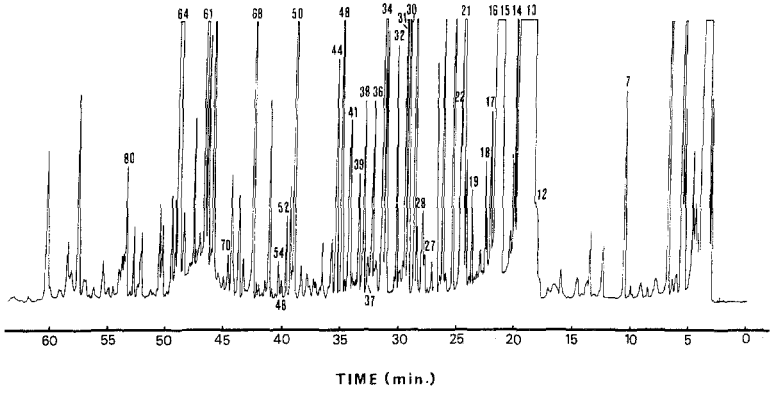

FIG. 4. Capillary GC analysis of sunflower-head volatile components from dichloromethane extraction (conditions are described by Etievant et al., 1984, and are reported in the text; peaks are numbered according to the notation of these authors).

Figure 3-B). Thus fraction C represented the new active fraction for bees, more limited as regards the number of chemicals than the previous one (i.e., the polar fraction of the global extract). Compounds identified from this fraction are presented in Table 2.

The mean number of individuals performing the tests (Table 1) was rather low compared to the number of recorded visits. These data show the high frequency of landings performed by each conditioned forager.

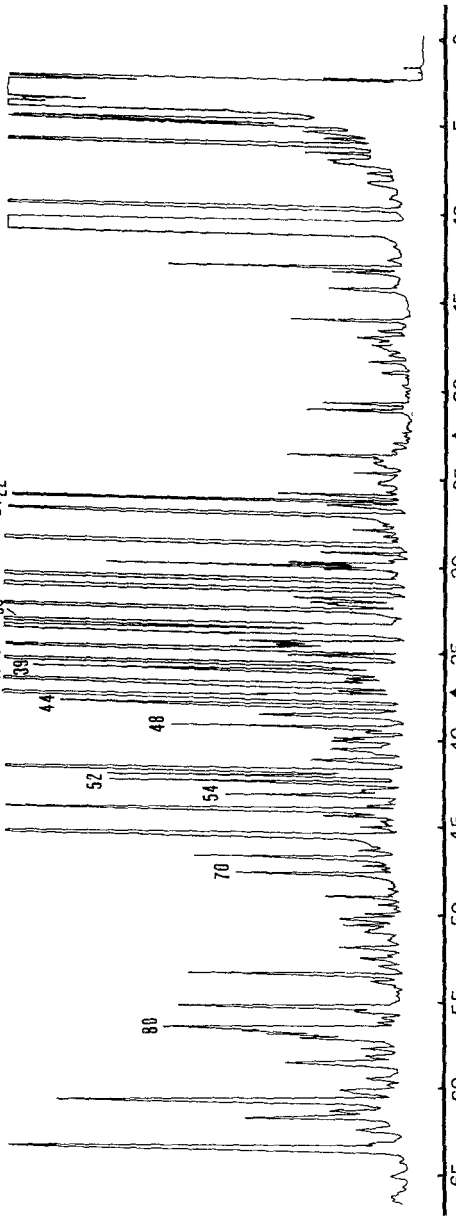

FIG. 5. Polar fraction of the global sunflower extract obtained from adsorption chromatography and then separated into three subfractions (A, B, C) according to conditions described in the text.

TABLE 2. VOLATILE SUNFLOWER COMPONENTS OF ACTIVE FRACTION

Peak number[a]	Compounds identified
43	verbenone
44	*trans*-carveol
46	unknown
48	ascaridole
49	unknown
50	bornylacetate
51	unknown
52	perillyl acetate
53	unknown
54	a 2,5-decadienal
55	unknown
68	eugenol
69	unknown
70	vanillin
71	methylcaprate
72	pentyl benzoate
73	geranyl acetone
74	unknown
75	8,9-dehydro-4,5-dihydrotheaspirone
76	2-tridecanone
77	δ-cadinol
78	unknown
79	unknown
80	propiovanillone
81	unknown
82	unknown
83	unknown
84	unknown

[a]See Etievant et al. (1984); numbers underlined are reported in the chromatogram of Figure 5 (C fraction).

Thus, this succession of experiments showed that a limited pool of molecules of the C fraction is sufficient for bees to identify a wider blend, including the active fraction. Successive steps leading to these results are summarized in Figure 6.

DISCUSSION

Data obtained from responses by foragers to different volatile extracts in an olfactory conditioning situation give a better understanding of the method used by bees to analyze complex olfactory information. In a complex aromatic

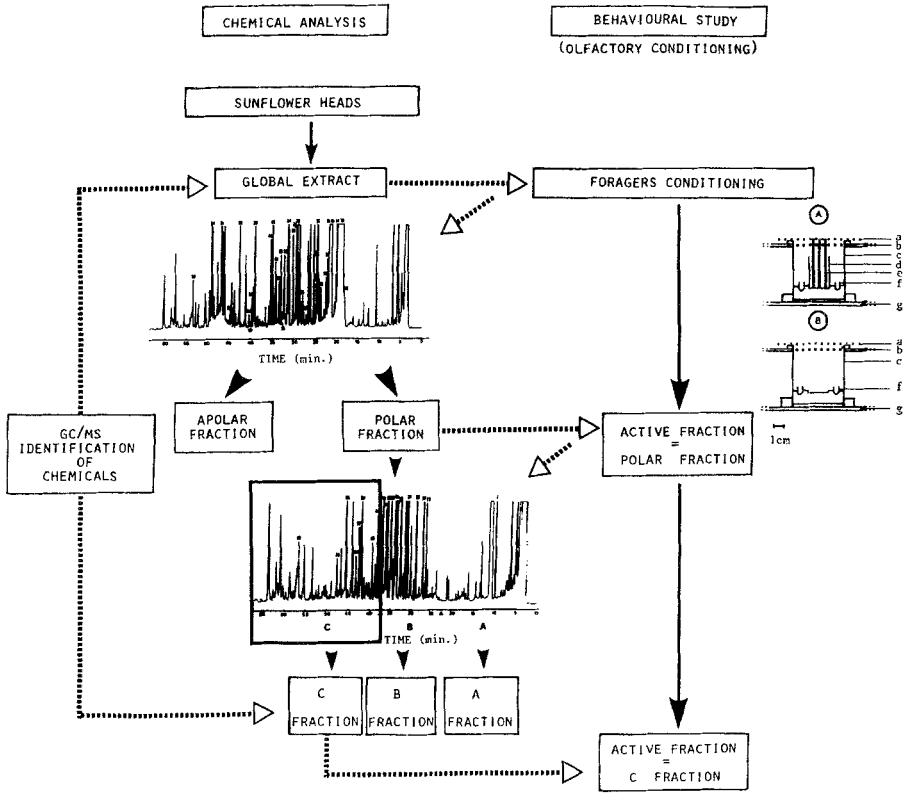

FIG. 6. Scheme summarizing the successive steps of combined chemical analysis and behavioral study leading to the identification of a limited fraction (fraction C) acting on bees foraging behavior. Artificial flower feeder: see Figure 2; A: conditioning device; B: testing device.

blend including hundreds of compounds, a limited fraction is extracted in a sufficient quantity to allow global olfactory information recognition. This fraction is acting as a "simplified aromatic pattern" of the plant.

While behavioral bioassays described in this work were carried out, 47 compounds were listed in the "mean aroma" obtained from the different sunflower genotypes collected. Through subsequent chemical analysis, Etievant et al. (1984) obtained 84 compounds among which 58 were identified.

The C fraction, identified as an active fraction in sunflower head aroma recognition, was quantitatively limited since it represented only about 2.5% of the global extract. Qualitatively, this fraction included monoterpenic alcohols and ketones as well as esters of these alcohols, a diunsaturated aldehyde, three phenols, one product of carotenoid degradation, and several sesquiterpenic al-

cohols, among which five are still to be identified and can be compared to the sesquiterpenic compounds identified in *Helianthus* genus by Gershenzon et al. (1981).

However, it must be noted that the main problem in the study of the bioactivity of complex chemical blends was to realize assays with microquantities of material for which composition was likely to alter with time. Thus, for further studies, stimulus control requires a theorical study of volatile release and the use of dynamic release processes in order to maintain a known and constant composition of the volatile fraction. Currently, in our laboratory, a multigate olfactory stimulating device is at our disposal; this device, set up by Masson et al. (in preparation), allows the automated stimulation of a biological material to be performed with a series of pure odorants in various dilutions of more or less complex blends in controlled quantities.

Through this device, a more analytical approach to the study of olfactory stimulus and resulting responses should be obtained. Biological activity resulting from this kind of stimulation should be quantified as peripheral nervous system responses, in the case of classical electrophysiological methods, and as behavioral responses, as registered in an adapted bioassay.

Improvement of such studies should allow us to complete our knowledge of the sunflower "aromatic pattern" composition, taking into account that the active fraction previously described was defined from a blend of different sunflower genotypes; aromatic pattern specificity as a function of plant genotypes should thus be determined, since qualitative and quantitative differences in volatile composition among sunflower cultivars have already been pointed out (Etievant et al., 1984).

In conclusion, such attractive fractions and plant-specific aromatic patterns should be useful cues for plant breeding. A joint genetic and chemical analysis could allow the determination of the genetic bases responsible for plant aromatic constituents biosynthesis (e.g., in *Mentha* genus according to Hefendehl and Murray, 1976). One should thus be able to create plant varieties with aromatic patterns attractive towards pollinating insects or, on the contrary, to improve chemical resistance to pests, linked to some terpenes, as suggested by Gershenzon et al. (1981) for sunflower. Such a genetic control could also allow the control of olfactory cues of discrimination between different cultivars and improve hybrid seed production linked to cross pollination.

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ROLE OF SECONDARY METABOLITES IN FEEDING ASSOCIATIONS BETWEEN A PREDATORY NUDIBRANCH, TWO GRAZING NUDIBRANCHS, AND A BRYOZOAN

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Abstract—The carnivorous nudibranch *Roboastra tigris* preys preferentially upon two nudibranchs, *Tambja abdere* and *T. eliora*, that in turn feed upon the bryozoan *Sessibugula translucens*. All four organisms contain tambjamines A–D (I–IV) that were shown to be fish feeding inhibitors. When attacked by *Roboastra, T. abdere* secretes a distasteful mucus containing a total of 3 mg of the tambjamines that sometimes causes the *Roboastra* to break off the attack. Under similar circumstances *T. eliora* attempts to swim away; it presumably contains insufficient of the tambjamines to deter *Roboastra. Roboastra* follows the slime trail of nudibranchs using contact chemoreception and reverses direction when the trail is broken. The slime trail of *T. abdere* contains low concentrations of the tambjamines. In Y-maze experiments, *T. eliora* was attracted towards seawater containing *S. translucens* and seawater containing 10^{-10} M tambjamines A and B (1 : 1) but was repelled by seawater containing $>10^{-8}$ M tambjamines A and B. At higher concentrations the mixture of tambjamines may be recognized as an alarm pheromone.

Key Words—*Roboastra tigris*, *Tambja abdere*, *Tambja eliora*, *Sessibugula translucens*, tambjamines A–D, chemical defense, alarm pheromones, trail-following behavior.

INTRODUCTION

Gastropod mollusks of the subclass Opisthobranchia do not seem to derive any physical protection from a shell which, if present, is often reduced or covered by soft tissue. In the Nudibranchia, a shell is completely lacking. The lack of a shell is correlated with alternative defense mechanisms (Faulkner and Ghi-

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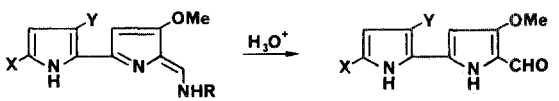
selin, 1983). Sea hares and dorid nudibranchs employ defense mechanisms based on dietary chemicals (Thompson *et al.*, 1982; Schulte and Scheuer, 1982) that are effective in deterring most potential predators. However, these chemical defense mechanisms can be penetrated by specialist predators such as the cephalaspidean *Navanax inermis* (Paine, 1963) and the nudibranch *Roboastra tigris* (Farmer, 1978). The added level of complexity in the association between a carnivorous nudibranch and the nudibranchs it eats led us to investigate the role of secondary metabolites in this relationship.

Roboastra tigris is a large carnivorous nembrothid nudibranch that is known to prey upon two smaller nembrothid nudibranchs, *Tambja abdere* and *Tambja eliora*. In their attempts to avoid predation by *R. tigris*, the two species of *Tambja* employ very different escape responses: *T. eliora* swims away by undulating the body from side to side while *T. abdere* secretes copious amounts of a yellow mucus from numerous goblet cells found in the skin (Farmer, 1978).

Chemical studies of *R. tigris*, *T. abdere*, and *T. eliora* revealed that all three nudibranchs contained a similar array of pharmacologically active metabolites that exhibited antimicrobial properties and inhibited cell division in the fertilized sea urchin egg assay (Carté and Faulkner, 1983). The metabolites were identified as the tambjamines A–D (I–IV) and the aldehydes V–VII (Figure 1). The aldehydes V–VII were subsequently shown to be artifacts produced by hydrolysis during the extraction procedure. By comparing the antimicrobial spectra of crude extracts, it became apparent that both species of *Tambja* obtained the tambjamines (I–IV) from a single dietary source, the bryozoan *Sessibugula translucens*. Field observations confirmed these associations. The quantitative distribution of the tambjamines (I–IV) and aldehydes V–VII in the four organisms is recorded in Table 1 (from Carté and Faulkner, 1983). These data establish that the bryozoan *S. translucens* is a principal component of the diets of both species of *Tambja* that are in turn preyed upon by *R. tigris*.

These observations raised several questions. What is the role of the tambjamines in the close association among the bryozoan and the three nudibranchs? How do the *Tambja* species locate their preferred food source? Why does *T. abdere* use an apparent chemical defense against predation by *R. tigris* while *T. eliora* attempts to swim away? Can *R. tigris* detect its prey by chemorecep-



I X = H Y = H R = H
II X = Br Y = H R = H
III X = H Y = H R = *i*-Bu
IV X = H Y = Br R = *i*-Bu

V X = H Y = H
VI X = Br Y = H
VII X = H Y = Br

FIG. 1 The structures of tambjamines A (I), B(II), C (III), and D (IV); and their hydrolysis products V (from I and III), VI (from II), and VII (from IV).

TABLE 1. DISTRIBUTION OF TAMBJAMINES A–D (I–IV) AND ALDEHYDES V–VII IN *Roboastra tigris, Tambja abdere, Tambja eliora,* AND *Sessibugula translucens* EXPRESSED AS WEIGHT/ANIMAL OR % DRY WEIGHT

	R. tigris	*T. abdere*	*T. eliora*	*S. translucens*
Tambjamine A (I)	0.56 mg	2.22 mg	0.48 mg	0.06%
Tambjamine B (II)	0.34 mg	0.94 mg	0.25 mg	0.083%
Tambjamine C (III)	0.54 mg	1.78 mg	0.22 mg	0.023%
Tambjamine D (IV)	0.36 mg	2.67 mg	0.51 mg	0.046%
Aldehyde V	0.43 mg	0.61 mg	0.23 mg	0.023%
Aldehyde VI	0.14 mg	0.5 mg	0.10 mg	0.071%
Aldehyde VII	0.25 mg	1.39 mg	0.17 mg	0.142%
Total wt/animal	2.66 mg	10.11 mg	1.96 mg	
Average wt	688 mg	296 mg	91 mg	
Percent dry wt	0.39%	3.42%	2.15%	0.45%

tion? In this paper we report both field observations and laboratory experiments that establish the chemical relationships between the four organisms.

METHODS AND MATERIALS

Major collections of specimens were made in several locations in the Gulf of California: Puerto Escondido, Baja California (May 1980)—*T. abdere* and *T. eliora*; Bahia de los Angeles, Baja California (May 1980 and April 1982)—*R. tigris*, *T. abdere*, *T. eliora* and *S. translucens* (1982 only); and Isla Partida (April 1982)—*T. eliora* and *S. translucens*. The animals were observed and collected at 2–6 m depth. Live specimens of all four organisms were collected at Bahia de los Angeles and transported to La Jolla in aerated seawater. All organisms were maintained in flowing seawater aquaria. *R. tigris* was maintained on a diet of *T. eliora*, the two *Tambja* species on a diet of *S. translucens*, and the bryozoan was fed daily with unicellular algae (*Isochrysis galbana*, *Anacystis marina*, *Rhodomonas lens*, and *Monochrysis lutheri*, all of which were cultured in GPM media under standard conditions).

Samples of tambjamines A–D (I–IV) were isolated and purified using the procedures described previously (Carté and Faulkner, 1983). In some experiments, mixtures of compounds were employed. The mixtures were obtained by omitting the final LC separation.

Feeding Inhibition Assays. Feeding inhibition was assayed by observing the feeding response of the spotted kelpfish *Gibbonsia elegans* toward food pellets ("San Francisco Bay Brand" freeze-dried euphausids) treated with pure compounds at concentrations ranging from 1 to 50 μg/mg. The kelpfish, collected intertidally at La Jolla, were kept in aquaria and were trained to accept

food from a glass tube. The test compounds were applied to the pellets (N = 10) in redistilled dichloromethane solutions, and the solvent was removed at 25°C under vacuum. Control pellets were treated with solvent only. Fish were fed the pellets in a random order and the response (eaten vs. rejected) recorded. The probability of obtaining the observed results was calculated using a chi-square distribution to determine the level at which a significant feeding avoidance was observed ($P < 0.5$ level). The following results were obtained: 3:2 mixture of tambjamine A (I) and tambjamine B (II); 10 μg/mg—7 of 10 pellets rejected, control—9 of 10 pellets eaten ($\chi^2 = 7.50$, $P < 0.01$); 5 μg/mg—3 of 10 pellets rejected, control—9 of 10 pellets eaten ($\chi^2 = 1.25$, $P < 0.5$). In a 3:7 mixture of tambjamine C (III) and tambjamine D (IV); 5 μg/mg—7 of 10 pellets rejected, control—9 of 10 pellets eaten ($\chi^2 = 7.50$, $P < 0.01$); 1 μg/mg—2 of 10 pellets rejected, control—9 of 10 pellets eaten ($\chi^2 = 0.39$, $P < 0.95$).

Analysis of Tambjamines A–D (I–IV) in Exudate and Slime Trail of T. abdere. A specimen of *R. tigris* was allowed to attack an average-sized specimen of *T. abdere* in a dish containing "Instant Ocean" synthetic seawater (100 ml). After the specimen had exuded copious quantities of a yellow mucus from glands on the dorsal surface, the animals were separated and removed. The dish and its contents were extracted with dichloromethane (3 × 75 ml), the combined extracts dried over anhydrous sodium sulfate and the solvent removed to obtain a green oil (4.8 mg).

Two specimens of *T. abdere* were allowed to crawl over a bed of aquarium dolomite that had previously been washed sequentially with water, dichloromethane, water, and synthetic seawater. The trails were marked with colored dolomite, the animals were carefully removed, and the dolomite on which the trails were laid was removed with a "lab scoop" spatula. The dolomite was washed with dichloromethane (3 × 200 ml), the combined extracts dried over anhydrous sodium sulfate, and the solvent evaporated to obtain an organic residue (3.1 mg).

The concentrations of the tambjamines A–D (I–IV) were determined by analytical LC. Separation of the tambjamines (I–IV) was accomplished by LC on an Alltech spherisorb 5-μm C18-ODS analytical column using a linear solvent gradient from 20% to 75% acetonitrile in 0.05 M pyridinium acetate buffer (pH 5.0) (see Figure 2). The tambjamines were detected by UV absorption at 400 nm. Standard response curves of concentrations vs. peak area (height × width at half height) for each pure compound were used to calculate concentrations of the tambjamines in the exudate and the slime trail (see Table 2).

Y-Tube Experiments. A Y-tube apparatus was constructed from clear Plexiglas tubing (3.75 cm ID) and "aged" in seawater. Prior to each experiment, the Y tube was thoroughly washed in natural seawater using a bottle brush to remove any attached materials. A coin flip was used to determine which arm of the Y tube would be the control arm. The control arm contained a stream of

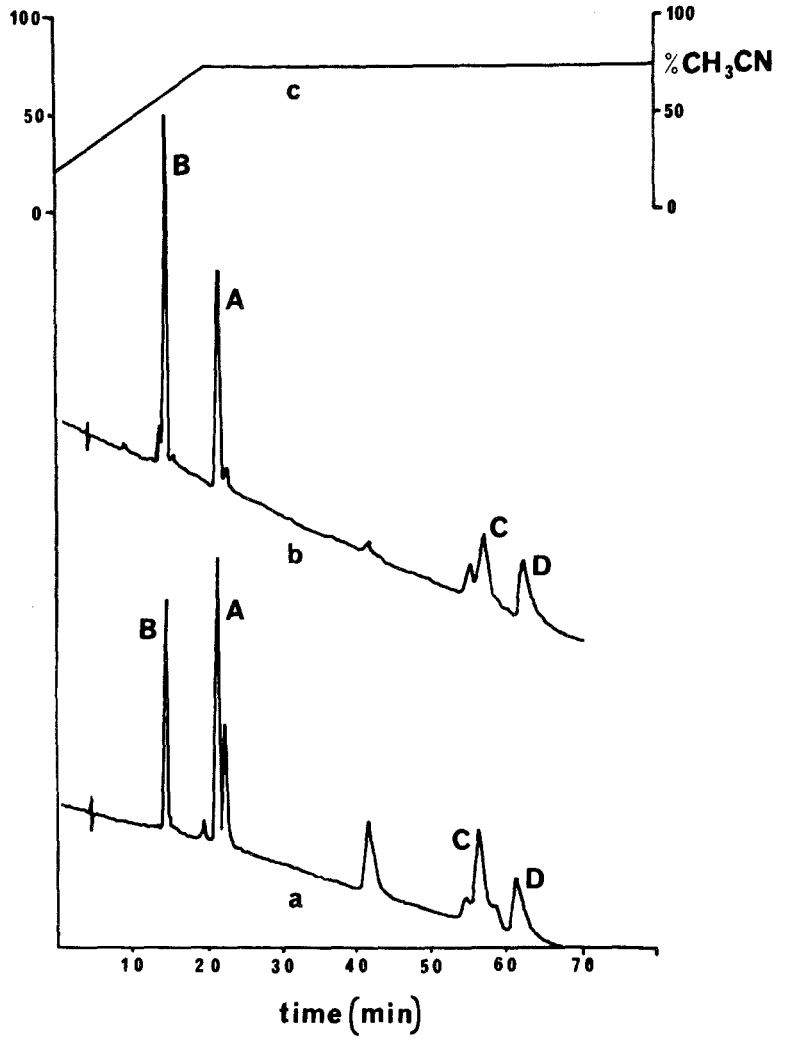


FIG. 2. HPLC analysis of (a) the defensive secretion of *Tambja abdere* compared with (b) a partially purified mixture of the tambjamines A–D isolated by extraction of whole animals. Compounds were identified by coinjection with pure samples. The samples were chromatographed on an analytical column of Alltech spherisorb 5 μm C18-ODS packing using a solvent gradient (c) from 20% to 75% acetonitrile in 0.05 M aqueous pyridinium acetate (pH = 5.0) at a flow rate of 1 ml/min.

either natural seawater or seawater containing ethanol (25 μl/liter of seawater). The test arm contained either seawater emanating from an aquarium containing the test organism or a solution of a 1 : 1 mixture of tambjamines A (I) and B (II) in ethanol (25 μl) in seawater (1 l) adjusted to produce final concentrations

TABLE 2. DISTRIBUTION OF TAMBJAMINES A–D (I–IV) IN *T. abdere* DEFENSIVE EXUDATION AND SLIME TRAIL

	Exudate (mg)	Trail
Crude	4.8	3.1 mg/50 cm
Tambjamine A (I)	0.85	0.06 μg/cm
Tambjamine B (II)	0.80	0.02 μg/cm
Tambjamine C (III)	0.79	0.13 μg/cm
Tambjamine D (IV)	0.56	0.15 μg/cm
Total	3.0	17.7 μg/50 cm

TABLE 3. RESULTS OF Y-TUBE EXPERIMENTS WITH *T. eliora*

Test sample	No. of Runs	Positive[a]	Negative[b]	Remarks
S. translucens	36	34 (94%)	2 (6%)	Attraction[d]
S. translucens (Δ 12 hr)[c]	20	9 (45%)	11 (55%)	Random[e]
Bugula neritina	10	5 (50%)	5 (50%)	Random[e]
Tambjamines A + B				
10^{-7} M	15	2 (13%)	13 (87%)	Avoidance[d]
10^{-8} M	15	3 (20%)	12 (80%)	Avoidance[d]
10^{-9} M	15	6 (40%)	9 (60%)	Random[e]
10^{-10} M	15	13 (87%)	2 (13%)	Attraction[d]
10^{-11} M	15	7 (47%)	8 (53%)	Random[e]

[a] Nudibranch crawls up the test branch of Y tube.
[b] Nudibranch crawls up the control branch of Y tube.
[c] Test sample consisted of water that was removed from an aquarium containing living colonies of *S. translucens* and allowed to stand at room temperature for 12 h.
[d] $P < 0.05$; binomial probability, $p = q = 0.5$.
[e] $P > 0.05$; binomial probability, $p = q = 0.5$.

ranging from 10^{-7} to 10^{-11} M tambjamines in seawater. Flow rates were adjusted to give equal flow rates (100–150 ml/min) in each arm of the Y tube. A specimen of *T. eliora* was introduced into the apparatus and the result recorded as positive if the animal crawled up the test arm of the Y tube. The results are recorded in Table 3.

RESULTS

Field and Aquarium Observations. There is a very close association between the three nembrothid nudibranchs and the bryozoan *S. translucens*. In particular, the nudibranchs *T. abdere* and *T. eliora* occurred only in locations where *S. translucens* was common, and individuals were generally found on or

near the bryozoan. Although *Roboastra tigris* was known to feed on a variety of smaller nudibranchs (J.R. Lance, personal communication), it prefers to prey on *T. abdere* and *T. eliora* and was most commonly found in areas where the *Tambja* nudibranchs, and therefore the bryozoan *S. translucens*, also occurred. However, a few specimens of *R. tigris* were encountered in habitats seemingly devoid of either *Tambja* species.

Field observations of the feeding habits of *R. tigris* suggested that the nudibranch could easily detect and follow fresh slime trails of *T. abdere*, *T. eliora*, and several other nudibranchs. The trail-following behavior is similar to that described by Paine (1963) for the Cephalaspidean opisthobranch *Navanax inermis*. To follow a slime trail, *R. tigris* "feels" for the trail with external, anterior located cephalic tentacles while sweeping its head from side to side. If the trail is broken, *R. tigris* will usually turn around and follow the trail in the reverse direction. It appears that *R. tigris* relies on direct contact between the cephalic tentacles and the slime trail and is not capable of detecting the resumption of the trail by distant chemoreception.

Once the prey has been located, *R. tigris* rapidly everts a blue buccal hood that engulfs the prey. In attempting to avoid predation by *R. tigris*, the two *Tambja* species use different escape strategies. *Tambja eliora* attempts to escape by writhing vigorously, while *T. abdere* excretes an apparently distasteful yellow mucus (Farmer, 1978). In aquarium situations, *T. abdere* was more successful in repelling attacks of *R. tigris*, but in field situations, where currents and wave motion were present, the swimming motion of *T. eliora* seemed equally effective because the slime trail is thereby broken.

Chemical Studies. We had previously found that specimens of *T. abdere* contained an average of approximately 10 mg of a mixture of tambjamines while specimens of *T. eliora* contained less than 2 mg of the same mixture of tambjamines (Carté and Faulkner, 1983). In order to demonstrate their deterrent value, mixtures of the tambjamines were assayed for feeding inhibition against the spotted kelpfish *Gibbonsia elegans* and were found to be effective at concentrations of 5–10 μg/mg pellet for a 3:2 mixture of tambjamines A and B and at 1–5 μg/mg pellet for a 3:7 mixture of tambjamines C and D. The aldehydes V–VII, which are formed by hydrolysis of the tambjamines, are not effective feeding inhibitors at concentrations less than 20 μg/mg. These data indicate that the tambjamines are relatively effective fish feeding inhibitors (cf., Thompson et al., 1982) and might be considered to provide protection against predators other than *Roboastra*. Since the differences in the escape responses of *T. eliora* and *T. abdere* might be an outcome of the different quantities of the tambjamines in the two animals, it was important to determine the quantity of tambjamines in the defensive secretion of *T. abdere*. LC analysis of the exudate from an average-sized specimen of *T. abdere* revealed that the defensive secretion contained a total of 3 mg of a mixture of tambjamines A–D (I–IV) (Table 2).

Since *R. tigris* had been observed to hunt *Tambja* species in preference to other nudibranchs, we suspected that the slime trails of *Tambja* might contain distinctive chemicals. A 50-cm length of the dolomite on which the trail of *T. abdere* had been secreted was extracted with dichloromethane to obtain a total of 18 μg of the tambjamines A–D (I–IV) (Table 2).

Although our field observations suggested that *R. tigris* could not detect the tambjamines by distant chemoreception, there was a good reason to believe that the *Tambja* species could find their food source, *Sessibugula translucens*, by using chemical detection. To test this hypothesis, a number of Y-maze experiments, modeled on those of Davenport and Hickock (1951), were performed using *T. eliora* as the test animal. *T. eliora* were clearly attracted by water that had been passed over *S. translucens*, but they were unaffected by water passing over *Bugula neritina*, a species of bryozoan that is common in areas populated by *Tambja* and that appears to be eaten only when *S. translucens* is unavailable. Water removed from an aquarium containing *S. translucens* and allowed to stand for 12 hr no longer elicited a positive response, indicating that the chemical responsible for attraction was not stable in seawater. These results suggested that the tambjamines (I–IV), which are easily hydrolyzed to the aldehydes V–VII, might be responsible for the observed chemotactic response of *T. eliora*. A 1 : 1 mixture of tambjamines A (I) and B (II) was tested at several concentrations from 10^{-7} M to 10^{-11} M. At very low concentrations, the tambjamines elicited no response, but the nudibranchs were attracted to a 10^{-10} M solution of tambjamines A and B in seawater. At concentrations greater than 10^{-9} M, *T. eliora* was repelled by the test solution (see Table 3).

DISCUSSION

Our previous chemical studies had established the presence of the tambjamines in the bryozoan *Sessibugula translucens* and the three nembrothid nudibranchs *Tambja abdere*, *Tambja eliora*, and *Roboastra tigris* (Carté and Faulkner, 1983). These results, together with our random field observations and those of Farmer (1978) and Lance (personal communication), clearly established that *T. abdere* and *T. eliora* favored the bryozoan *S. translucens* as a food source and were, in turn, eaten by *R. tigris*. The Y-maze experiments demonstrate that the *Tambja* species can locate their preferred food source by detection of low concentrations of the tambjamines that are produced by the bryozoan *S. translucens*. While it is not firmly established that *R. tigris* can distinguish the slime trails of *Tambja* species from those of other species by detecting tambjamines, their presence in low concentrations in the slime trail of *T. abdere* certainly suggests that this is the case. It would be difficult to test this hypothesis because we would require a synthetic slime trail sufficiently similar to the natural material that it would be followed by *Roboastra*.

The exudate of *T. abdere* contains a very large quantity of the mixture of tambjamines, representing approximately 30% of the metabolites stored by a typical animal. The exudate is clearly distasteful enough to deter most potential predators and can sometimes repel the specialist predator *R. tigris* (Farmer, 1978). However, the use of such a great proportion of the stored tambjamines to repel a single attack must render the animal susceptible to repeated attacks if it cannot rapidly replace the active ingredients of its defensive secretion. Since the entire tambjamine content of an average specimen of *T. eliora* is less than that used by *T. abdere* during a single, sometimes unsuccessful encounter with *R. tigris*, it is not surprising that *T. eliora* has adopted an escape strategy that is not dependent on a chemical feeding inhibitor.

The Y-maze experiments demonstrated that *T. eliora* avoided a 10^{-8} M solution of a 1 : 1 mixture of tambjamines A and B in seawater but were attracted by a 10^{-10} M solution of the same compounds. The ability to detect a two order of magnitude difference in the concentration of tambjamines in seawater may assist *T. eliora* in avoiding predation. While it is unlikely that *R. tigris* exudes a sufficient quantity of the tambjamines to be detected at a distance, an encounter between *Roboastra* and a *Tambja* species might result in the localized release of tambjamines, thus warning other potential prey of the presence of a predator, similar to a situation found among some fishes (von Frisch, 1941) and for the marine mud snail *Nassarius obsoletus* (Atema and Stenzler, 1977). Concentration-dependent properties have also been observed for ant trail pheromones (Tumlinson et al., 1971).

The ease with which the tambjamines (I–IV) are hydrolyzed to obtain the less active aldehydes V–VII provides a natural mechanism to remove old trails and exudations that might otherwise provide false information. This is considered an important factor in maintaining the efficacy of a chemical messenger.

Acknowledgments—We thank James R. Lance for providing information on the distribution and behavior of the nudibranchs. The feeding inhibition assay was devised by Joseph R. Pawlik who has also critically reviewed this manuscript. The research was supported by a grant from the National Science Foundation (CHE81-21471).

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Book Review

Semiochemistry: Flavors and Pheromones. Proceedings of an American Chemical Society Symposium, Washington, D.C., U.S.A., August 1983. Terry E. Acree and David M. Soderlund (eds.). Berlin, Walter de Gruyter, 1985. DM 160, 289 pp. (ISBN 3 11 010120 3; 0 89925 008 4)

The pheromone chemist becomes curious about how insects or mammals perceive odor and turn it into a nervous signal and that leads his curiosity on to theories of odor. This brings pheromone and flavor chemists onto common ground, and I find the increasing signs of the mingling of these disciplines exciting and informative. Cross-fertilization of this kind will help us to understand the mechanism of odor. Here lies the value of such mingling rather than the exchange of technical methods of chromatography or bioassay.

This is a very readable book, an account of one of these exchanges of information, a splicing of knowledge at an ACS symposium. The first chapter, by Miller and Harris described how the onion fly uses chemical and visual (shape and color) clues to find the onion plant for laying its eggs. They describe a number of neatly designed experiments which illustrate the case. The subsequent theoretical treatment with the cumbersome (and is it really necessary?) term "across-modality stimulus summation" puts the case that taste, smell, visual, and tactile signals all interlock to release a particular behavior such as movement or oviposition. They rightly point to the danger of trying to narrow down a behavioral pattern to a single stimulus such as a chemical odor. The next contribution, by Acree and Nishida, illustrates the effect of chirality on odor with respect to the methyl jasmonate isomers. One isomer (the one normally found in nature) has a much lower detection threshold (1000×) than any other. Another paper describes the interaction of various female- and male-derived pheromones, described as a chemical dialog, which produces copulation in the Oriental fruit moth.

The pheromone emphasis is on insects, but there is one interesting contribution by R.W. Bullard on the quite different problem of devising attractants and repellents for birds and wild animals. A point which emerges here and elsewhere is that in many cases (not all) the same compounds are odorous for us, mammals and insects. It is unfortunate there were no data anywhere in the book about comparative detection thresholds for different animals. (+)-α-Terpineol, which may be a pheromone for a pentatomid bug is shown to be an

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attractant for its predators and parasites. Silverstein gives a general account of chirality and its effect on pheromones and odor, which makes very pleasant reading. Most researchers are now thoroughly alert to the importance of chirality in this respect, but this chapter will be a very useful guide for beginners in the subject.

There is a discussion of bitter compounds in citrus fruit, on the attractiveness of sunflowers to insects, and the volatile flavor substances of the cherimoya fruit. T.H. Parliment describes an interesting experiment where he mixed 18 synthetic flavor chemicals and then fractionated them on various HPLC phases and obtained fractions with distinctly different flavors. Inevitably, at a symposium, not all contributions will be equally addressed to the topic, and some are more technical and limited in their view than others, but overall, the 16 contributions make interesting and easy reading. Papers with emphasis on technical detail, such as the bioassay of lepidopteran sex pheromones, were less interesting to me than those with a more wide-ranging discussion.

It is true, as the editors point out, that researchers into flavors, fragrances, and pheromones often find themselves confronted by identical problems of separation and characterization. I have the impression we are usually quick to adopt or adapt techniques, once we have heard of them, but the theoretical understanding is where we have most to gain by contact. It is therefore important that we speak the same language, as far as possible, and avoid the use of too much specialized jargon.

This book is for browsing, rather than for reference. Its 289 pages are double-space typewritten, with justified margins. There are not many words to the page, and the style is often cursive; it does not take long to read. Unfortunately the price means that many will read the library copy, but it is certainly worth seeking out. Like a flavor, this book is intended to stimulate. It does.

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Editors' Note

This special issue is devoted to a symposium on interactions between insects and photoactive chemicals. It includes invited papers presented at the December 1984 national meeting of the Entomological Society of America in San Antonio, Texas, and several subsequent related contributions. Dr. May Berenbaum, who has written an introduction to the issue, served as coordinator of the symposium and as a reviewer of the manuscripts. Others serving as reviewers were: A. Eisenstark, C.S. Foote, J.R. Heitz, G.W. Ivie, T.W. Mize, D. Sharp, G.H.N. Towers, R.W. Tuveson, and S.J. Yu.

We acknowledge the cooperation of Plenum Publishing Corporation in producing extra copies of this issue to accommodate registrants of the symposium and others interested.

R.M. Silverstein
J.B. Simeone, Editors

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Introduction

Many plants, including several economically important weeds and crop plants, are able to produce blistering, swelling, and erythema (reddening) upon contact or ingestion in a variety of organisms (Galitzer and Oehme 1978). These effects were, of course, of most concern in humans and the pharmacological applications of these plants are legion in human history, dating back at least to 1400 BC, when the Atharva Veda, a Hindu sacred book, refers to the use of certain black seeds, thought to be *Psoralea corylifolia*, in the treatment of certain skin diseases (Fitzpatrick and Pathak, 1959). Early herbalists such as Mattioli and Gerard cautioned readers against the hazards of collecting rutaceous plants such as *Ruta*, which "causes blisters and pimples on the hands and reddens them" (Knowlton and Ockert, 1950). In 1905, Busck first suggested that sunlight may be a critical component in this and similar reactions, known collectively as phytodermatitis; it was not, however, until 1939 that Jensen and Hansen established that only those solar wavelengths between 320 and 400 nm were capable of effecting pigmentation and erythema (Clare 1952). This range comprises the near-UV (NUV; 300–400 nm) or UV-A region of the solar spectrum.

Ultraviolet light can have dramatic effects when absorbed by a biomolecule. Absorption of photon energy can change the reactivity of a chemical by altering its electron configuration. Under standard conditions, molecules tend to stay in their least reactive electron state, called the ground state. Higher states result from absorption of a photon and subsequent movement of an electron from a low-energy orbital to a higher energy orbital. Each electron has a spin of + or $-\frac{1}{2}$; when the spins are parallel, the configuration is referred to as a triplet and if the spins are opposite, the state is referred to as a singlet. Excited states are generally reactive and short-lived. Molecules in excited states can dispose of their surplus energy in several ways; they can relax and return to ground state by emitting a photon, resulting in fluorescence; they can return to ground state and emit heat; or they can undergo intersystem crossing—that is, an excited singlet can form an excited lower-energy triplet state, which can then go on to transfer energy to another molecule in ground triplet state.

In the case of the furanocoumarins, photosensitizing substances in the Umbelliferae and Rutaceae, excited triplets interact with DNA, particularly pyrimidine DNA bases, to form irreversible cycloadducts which then interfere with transcription and template formation (Scott et al., 1976). In the case of many other photosensitizers, the excited triplet state interacts with molecular oxygen. In its ground state, oxygen is a triplet, which can take the energy of an absorbed

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photon to form a singlet state. Singlet oxygen is highly reactive and can proceed to react with other biomolecules, including proteins, lipids, and DNA, to cause oxidative damage. As a diradical, ground-state oxygen can also form superoxide free radicals in the presence of a photosensitizer. Superoxide can peroxidize lipids, denature enzymes, cleave DNA, depolymerize polysaccharides, and otherwise interfere with basic biological processes (Larson, 1978).

The presence of photoexcitable compounds in plants remained something of a scientific curiosity (and nuisance to cattle, horse, and sheep ranchers whose stock was prone to periodic phytodermatitis as a result of ingesting photoactive species; Ivie, 1977) long after the actual mechanism of photosensitization was described. In 1959, however, Fraenkel introduced the idea that plant secondary substances, compounds—such as photoactive biomolecules—with no known physiological function in the plants producing them, actually serve plants as in situ insecticides; as a result of the intensive selection pressure exerted over time by herbivorous insects, these chemicals evolved to reduce losses to insect herbivores. Almost 20 years later, the importance of ultraviolet light as an ecological factor mediating the toxicity of plant secondary substances to insects was first demonstrated (Berenbaum, 1978); since that time, considerably more evidence has been amassed to suggest that, over evolutionary time, many plants have exploited two ubiquitous components of their physical environment—sunlight and oxygen—to enhance the efficacy of their defensive armamentarium against insects.

In December 1984, the Entomological Society of America sponsored the first symposium focusing on interactions between insects and photoactive plant chemicals. Seven papers were presented at the meeting in San Antonio, Texas; since that time, several other investigators in the field have consented to contribute to the published proceedings of the symposium. The contributors address the interactions among insects, allelochemicals, and light at levels ranging from the cell to the ecosystem. Towers provides an overview of the distribution of photoactivity among angiosperm plants; Downum and Rodriguez expand upon known mechanisms of phototoxicity and the ecological correlates of photoactivity in plants. Champagne et al. provide a thorough study of the toxic properties of photoactive chemicals in the Asteraceae, documenting the efficacy of these chemicals as deterrents and toxicants toward ecologically appropriate insect herbivores. As Ehrlich and Raven (1964) suggest, insects can and do, by the process of coevolution, develop resistance to secondary substances; photoactive compounds are no exception. Larson, in his article, describes the distribution of insect chemicals whose structures suggest a defensive function against toxic oxygen species such as would be generated by photoactive plant compounds; Ivie et al. and Bull et al. describe in detail the elaborate biochemical defense of one insect—the black swallowtail caterpillar (*Papilio polyxenes*)—that feeds almost exclusively on photoactive plants.

Since the phenomenon of photosensitization was first documented, its potential applications in insect control have been clearly recognized. Barbieri (1928) demonstrated that several photosensitizing dyes, including rose bengal, eosin, and erythrosin, were effective against mosquito larvae. Contributors to this symposium, however, graphically demonstrate the practical potential of natural photoactive plant compounds for insect control. Philogène et al. report that α-terthienyl, a natural product widespread among members of the Asteraceae, is not only equal or superior to commercial synthetic organic insecticides for control of *Aedes intrudens*, it is considerably less toxic to nontarget organisms than are many synthetic alternatives. Kagan et al. describe details of the phototoxicity of secondary substances to mosquitoes from species of Asteraceae and Apiaceae; effects range from immediate death to delayed mortality and impaired development depending upon dosage.

Since phototoxins do not discriminate on the basis of phylogenetic status, affecting living organisms ranging from one-celled protists to humans and other mammals, rapid and sensitive bioassays with relatively simple organisms, such as bacteria, can be used to identify and purify photoactive substances. These compounds, once isolated and purified, can be used both for ecological investigations designed to elucidate the evolutionary aspects of phototoxin production in plants and for screening programs designed to identify potential control chemicals for pest insects. Ashwood-Smith et al. describe a bacterial bioassay based on a DNA-repair-deficient mutant *Escherichia coli* that is particularly sensitive to photodamage to DNA; this assay, as they demonstrate, can be used not only to detect phototoxic plant constituents but also to detect metabolism of photoactive compounds in resistant insects. Tuveson et al. have developed a series of mutant *E. coli* strains that are differentially sensitive to DNA damage and to oxidative damage associated with photoactivity; these strains can therefore be used to elucidate the mechanism of phototoxicity and estimate the mutagenicity of potential control chemicals.

That over 25 people contributed to the papers in this issue is evidence of increasing interest in insects and photoactive plants. Light quality is rarely controlled in routine assays of plant constituents for insecticidal properties—it might well be that phototoxicity is far more widespread as a defensive strategy than is indicated here. Aside from merely documenting its occurrence, plant phototoxicity to insects offers outstanding opportunities for research in the areas of toxicology, ecology, and evolutionary biology. It is our hope that this issue will stimulate research in this area; we could learn from plants about the tremendous potential of solar energy when it comes to insect control.

May R. Berenbaum
Guest Editor

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SIGNIFICANCE OF PHOTOTOXIC PHYTOCHEMICALS IN INSECT HERBIVORY

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Abstract—The significance of the wide range of phototoxins, occurring in many plant species, with respect to their role in insect herbivory and on insects, is not fully understood. The types of compounds include polyacetylenes, quinones, furanocoumarins, tryptophan- and tyrosine-derived alkaloids and are distributed throughout some of the major families of flowering plants. Efforts are being made to determine the mechanisms of cellular toxicity of these compounds at the cellular and the organismal levels.

Key Words—Furanocoumarins, furanoquinolines, polyacetylenes, alkaloids, quinones, photosensitizers, photodynamic action, phototoxicity.

The effects on insects of phytochemicals which require light for the full expression of their toxicities was briefly reviewed recently with an emphasis of the role of photoactive polyacetylenes (Arnason et al., 1983). With our increasing understanding of the ecological importance of secondary plant chemicals, it is interesting now to examine more carefully the possible significance of these compounds, especially in relation to insect herbivory. This symposium is therefore most timely.

In addition to photodynamic synthetic xanthene dyes (Carpenter et al., 1984), an increasing variety of photochemicals, including quinones, acetylenes, and alkaloids have been shown in recent years to be phototoxic to virus, bacteria, fungi, nematodes, and insects (Towers, 1984). The accumulation, in conspicuous quantities in plants, of these phytochemicals, in resin canals or glandular trichomes, suggests that they may play a role in deterring or destroying other organisms, including insects. These photoactive chemicals otherwise have no known metabolic or physiological functions in plants. For example, hyper-

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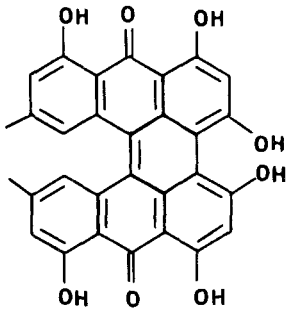
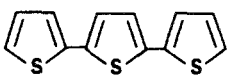


I

icin (I), a quinone photosensitizer which accumulates in specialized glands in leaves of species of St. John's wort [*Hypericum* spp. (Clusiaceae)] and which is responsible for photodermatitis in grazing animals is not toxic to some species of beetles in the genus *Chrysolina* (Daloze and Pasteels, 1979). In fact, one species, *C. brunsvicensis*, stores hypericin (Rees, 1969). What role, if any, does this phototoxic chemical have in relation to other insects? We simply do not know.

Phototoxic compounds or photosensitizers are chemicals which, when excited to a new electronic or electric state by absorption of a photon, react with other molecules in a given system. The resulting chemical changes in cells are often sufficiently severe to result in cell death. In many photoreactions of this type the excited photosensitizer transfers the excitation energy to oxygen which subsequently brings about an oxidation of another molecule. In this so-called photodynamic reaction, singlet oxygen, which may be the reactive species formed, may react with phospholipids, proteins, and sterols of plant membranes. For example, α-terthienyl (II), a not uncommon thiophene product of acetylene metabolism in members of the Compositae or Asteraceae, is a very efficient singlet oxygen producer (Reyftmann et al., 1985). Photosensitizers like α-terthienyl damage cell membranes (Yamamoto et al., 1984). Other photosensitizers, with equal potential for cellular destruction, penetrate cells to react with organelles including nuclei. Compounds of this type do not require oxygen. They form covalent adducts with bases in nucleic acids and may be termed photogenotoxic (Towers and Abramowski, 1983). There exist additional photosensitizers for which cellular targets have not yet been defined.

Many polyacetylenes, or polyines as they are more correctly called, and their thiophene derivatives have recently been found to display phototoxicities.

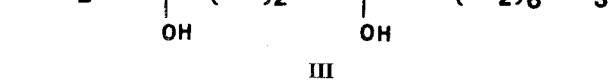


II

This large class of phytochemicals, characteristic of the Compositae (Asteraceae), Campanulaceae, Umbelliferae, and a number of other families of flowering plants (Bohlmann et al., 1973) include photosensitizers which have been shown to be phototoxic to many types of cells as well as to mosquito larvae (Arnason et al., 1981). On the other hand, none of the polyacetylenes of the Campanulaceae nor the characteristic acetylenes of the Umbelliferae and Araliaceae, namely falcarinone, falcarindione, falcarinol, or falcarindiol (III), appears to be phototoxic. Falcarindiol, however, in the leaf cuticle of the cultivated carrot, apparently stimulates oviposition in the female carrot fly, *Psila rosae* (Stadler and Buser, 1982). Concentrations of polyacetylenes in species of Compositae may be as high as 1% of the fresh weight, especially in young rhizomes at certain times of the year. The range of compounds usually differs between flowers, seeds, leaves, stems, and roots. Often the roots contain high levels. Is it possible that insect larvae, feeding on acetylene-rich roots or rhizomes, in the soil, may, on subsequent exposure to sunlight, either as larvae or as adults, suffer from phototoxicity? This has not been examined. Alpha-terthienyl, which has been studied most intensively, is so phototoxic to insect larvae that its potential as a commercial larvicide has been evaluated in stimulated pond trials. The cellular target of α-terthienyl and of polyacetylenes appears to be membranes but not all of them require oxygen for their activity (McLachlan et al., 1984). Serious field studies remain to be done with respect to this large class of chemicals, many of which are demonstrably phototoxic to insects under laboratory conditions. Especially significant would be comparable analyses of their possible effects on phytophagous insects along the lines of the research on phototoxic furanocoumarins.

The furanocoumarins, derivatives of phenylalanine, are characteristic of the Rutaceae and the Umbelliferae (Apiaceae). They also occur in members of the Leguminosae, Moraceae, Solanaceae, Pittosporaceae, Thymeleaceae, and Orchidaceae (Murray et al., 1982). Many of these tricyclic, planar compounds are phototoxic but perhaps the majority are not. Two nontoxic examples are 8-hydroxypsoralen and isopimpinellin. Common plant species in which phototoxic linear furanocoumarins occur are given in Table 1.

Many, but not all, of the biological effects of these much-studied compounds can be explained by photoinduced modification of DNA. The furanocoumarin intercalates in DNA and, on subsequent irradiation, reacts with pyrimidine bases to form covalently bound adducts. The photochemical reaction(s) is dependent on the presence of double bonds in the pyrone and in the furan rings. Some simple coumarins, however, e.g., 5,7-dimethyoxycoumarin are also



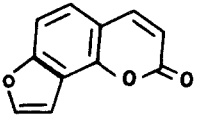
III

TABLE 1. LINEAR FURANOCOUMARINS

Psoralen (ficusin)	*Ficus carica* (Moraceae)
	Psoralea corylifolia (Leguminosae)
5-Methoxypsoralen (bergapten)	*Ficus carica*
	Citrus bergamia (bergamot) (Rutaceae)
	Citrus limonum (lemon) (Rutaceae)
	Heracleum spp. (Umbelliferae)
	Ammi majus (Umbelliferae)
	Seseli indicum (Umbelliferae)
	Petroselinum sativum (Umbelliferae)
8-Methoxypsoralen (xanthotoxin)	*Ammi majus*
	Angelica spp. (Umbelliferae)
	Pastinaca sativa (Umbelliferae)
	Ficus carica
	Ruta graveolens (Rutaceae)

photosensitizers. The formation of bi-adducts by furanocoumarins occurs only with certain linear types and depends on nucleotide sequences, poly(dA–dT) · poly(dA–dT) sequence regions being the most favorable sites for intercalation and photocyclization. In the photoconjugation of furanocoumarins with pyrimidine bases there is no involvement of oxygen. It has been suggested, however, that with certain furanocoumarins such as angelicin (IV), reactive species of oxygen may play a major role in their carcinogenic effects (Joshi and Pathak, 1983).

Plants usually store furanocoumarins as the photochemically active aglycones, the major sites of accumulation being oil channels or ducts present in leaves, stems, roots, and flower parts. They may also be found in the cuticle (Stadler and Buser, 1982). Many of them accumulate in roots, as with acetylene reservoirs in roots of Compositae, but their ecological importance as phototoxic defense compounds in not obvious as ultraviolet light does not penetrate more than a few millimeters below the soil surface. Why do they accumulate in roots? Insect predation of the leaves of umbellifers has been studied in some detail, and at least one species of butterfly, *Papilio polyxene*, elaborates furanocoumarin detoxifying enzymes (Ivie et al., 1983). Some species of *Papilio* actually appear to select species of umbellifers or members of the Rutaceae which con-



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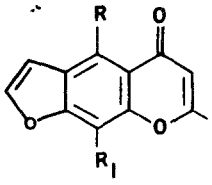


$R=H;\ R_1=OCH_3$

V

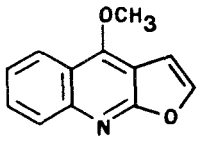
$R=OCH_3;\ R_1=OCH_3$

VI

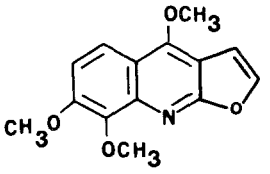
tain linear furanocoumarins as food sources (Berenbaum, 1981). The possible ecological importance of the furanocoumarins will be presented in later papers.

The furanochromones khellin (V), and visnagin (VI), which are of polyketide origin, cooccur with furanocoumarins in species of *Ammi* (Umbelliferae) (Schonberg and Sinha, 1950; Spath and Gruber, 1941) and, like them, are phototoxic to bacteria and to fungi. They cause chromosomal damage to CHO cells in UV-A (Abeysekera et al., 1983). By analogy with the furanocoumarins (Kanne et al., 1982), khellin could be expected to react with a base such as thymine in a 2 + 2 photoaddition involving either the 2–3 or the 6–7 double bond of khellin and the 5′–6′ double bond of thymine. UV-A irradiation of a frozen aqueous solution of khellin and thymine in fact yields photoadducts including one between the 2–3 double bond of khellin and the 5′–6′ double bond of thymine (Abeysekera et al., 1983). The effects of these compounds on phytophagous insects has not been studied so far, nor have the polyketide benzofurans, 6-methoxyeuparin as well as encecalin and 7-hydroxyencecalin from species of *Encelia*, although these compounds have been shown to be phototoxic to bacteria and to fungi (Proksch et al., 1983).

Some species in the Rutaceae, such as *Skimmia japonica*, *Dictamnus alba*, and even species of *Citrus*, contain, in addition to appreciable concentrations of furanocoumarins such as 5- and 8-methoxypsoralens, photosensitizing furanoquinoline alkaloids, derivatives of anthranilic acid. These tricyclic planar compounds, such as dictamnine (VII) and skimmianine (VIII), intercalate in



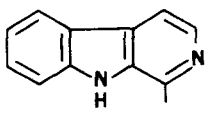
VII



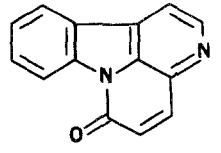
VIII

DNA in the dark and subsequently undergo photobinding in UV-A. Dictamnine binds preferentially to the same sites as 8-methoxypsoralen (Pfyffer et al., 1982). Dictamnine, as well as a number of other furanoquinolines and certain other alkaloids, is mutagenic and causes gross chromosomal abnormalities in CHO cells in UV-A (Towers and Abramowski, 1983). Their importance in relation to insect predation is unknown as is the significance of the cooccurrence of two biogenetically unrelated phototoxic compounds in one species.

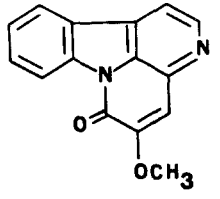
A second group of alkaloids which has been found to be photogenotoxic to CHO cells (Towers and Abramowski, 1983) and phototoxic to larvae of *Aedes atropalpus* is the β-carbolines or harman compounds, e.g., harman (IX) (Arnason et al., 1983). These are very widely distributed, occurring in about 26 families of plants (Allen and Holmstedt, 1980). Additional tryptophan-derived alkaloids, such as 6-canthinone (X) and 5-methoxy-6-canthinone (XI) of *Zanthoxylum* (Rutaceae) and brevicolline (XII), an *N*-methylpyrolidine-substituted harman alkaloid which occurs in the sedge, *Carex brevicollis*, have also been shown to be phototoxic to bacteria, fungi, and CHO cells (Towers and Abramowski, 1983) but their effects on insects remain unstudied. Berberine, a well-known phenylalanine-derived alkaloid is also phototoxic to mosquito larvae but



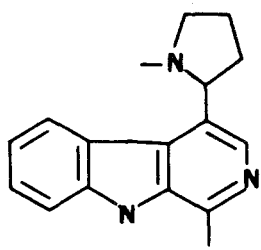
IX



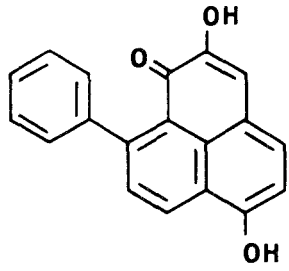
X



XI



XII



XIII

its mechanism of action is not known (Philogene et al., 1984). There are undoubtedly very many other alkaloids with this type of bioactivity awaiting research by chemical ecologists with an understanding of photobiology.

The excreta of the silkworm (*Bombyx mori*), used in traditional Chinese medicine, contains cytostatic/cytotoxic porphyrins, including the phototoxic methyl esters of pheophorbides (Nakatani et al., 1981). The generation of singlet oxygen in light by excited states of certain porphyrins is responsible for their phototoxicity. How many kinds of phytophageous insects produce phototoxic frass? The ecological significance of phototoxic frass may be of interest in relation to microbial colonization of this material but is this phototoxicity fortuitous and is it really important ecologically?

One can extend these questions to include the many other miscellaneous compounds of plant origin such as lachnanthocarpone (XIII), in species of *Lachnanthes* of the Hemodoraceae which are phototoxic to bacteria and even to vertebrates (Kornfeld and Edwards, 1972). How important are they to phytophagous insects?

Some of these problems have been addressed in work reported in this symposium but much remains to be done; it seems to be clear from research so far that interesting and important new ecological concepts concerning the relations between insects, phytochemicals, and light remain to be discovered.

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TOXICOLOGICAL ACTION AND ECOLOGICAL IMPORTANCE OF PLANT PHOTOSENSITIZERS

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Abstract—This review discusses the biochemical action and ecological significance of phototoxic phytochemicals. Mechanistic details of photosensitization as well as cellular and molecular targets of plant-derived phototoxins in model biological systems (microbial and in vitro) and in herbivorous insects are described. Findings from these studies suggest the potential importance of phototoxic plant metabolites in plant–insect interactions. Aspects of phototoxin distribution and significance in diverse ecosystems are considered, and areas for future research are suggested.

Key Words—Phototoxic phytochemicals, insect photosensitization, mechanisms of photosensitization, plant–insect interactions.

INTRODUCTION

Many phytochemicals capable of photosensitizing action have been isolated from plants (Towers, 1984; Knox and Dodge, 1985; Downum, 1986). Most are potent biocidal agents that effect an array of organisms in the presence of sunlight or UV-A irradiation (320–400 nm). In addition to killing or inactivating viruses, bacteria, fungi, nematodes, and assorted other organisms (see review by Towers, 1984), the susceptibility of herbivorous insects to some of these chemicals has also been demonstrated (Berenbaum, 1978; Kagan et al., 1983; Downum et al., 1984; Champagne et al., this issue). This review discusses aspects of the toxicology of plant photosensitizers (or phototoxins) toward herbivorous insects that might encounter such chemicals in their diets. In addition, we also address

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questions concerning the ecological importance of photosensitizing chemicals in nature.

MECHANISMS OF PHOTOSENSITIZATION

Two general types of photosensitizers or phototoxins are recognized: those that participate in radical or electron transfer reactions (type I mechanisms) and those that catalyze the formation of singlet oxygen ($^1O_2$) via a type II mechanism (Spikes, 1977; Krinsky, 1985). The contrasting mechanisms and the molecular targets of important classes of phototoxic phytochemicals are reviewed in detail elsewhere (Towers, 1984; Knox and Dodge, 1985) and will be mentioned only briefly here to aid the reader in subsequent discussions.

Both type I and type II reactions result from the excitation of a ground-state sensitizer (S) to its singlet-excited state (^1S) following absorption of a photon ($h\nu$) (equation 1). ^1S is highly unstable and readily undergoes electron rearrangement (intersystem crossing) to form a triplet-excited sensitizer (^3S) (2), a longer-lived species that is responsible for catalyzing most biological photosensitizations.

$$S \xrightarrow{h\nu} {}^1S \tag{1}$$

$$^1S \longrightarrow {}^3S \tag{2}$$

In type I mechanisms, the triplet-excited sensitizer ^3S generally bonds covalently to some biomolecule. DNA is one of the most important targets of type I photosensitizers (Dall'Acqua et al., 1971; Song and Tapley, 1979; Towers, 1984), although tRNA and cellular proteins also represent potentially significant targets (Ou and Song, 1978; Averbeck et al., 1978; Veronese et al., 1982; Granger and Helene, 1983). Type II mechanisms, in contrast, involve transfer of excitation energy between ^3S and ground-state or molecular oxygen (which exists in the triplet state, $^3O_2$) (3). Bimolecular reactions such as these result in the formation of singlet oxygen ($^1O_2$), a highly reactive oxygen species, and S which is free to catalyze additional reactions if reexcited by other photons. Membranes are important targets of $^1O_2$. Damage to these cellular structures results from photooxidation of unsaturated fatty acids (Rawls and Van Santen, 1970; Rahimtula et al., 1978), sterols (especially cholesterol) (Teng and Smith, 1973; Suwa et al., 1978; Smith and Stroud, 1978), free amino acids (Fisch et al., 1971), and amino acid residues in proteins (Lamola and Doleiden, 1980; Dubbelman et al., 1980).

$$^3S + {}^3O_2 \longrightarrow S + {}^1O_2 \tag{3}$$

The mechanisms of photosensitization for the major classes of phototoxic phytochemicals are listed in Table 1.

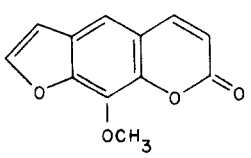
TABLE 1. MECHANISMS OF MAJOR CLASSES of PHOTOTOXIC PHYTOCHEMICALS

	References
Type I	
Acetophenones	Proksch and Rodriguez, 1983
β-Carboline alkaloids	Towers and Abramowski, 1983
Furochromones	Abeysekera et al., 1983
Furocoumarins	Song and Tapley, 1979
Furoquinoline alkaloids	Ashwood-Smith et al., 1982
	Pfyffer et al., 1982
	Pfyffer and Towers, 1982
Isoflavonoid phytoalexins	Bakker et al., 1983
Intermediate	
Acetylenes	Arnason et al., 1980
	Kagan et al., 1984
	McLachlan et al., 1984
Type II	
Extended quinones	Knox and Dodge, 1985
Isoquinoline alkaloids	Philogene et al., 1984
Thiophenes	Bakker et al., 1979
	Arnason et al., 1981a
	Downum et al., 1982

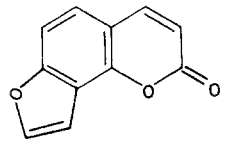
INSECT PHOTOSENSITIZATION

Phototoxic constituents occur in a wide variety of plant families (Downum, 1986). Many of these natural products are toxic to insects as well as to numerous other organisms following excitation by light (Towers, 1984). Although the mechanisms and the molecular targets of quite a few phototoxic phytochemicals have been established using model cell and in vitro systems, parallel investigations with herbivorous insects that might encounter photosensitizers in their food plants are just beginning. Furocoumarins, polyines, quinones, β-carboline and isoquinoline alkaloids (Figure 1) are the major classes of plant-derived photosensitizers which have been shown to interfere with insect growth and development.

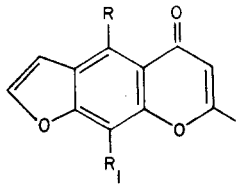
Berenbaum (1978) was the first to demonstrate the light-activated toxicity of a plant chemical to an herbivorous insect. She reported that southern armyworm larvae (*Spodoptera eridania*) reared on diets containing the furocoumarin xanthotoxin (also known as 8-methoxypsoralen or 8-MOP) did not mature past the second instar under simulated daylight conditions but that 40% of the larvae survived through pupation to adult emergence when UV-A wavelengths were excluded. Enhanced larval survival in the absence of UV-A is consistent with



I.

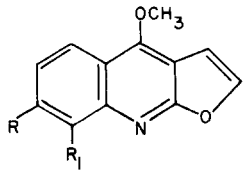


II.



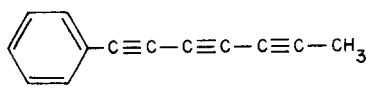
III. R = H; R_1 = OCH_3

IV. R = OCH_3; R_1 = OCH_3

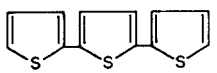


V. R = H; R_1 = H

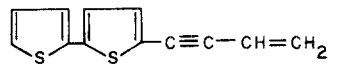
VI. R = OCH_3; R_1 = OCH_3



VII.



VIII.



IX.

FIG. 1. Chemical structures of important phototoxic phytochemicals. I, 8-methoxypsoralen (a linear furocoumarin); II, angelicin (an angular furocoumarin); III and IV, khellin and visnagin, respectively (furochromones); V and VI, dictamnine and skimmianine, respectively (furoquinoline alkaloids); VII, phenylheptatriyne (an acetylenic polyine); and VIII and IX, 2,2′:5′,2″-terthiophene (alpha-terthienyl) and 5-(3-buten-1-ynyl)-2, 2′-bithiophene, respectively (thiophenic polyines).

the type I photomechanism previously demonstrated for 8-MOP (Song and Tapley, 1979). Although formation of 8-MOP/DNA photoadducts could be responsible for much of the larvicidal activity of this common furocoumarin, the susceptibility of *S. eridania* in the absence of UV-A suggests that at least part of its toxic action is not light dependent. Frameshift mutations resulting from noncovalent DNA interactions occur in bacteria exposed to 8-MOP in the dark (Ashwood-Smith, 1978). Similar interactions might explain some of the non-UV-A-induced toxicity of 8-MOP reported by Berenbaum (1978), but this remains to be demonstrated. Light-independent mechanisms have been demonstrated with other phototoxic chemicals from plants (McLachlan et al., 1982; Champagne et al., this issue) as well as with synthetic xanthene dyes (Heitz, 1982).

Discovery of 8-MOP phototoxicity to southern armyworm larvae stimulated research into the photoactivity of other plant compounds toward herbivorous and nonherbivorous insects. The larvicidal activity of 27 polyines (acetylenes and thiophenes) to first and fourth instar mosquito (*Aedes aegypti*) and blackfly (*Simulium vitattum*) larvae in sunlight and UV-A was the subject of two complementary studies (Wat et al., 1981; Arnason et al., 1981b). Exposure of *A. aegypti* larvae to 16 of the compounds and light resulted in substantial mortality. Four of the 14 phytochemicals tested against *S. vitattum* were lethal in light, but three killed larvae in the dark. Alpha-terthienyl (alpha-T) and phenylheptatriyne (PHT) were among the most powerful of the plant-derived photosensitizers tested in these studies.

Alpha-T and PHT, as well as several biosynthetically related derivatives of each, are also potent feeding inhibitors of herbivorous larvae including: the tobacco hornworm (*Manduca sexta*); the dark-sided cutworm (*Euxoa messoria*); and the European cornborer (*Ostrinia nubilalis*) (McLachlan et al., 1982; Champagne, this issue). Feeding inhibition in *M. sexta* and *E. messoria* is UV-A-mediated, but substantial deterrence in the absence of UV-A wavelengths also occurs (Champagne, this issue). *O. nubilalis*, an herbivore that specializes on polyine-containing members of the Asteraceae (sunflower family), is least affected by polyine-containing diets. This may indicate that *O. nubilalis* larvae have an evolved tolerance to these potent light-activated phytotoxins which allows them to feed on plants containing such phototoxins.

Alpha-T, in addition to the above effects, elicits severe toxic responses from tobacco hornworm larvae (*M. sexta*) (Downum et al., 1984). Larval growth and development are drastically altered following ingestion of the thiophene in artificial diets and subsequent exposure to UV–A irradiation. The pupal abnormalities that result from larval treatment with this allelochemical are quite similar to the deformities caused by the action of L-dopa on *S. eridania* (Rehr et al., 1973). Rehr and coworkers suggested that failure of their pupae to sclerotize completely may have resulted from interference of tyrosinase (i.e., phenoloxi-

dase), an enzyme essential for hardening and darkening of the insect cuticle, by the nonprotein amino acid. Singlet oxygen produced by UV-A-activated alpha-T cross-links membrane proteins in *E. coli* (Downum et al., 1982) and inactivates human erythrocyte enzymes (Yamamoto et al., 1979; Wat et al., 1980). Similar effects caused by $^1O_2$ in the integument of *M. sexta* might be responsible for impaired sclerotization and melanization. Preliminary studies of *M. sexta* integument proteins, however, show no pronounced protein cross-linking or enzyme inactivation immediately following treatment with alpha-T/UV-A (Downum et al., 1984).

The extended quinone hypericin, assorted β-carboline alkaloids, and the isoquinoline alkaloid berberine are phototoxic to mosquito larvae (Arnason et al., 1983; Philogene et al., 1984). In addition to killing nonherbivorous insect larvae, hypericin is also phototoxic to the tobacco hornworm *M. sexta* (Knox and Dodge, 1985). Numerous other classes of phytochemicals (e.g., furochromones, furoquinoline alkaloids, lignans, etc.) mediate photosensitized reactions in microbial and in vitro systems (Towers, 1984; Knox and Dodge, 1985; Downum, 1986); however, their phototoxic mechanisms and cellular targets in insects have yet to be established.

ECOLOGICAL IMPORTANCE OF PHOTOSENSITIZING PHYTOCHEMICALS

Considerable information is available on the types of plant secondary metabolites capable of photosensitization and the nonspecific nature of their biocidal action, but little is known about the distribution, frequency, and variation of photosensitizers evolved by plants in different environments. A recent review by Downum (1986) reveals that phototoxic constituents occur in at least 30 plant families. The potential importance of these naturally derived phototoxins in ecological interactions can be appreciated when one realizes that the major plant families synthesizing these light-activated compounds (e.g., Apiaceae, Asteraceae, Fabaceae, Liliaceae, Moraceae, Rutaceae, Solanaceae, and Zygophyllaceae among others) are widely distributed in nature (Heywood, 1978).

In a recent survey of more than 100 plants from North American deserts, we found that species which contained photosensitizers belonged to 13 genera from two of the most successful families of arid/semiarid plants: the Asteraceae and the Zygophyllaceae (Downum et al., 1986). Table 2 lists several of the dominant desert genera which were found to produce phototoxins. All of the Asteraceae genera examined belonged to the tribe Heliantheae (as revised by Robinson, 1981), while the creosote bush *Larrea tridentata* was the only genus in the Zygophyllaceae to give a positive phototoxic response.

Acetylenic and thiophenic polyines which are common phytochemical constituents (Bohlmann et al., 1973) and potent photosensitizers in the Asteraceae (Camm et al., 1975; Towers et al., 1977) are probably responsible for the ma-

TABLE 2. IMPORTANT PHOTOTOXIN-CONTAINING GENERA FROM ARID AND SEMIARID REGIONS OF SOUTHWESTERN UNITED STATES AND MEXICO FOUND IN A RECENT SURVEY (Downum et al., 1986)[a]

Asteraceae	Zygophyllaceae
Adenophyllum (Pectidinae)	*Larrea*
Ambrosia (Ambrosiinae)	
Chrysactinia (Pectidinae)	
Dyssodia (Pectidinae)	
Encelia (Ecliptinae)	
Helianthus (Helianthinae)	
Hymenantherum (Pectidinae)	
Nicolletia (Pectidinae)	
Pectis (Pectidinae)	
Porophyllum (Pectidinae)	
Thelesperma (Coreopsidinae)	
Viguiera (Helianthinae)	

[a] All genera in the Asteraceae belong to the tribe Heliantheae and are followed by their subtribal affiliations in parentheses (after Robinson, 1981).

jority of positive bioassay responses elicited by species in the Heliantheae. Thiophenic polyines are characteristic of most genera in the subtribe Pectidinae (Asteraceae; Heliantheae), although such allelochemicals do not occur in *Pectis*, the largest genus in the Pectidinae (Downum and Towers, 1983; Downum et al., 1985). Despite the lack of thiophenic metabolities in *Pectis*, crude extracts from 12 species are phototoxic to *E. coli* and the yeast *S. cerevisiae* (Downum et al., 1986). The chemical(s) responsible for this activity have yet to be identified, but it is clear from our preliminary work that they do not belong to currently recognized photosensitizer classes from the Asteraceae.

Benzofurans and benzopyrans are another group of phototoxic compounds produced by various genera in the Asteraceae (Proksch and Rodriguez, 1983; Proksch et al., 1983). These acetophenone-derived compounds are effective insect antifeedents (Wisdom et al., 1983; Proksch and Rodriguez, 1983), elicit antijuvenile responses in insects (Bowers, 1982), and recent studies by Aregullin (1985) establish that many naturally occurring derivatives are also phototoxic to insects and to fungi. Several common desert genera in the Heliantheae (e.g., *Encelia*, *Enceliopsis*, *Flourensia*, *Helianthella*, and *Gereae*) produce substantial quantities of these secondary metabolites. Although their exact mode of action has not been determined, they may form quinone methide intermediates which might alkylate important metabolic enzymes and/or DNA in susceptible organisms (Proksch and Rodriguez, 1983; Aregullin, 1985).

The creosote bush *L. tridentata* (Zygophyllaceae) is perhaps the most im-

portant phototoxin-containing plant found in our survey of North American arid-land plants (Downum et al., 1986). The photosensitizer, identified as nordihydroguiaretic acid (NDGA) (Downum et al., in preparation), occurs primarily on external leaf surfaces where it comprises 20–30% of the total leaf resin and up to 5–10% of the leaf dry weight (Mabry et al., 1977). Although the leaf resin of the creosote bush has been shown to deter leaf-chewing insects (Rhoades, 1976, 1977), the involvement of photoactivated NDGA in this herbivore deterrence was not studied and remains unknown. The potential importance of NDGA is apparent when one considers that *L. tridentata* is the dominant woody shrub throughout the Chihuahuan, Mojave and Sonoran deserts of North America (Hunziker et al., 1977). Closely related species *L. ameghinoi*, *L. cuneifolia*, *L. divaricata*, and *L. nitida* (which also synthesize NDGA) are dominant components of South American deserts. Other genera of the Zygophyllaceae such as *Guaiacum*, *Kallstroemia*, and *Viscainoa* from Chihuahua and Baja California either do not contain NDGA or lack sufficient quantities to elicit phototoxic activity (Downum et al., 1986).

Tropical plants, unlike arid and semiarid plants from warm deserts, have not yet been systematically investigated for naturally occurring photosensitizers. This is rather surprising, since one might predict that tropical plants which can be exposed to high levels of solar irradiation throughout the year might elaborate a plethora of light-activated chemicals for defensive purposes (assuming that photosensitizers do in fact have a defensive role). Survey studies are currently underway to establish the significance of plant-derived photosensitizers in tropical ecosystems.

CONCLUSION

It is clear that a considerable amount of toxicological information is available concerning the mechanisms and the targets of plant photosensitizers; however, most of the data result from studies of their action in model cell systems. Efforts to elucidate the toxicology of phototoxic phytochemicals in herbivorous insects have only recently begun. Detailed information in this area is essential to clarify the influence such chemicals might have on the interactions between plants and their insect herbivores.

It is also clear that much remains to be learned about the ecology of phototoxin-containing plants. Studies involving the distribution and quantitative significance of these allelochemicals in a range of diverse habitats as well as their effect on herbivory patterns are areas that remain to be explored. Until such investigations are in progress, the ecological importance and the evolutionary implications of phototoxic phytochemicals must remain highly speculative.

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LIGHT-MEDIATED ALLELOCHEMICAL EFFECTS OF NATURALLY OCCURRING POLYACETYLENES AND THIOPHENES FROM ASTERACEAE ON HERBIVOROUS INSECTS

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Abstract—Polyacetylenes and their thiophene derivatives, characteristic secondary metabolites of the Asteraceae, were examined for their effects on herbivorous insects. Three thiophenes (a monothiophene, a bithiophene, and α-terthienyl) and four polyacetylenes (phenylheptatriyne, phenylheptadiynene, phenylheptadiyene acetate, and matricaria lactone) were studied for their phototoxicity and light-independent toxicity to (1) a polyphagous lepidopteran, *Ostrinia nubilalis*, whose host range includes a number of phototoxic Asteraceae, (2) a polyphagous lepidoteran, *Euxoa messoria*, whose host range includes very few species of Asteraceae, and (3) an oligophagous lepidopteran, *Manduca sexta*, which is a specialist on Solanaceae. Several compounds were phototoxic to *M. sexta* and *E. messoria* even at very low irradiance levels, but behavioral adaptations, including spinning silk and boring into diet, allowed *O. nubilalis* to avoid photosensitization. Light-independent activity of the compounds to all three species involved feeding deterrence increasing in the order *O. nubilalis*, *E. messoria*, and *M. sexta*, and long-term metabolic toxicity in the form of impaired nutrient utilization. The biosynthetically derived thiophenes were more toxic than their acetylenic precursors, and toxicity increased with increasing number of thiophene rings. The results are discussed in terms of plant–insect coevolution.

Key Words—Polyacetylenes, thiophenes, Asteraceae, phototoxicity, *Ostri-*

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nia nubilalis, *Euxoa messoria*, *Manduca sexta*, Lepidoptera, Pyralidae, Sphingidae, Noctuidae, coevolution, photosensitization, feeding deterrence.

INTRODUCTION

Studies of the allelochemical effects of plant secondary metabolites on herbivorous insects have usually examined the activity of these compounds in the ground state. Recently, it has been realized (Towers, 1980) that many natural products of plant origin interact with light, yielding excited state molecules with the capacity to undergo a variety of toxic high-energy processes. Most of these naturally occurring photosensitizers have been examined for their effects on microorganisms, with insects only recently receiving attention. Furanocoumarins of the Apiaceae (Berenbaum, 1978, 1981, 1983; Berenbaum and Feeny, 1981) are the only photosensitizers that have been thoroughly investigated for their role in plant–insect relations.

Among the more active natural photosensitizers are the polyacetylenes and their thiophene derivatives (Towers, 1980, Arnason et al., 1983a). These are characteristic secondary metabolites of the Asteraceae, where they are diverse and abundant, but they also occur in several other plant families (Bohlmann et al., 1973). Simple aliphatic polyacetylenes have an oxygen-independent (nonphotodynamic) mechanism of action which may involve the formation of free radicals upon photoexcitation, whereas the biosynthetically advanced thiophenes are type II photodynamic photosensitizers which damage biological targets via the catalytic production of singlet oxygen (McLachlan et al., 1984; Downum et al., 1982; Arnason et al., 1981a). Partly cyclized polyacetylenes, intermediate in structure between the aliphatic acetylenes and the thiophenes, apparently have access to both photodynamic and nonphotodynamic mechanisms of action (McLachlan et al., 1984). Target sites appear to involve membranes (Downum et al., 1982; Wat et al., 1980; Yamamoto et al., 1979).

Some polyacetylenes and thiophenes are highly phototoxic to insects in bioassays using mosquito (*Aedes aegypti*) and blackfly larvae (Arnason et al., 1981b; Wat et al., 1981). The thiophene α-terthienyl (α-T) is not only phototoxic to blood-feeding insect larvae (Philogène et al., 1985) but also to larvae of the phytophagous lepidopterans *Euxoa messoria* (Champagne et al., 1984) and *Manduca sexta* (Downum et al., 1984). Polyacetylenes and thiophenes may photosensitize a variety of other organisms, including pathogenic fungi (Bourque et al., 1985), vascular plants (Campbell et al., 1982), nematodes (Wat et al., 1981; Gommers and Geerligs, 1973; Bakker et al., 1979), trematodes (Graham et al., 1980), and numerous prokaryotic and eukaryotic microorganisms (Chan et al., 1975; Wat et al., 1979; Towers, 1980; Arnason et al., 1981b).

The toxic action of these natural products is not confined to light-mediated reactions, as a number of polyacetylenes and thiophenes are antibiotic. Gener-

ally phytoalexin polyacetylenes are toxic to fungi without photosensitization (e.g., DeWitt and Keddie, 1981), although their activity may possibly be enhanced in the presence of near-UV light. Light-independent toxicity of acetylenes to phytophagous insects has also been described. Binder et al. (1979) found that matricaria ester, a widespread aliphatic polyacetylene in the Asteraceae, was toxic to three species of herbivorous lepidopterans, and McLachlan et al. (1982) reported that phenylheptatriyne (PHT), a partially cyclized polyacetylene, reduced the growth of the cutworm *Euxoa messoria*. In both cases, feeding deterrence was suggested to be a major component of the mechanism of action. Some matricaria ester derivatives are antifeedants to the Colorado potato beetle (Jermy et al., 1981). A polyacetylene from an insect, *Chauliognathus pennsylvanicus*, is also a powerful antifeedant, conferring protection from carnivores (Eisner et al., 1981)

The effects of polyacetylenes and thiophenes on herbivorous insects and the role of photosensitization in this activity, are as yet poorly understood. In this study, we examine the effects of seven polyacetylenes and thiophenes, administered orally, on the survivorship, growth, and feeding behavior of three phytophagous insects: the darksided cutworm, *Euxoa messoria* (Lepidoptera: Noctuidae), the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae), and the tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphingidae). These insects cover a range of feeding habits from specialization on nonphototoxic Solanaceae (*M. sexta*), through polyphagy excluding phototoxic Asteraceae (*E. messoria*), to polyphagy including phototoxic Asteraceae (*O. nubilalis*). The compounds selected for the study (Figure 1) are representative of the Asteraceae generally and are found in many species in the tribes Heliantheae, Cynareae, Helenicae, and Asteraceae.

METHODS AND MATERIALS

Source of Compounds. PHT was extracted as described by Wat et al., (1979) from leaves of *Bidens pilosa* L. from Tampa, Florida. Alpha-terthienyl was prepared synthetically (Philogène et al., 1985). All other compounds were obtained by one of us (J. Lam) as described in McLachlan et al. (1984) from *Dahlia* spp., *Echinops* spp., and *Solidago vigaurea*.

Insect Cultures. Insects were reared on meridic diets as described by Stewart and Philogène (1983) for *M. sexta*, Guthrie (1971) for *O. nubilalis*, and Devitt et al. (1980) for *E. messoria*. Growth conditions were 27°C, 80% relative humidity and a photoperiod of 18:6 light–dark under fluorescent Vitalites® (100 W/m^2).

Test Diets. Appropriate amounts of the test compounds in 95% EtOH were placed in 50- or 100-ml beakers, to which was added warm liquid diet. The temperature of the diet was not more than 35°C at this time in order to prevent

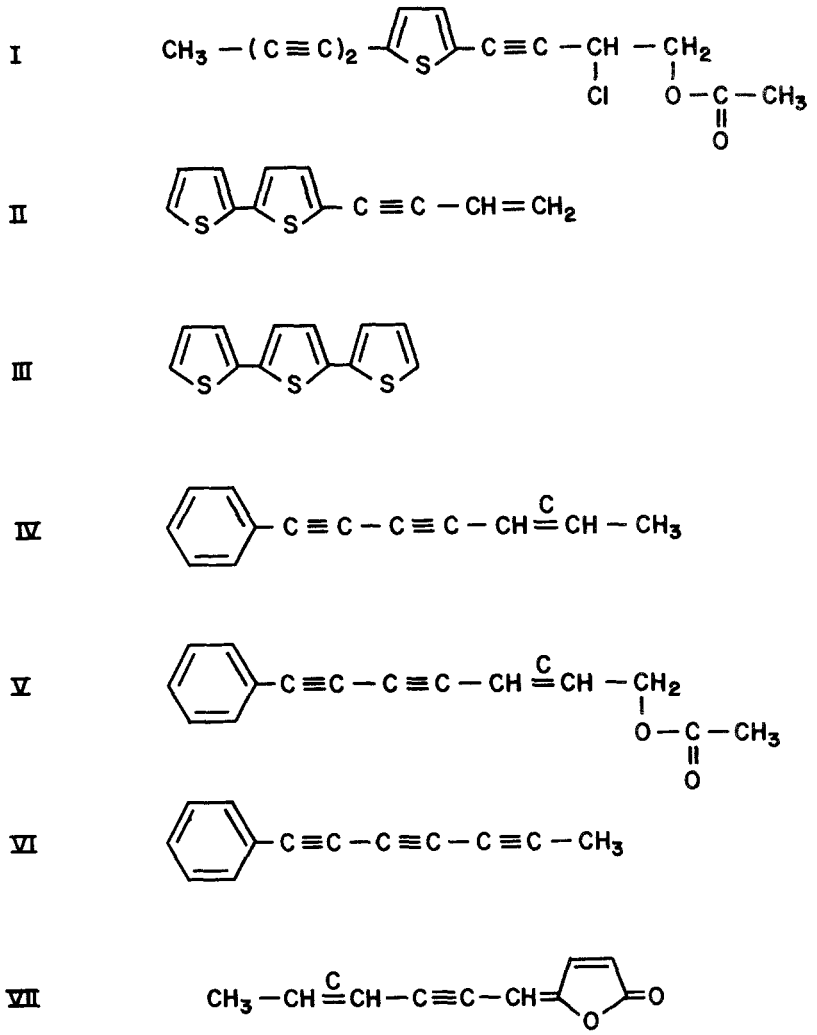

FIG. 1. Polyacetylenes and thiophenes used in the study. Compound III is known by the trivial name α-terthienyl and compound VI, phenylheptatriyne.

degradation of the acetylenes. The diet and acetylenes in EtOH (or EtOH alone in the case of controls) were mixed vigorously by hand for about 2 min; the diet was then poured into 1 × 1 × 0.6-cm molds to cool. The amount of 95% EtOH carrier in the diets did not exceed 2.5% (0.5 ml 95% EtOH in 25 g diet). Fresh diet for all insect species was prepared weekly and refrigerated at about 5°C until used.

Growth Studies with Manduca sexta. Thirty or forty mature eggs of *M. sexta* were placed into each Petri dish with an appropriate diet cube containing

100 μg/g PHT or α-T. The dishes were examined 24 h later, and unhatched eggs and larvae in excess of 25 were removed. Irradiation was begun on day 5 of the test, to allow larvae to accumulate the test compounds and to facilitate distinction of light-independent effects from phototoxicity. Larvae were weighed on days 5, 7, 10, 14, and 15 post-hatch, at which time the experiment was terminated. Diets were changed every 2–3 days. The experiments were repeated twice, and each experiment included two replicates of the α-T and PHT treatments and three of the control.

The light system used for these experiments included a bank of eight Vitalites augmented by four black light blue (BLB) lamps, 54 cm above the insects. The near-UV contributed by the Vitalites was negligible as these lamps were located above a UV-opaque Plexiglas partition in the growth cabinet. The near-UV was contributed by the BLB lamps and was measured at 2.0 W/m^2.

Growth Studies with Euxoa messoria. Three thiophenes (compounds I–III) and four polyacetylenes (compounds IV–VII) were screened for allelochemical effects against *Euxoa messoria*.

Diet cubes (two to four, depending on the age and size of the insects containing 100 μg/g test compound), were cut in two and placed in a Petri dish; the perimeter of the dish was lined with Whatman No. 1 glass fiber filter paper. Twenty-five larvae of *E. messoria* were placed in each dish, which was sealed with polyethylene film. One dish was prepared for each combination of test compound and light treatment (2 W/m^2 near-UV) because of limitations in the available amount of test compounds. The experiment was subsequently repeated but followed for two, instead of three weeks. Diets were changed, dead larvae counted and removed every second day, and larvae were weighed every fourth day. The larvae were reared individually from day 18 to day 21, at which time the experiment was terminated.

The same light system as described for *M. sexta* was used in these experiments (2 W/m^2 near-UV).

For experiments at low-light intensity, groups of 30 larvae were treated with combinations of 0.5 W/m^2 near-UV and diet containing 10 or 100 μg/g α-T. Larvae were reared individually in ventilated polystyrene vials. Fresh diet was provided, larvae weighed, and exuvia checked every two days until all individuals in each treatment group had entered the prepupal stage. Prepupae were transferred to vials containing moist peat moss and checked every two to three days until pupation occurred, at which time pupae were weighed. Darkened pupae were transferred to 62 × 60 × 30-cm oviposition cages for adult emergence.

Light was provided by a bank of 12 Vitalite fluorescent tubes 50 cm above the insects. The near-UV intensity to which the larvae were exposed was measured at 0.5 W/m^2.

Growth Studies with Ostrinia nubilalis. The toxicity of three polyacetylenes (IV, V, and VI) and three thiophenes (I, II, and III) to *Ostrinia nubilalis* at 100 μg/g in diets was examined.

TABLE 1. EFFECT OF POLYACETYLENES AND THIOPHENES ON FEEDING BEHAVIOR OF NEONATE *Manduca sexta, Euxoa messoria,* and *Ostrinia nubilalis* IN NO-CHOICE TEST[a]

Compound	*Manduca sexta*	*Euxoa messoria*	*Ostrinia nubilalis*
Control	100 a	100 a	100 a
I		80.8 a	91.2 a
II		38.0 b	72.8 b
III	0 b	38.0 b	64.8 b
IV	5.0 b	32.8 b	103.2 a
V		25.1 b	108.0 a
VI		16.3 b	85.6 b
VII		80.1 a	94.4 a

[a] Feeding rate is given as percent of control; all compounds were tested at 100 μg/g and without near-UV irradiation. Values in a column followed by the same letter are not significantly different (Duncan's multiple-range test, $P \leq 0.05$).

To screen compounds I–VI (Table 1), egg masses at the blackhead (hatching) stage, containing 10–30 eggs each, were pinned to appropriately treated diet cubes in glass scintillation vials and stoppered with a cotton plug. Hatching neonates moved onto the diet cubes and began feeding. The following morning (15 hr later), larvae were distributed to six vials with appropriate diet for each treatment group, five larvae to a vial. Thus, all of the 30 larvae in each treatment group were of the same age, within 15 hr. Diets were changed and larvae weighed every four to five days over a 20-day period.

Feeding Deterrency Tests. To test the effect of thiophenes and polyacetylenes on neonate feeding behavior, test compounds were applied in acetone to both surfaces of a leaf disk. Control disks were treated with acetone alone. Lettuce (*Lactuca sativa*) leaves were used for *E. messoria*, corn leaves (*Zea mays* cultivar Silver Queen) for *O. nubilalis*, and tobacco (*Nicotiana tabacum* cultivar Delhi 77) leaves for *M. sexta*. Leaf disks were weighed and placed in glass scintillation vials; five *E. messoria* and *O. nubilalis* or two *M. sexta* neonates were placed on each leaf disk and the vials were loosely capped to allow ventilation. Experiments were conducted in a Conviron ET growth cabinet at 26°C, 80% relative humidity, and a photoperiod of 18:6 hr light–dark. After 48 h, the surviving larvae in each vial were counted and the leaf disks were dried for 24–48 h at 80°C in a drying oven and weighed. Consumption was calculated as the difference in leaf dry weight; initial dry weights were calculated from regression equations based on not less than 10 dry weight/fresh weight determinations for each plant species. All compounds were tested at 100 μg/g (and in some cases 10 μg/g) concentrations. At least five replicates of each treatment were obtained. The design of these experiments was "no-choice."

More mature *E. messoria* and *M. sexta* larvae were tested in no-choice and choice experiments. For no-choice tests, preweighed fourth and fifth instar unstarved larvae were presented with an appropriately treated weighed cube of artificial diet. Consumption and frass production were measured after 24 and 48 hr, and larvae were reweighed. Diet weight loss due to evaporation was corrected for on the basis of diet cubes not exposed to insect feeding; evaporation accounted for a daily weight decline of about 16%. At least ten replicates of each concentration were obtained. For *E. messoria* fed 100 μg/g α-T, trials with and without near-UV (4 W/m^2) were conducted. For choice tests, eight diet cubes were pinned around the perimeter of a 14-cm-diameter desiccator. Each experiment included three control and five dosed diet cubes; the dosed cubes included two at each of two concentrations plus one at a third concentration. Four *E. messoria* larvae were released in the center of the desiccator. Experiments were conducted with both starved (18 hr) and unstarved larvae, but no difference in the behavior of the two groups was apparent. Both α-T and PHT were tested at 0.1, 1.0, 10.0, and 100 μg/g, each concentration at least seven times. Feeding larvae were exposed to a regime of visible light without UV of 18:6 hr light–dark.

Nutritional Indices. Nutritional indices determined (Reese, 1979; Waldbauer, 1968) included the consumption index (CI), the approximate digestibility (AD), the efficiency of conversion of ingested food (ECI), and efficiency of conversion of digested food (ECD).

Larvae of *E. messoria*, from the test in which three thiopenes and four polyacetylenes were examined, were weighed on day 18 of that test, then transferred to individual 3.5 × 6.0-cm clear polystyrene vials with a fresh, weighed diet cube dosed with the same compound (100 μg/g) to which the insects had been exposed. Larval weights, frass, and unconsumed diet were determined daily for the next three days. The same procedure was followed with fourth instar larvae from the stock culture: such larvae were "naïve" in that they had not previously been exposed to the test compounds. Remaining diet was dried at 80°C in a drying oven. Frass was dried for 48 hr under vacuum in a desiccator. The initial dry weight of the diet was calculated from the dry weight/fresh weight ratio (=0.2624), as were both the initial and final dry weights of the larvae (for unstarved larvae, dry weighted = 0.1415 fresh weight).

Data Analysis. Data sets from all treatment groups were log transformed and tested for homogeneity of variance with the Bartlett box F test before proceeding to Duncan's multiple-range test.

RESULTS

Growth Studies with M. sexta. When a model thiophene, α-T (III) and a model acetylene PHT (IV) were added to insect diets, both had a light-independent and phototoxic effect on growth and survival of *M. sexta* larvae (Figure 2).

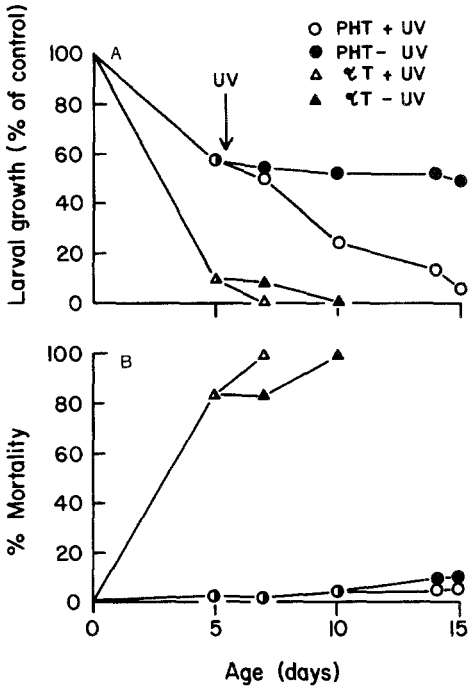

FIG. 2. *Manduca sexta* growth (A) and mortality (B) in response to combinations of 2 W/m^2 near-UV and diet containing 100 $\mu g/g$ α-T or PHT. The arrow indicates the initiation of radiation on day 5. Standard errors were less than 5% and are omitted.

Neonates fed 100 $\mu g/g$ α-T or 100 $\mu g/g$ PHT without photosensitizing radiation had significantly ($P \leq 0.05$) lower growth (Figure 2A) than controls at five days. α-T had greater growth reducing effects than PHT. During the first five days of growth, mortality occurred only in the α-T-treated insects (Figure 2B). Larvae in these groups consumed little diet and produced few fecal pellets, suggested that starvation contributed to mortality.

Subjecting half the insects at day 5 to photosensitizing near-UV led to a separation of the growth and survival curves. With PHT, irradiated larvae had significantly ($P \leq 0.05$) less growth than nonirradiated larvae from day 10 on. With α-T, the same trends were observed in mean growth, although the photosensitized and nonphotosensitized groups were not significantly different. Increase mortality trends were also observed in the irradiated group with α-T as compared to nonirradiated larvae.

Irradiated larvae developed black necrotic lesions on the dorsal surface, particularly along the mid-dorsal vein, which were never seen in unirradiated larvae treated with α-T or PHT or in larvae given control diets and irradiated. Lesions were more evident in PHT-treated than α-T-treated insects. Larvae

from one replicate of each experiment were exposed to direct sunlight for 50 min, and the number of lesions was seen to increase substantially during this period. Small lesions caused the larvae no immediate observable distress. However, at moulting they were often unable to ecdyse past the lesion. The partially moulted cuticle constricted the larvae, preventing passage of the gut contents and restricting circulation of the hemolymph. Eventually, the anterior part of the larvae became turgid, and the larvae stopped feeding and died.

Growth Studies with E. messoria. With this insect, the full range of seven compounds available was tested at 100 μg/g in insect diets with and with near-UV treatments (Figure 3). Near-UV irradiation alone, without the compounds, clearly had no effect on growth as compared to controls. Three compounds: α-T (III), PHD (IV), and PHT (VI) were clearly phototoxic and caused more growth reduction under irradiated than nonirradiated conditions. Little difference between irradiated and nonirradiated groups was seen with control diets or with compound II, while compounds I, V, and VII showed reduced activity over part of the growth curve in irradiated groups. These latter compounds are highly unstable in the presence of near-UV (McLachlan et al., 1984), and their loss of activity can be explained by photodegradation to inactive polymers.

The sublethal effects of α-T on *E. messoria* were further investigated when larvae were reared on diet containing 100 and 10 μg/g α-T and exposed to a low intensity (0.5 W/m^2) of near-UV (as compared to 2 W/m^2 in the previous experiment or 4 W/m^2 as described in Champagne et al., 1984). These low levels of near-UV actually stimulated growth of insects given control diets, a phenomenon not observed at higher intensities.

Larvae fed 100 μg/g α-T showed significantly depressed growth relative to the irradiated and nonirradiated controls (Figure 4) and development time to the prepupal stage (indicated by the leveling off of growth curves in Figure 4) was increased from 34 to 50 days ($P \leq 0.01$). Maximum larval weight decreased from 478 mg to 385 mg ($P \leq 0.05$) (without irradiation) or 338 mg (irradiated) ($P \leq 0.01$). No increase in the number of individuals entering supernumerary instars was noted. Pupal weights were similarly reduced. The mean weight of larvae treated with α-T and near-UV was consistently lower than that of larvae exposed to α-T alone, and the observed weights were lower than would be expected, if α-T and near-UV light did not interact, from day 28 on.

Similar results were found when larvae were fed diet containing 10 μg/g α-T (data not shown). The growth of these larvae was depressed to a rate intermediate between the control groups and the larvae fed 100 μg/g α-T. These larvae reached the prepupal stage four days earlier than larvae treated with α-T at 100 μg/g but significantly later than control larvae ($P \leq 0.05$). Maximum larval weights were similar to those observed for larvae treated at the higher α-T concentration. Pupal weights were significantly ($P \leq 0.05$) lower than the controls. There were no significant differences in mortality rates between any of the treatment groups.

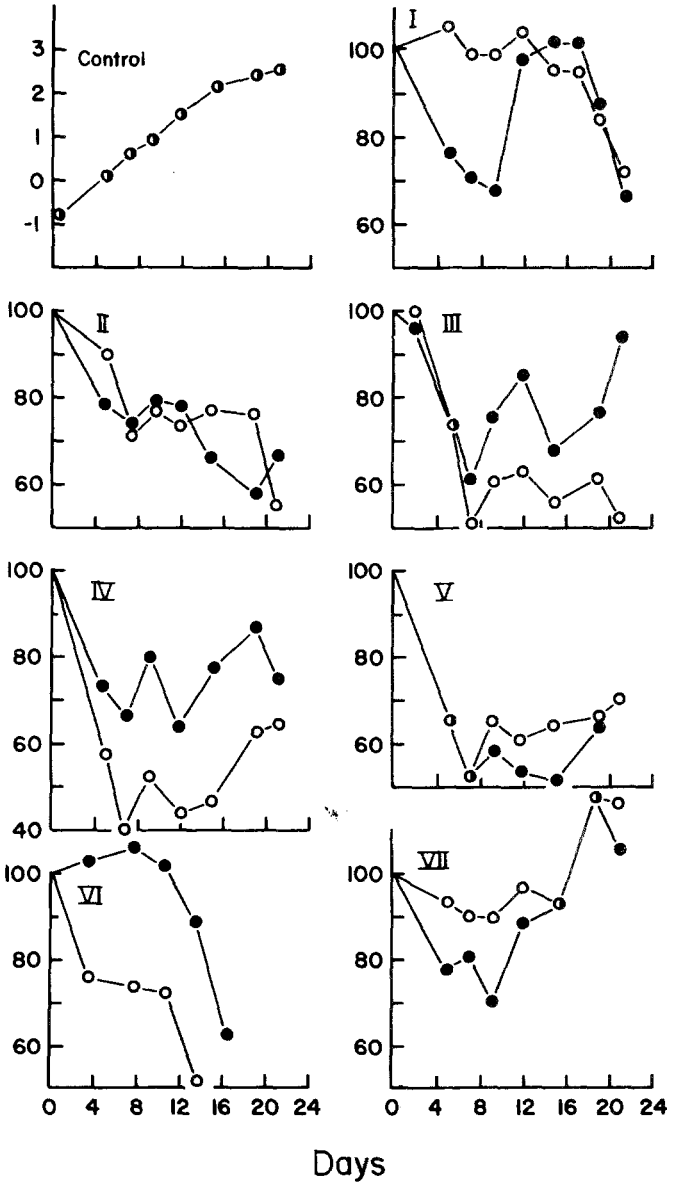

FIG. 3. *Euxoa messoria* growth as percent of control with exposure to 2 W/m^2 near-UV and diet containing 100 μg/g compounds I–VII. The growth of control larvae, as log wt (mg), is given in the top left figure. Standard errors were generally less than 10% and are omitted. ○, irradiated; •, unirradiated.

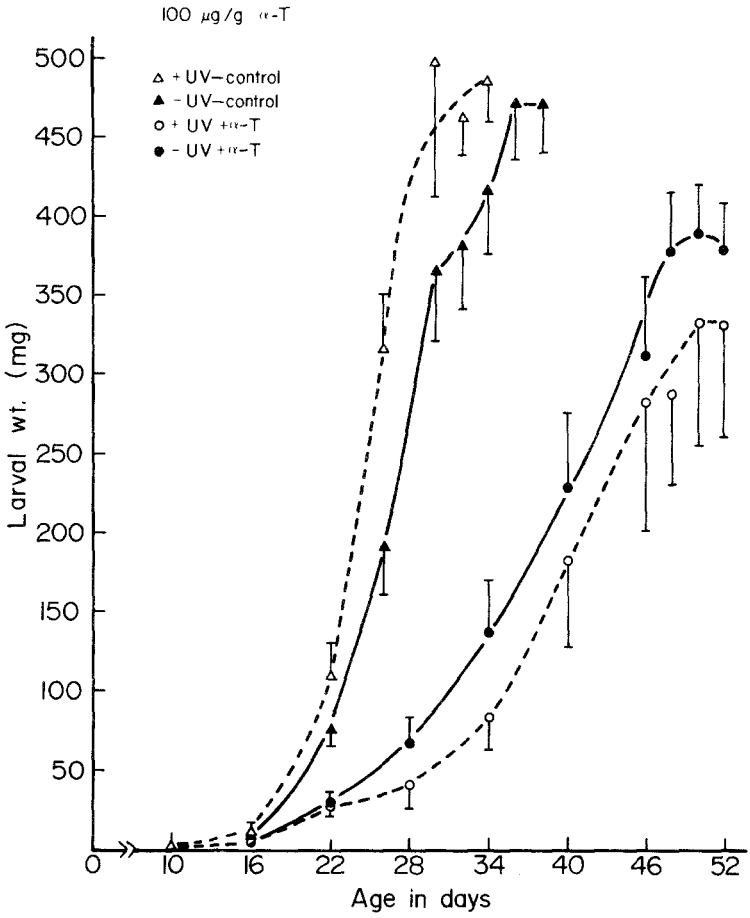

FIG. 4. *Euxoa messoria* growth with exposure to combinations of 100 μg/g α-T and very low UV irradiation (0.5 W/m^2 near-UV). Bars represent one standard deviation.

Growth Studies with O. nubilalis. All larvae lined the rearing vials with UV-opaque silk or bored into diets, thus preventing an investigation of the effects of near-UV irradiation. Nonphotosensitizing effects resulting in growth reduction (Figure 5) were found with all seven compounds tested at 100 μg/g in diet. The thiophenes I–III were more inhibitory than the nonthiophenic acetylenes (IV–VII), with inhibition increasing with the number of thiophene rings. High mortality was seen with compounds II (67%) and III (90%) at the end of the trial as compared to controls (20%), but with compounds I, IV, V, VI, and VII mortality was not different from controls.

Antifeedant Activity. The rapid initial decline in relative weight of neonate *E. messoria*, *O. nubilalis*, and *M. sexta* seen with most of the acetylenes and

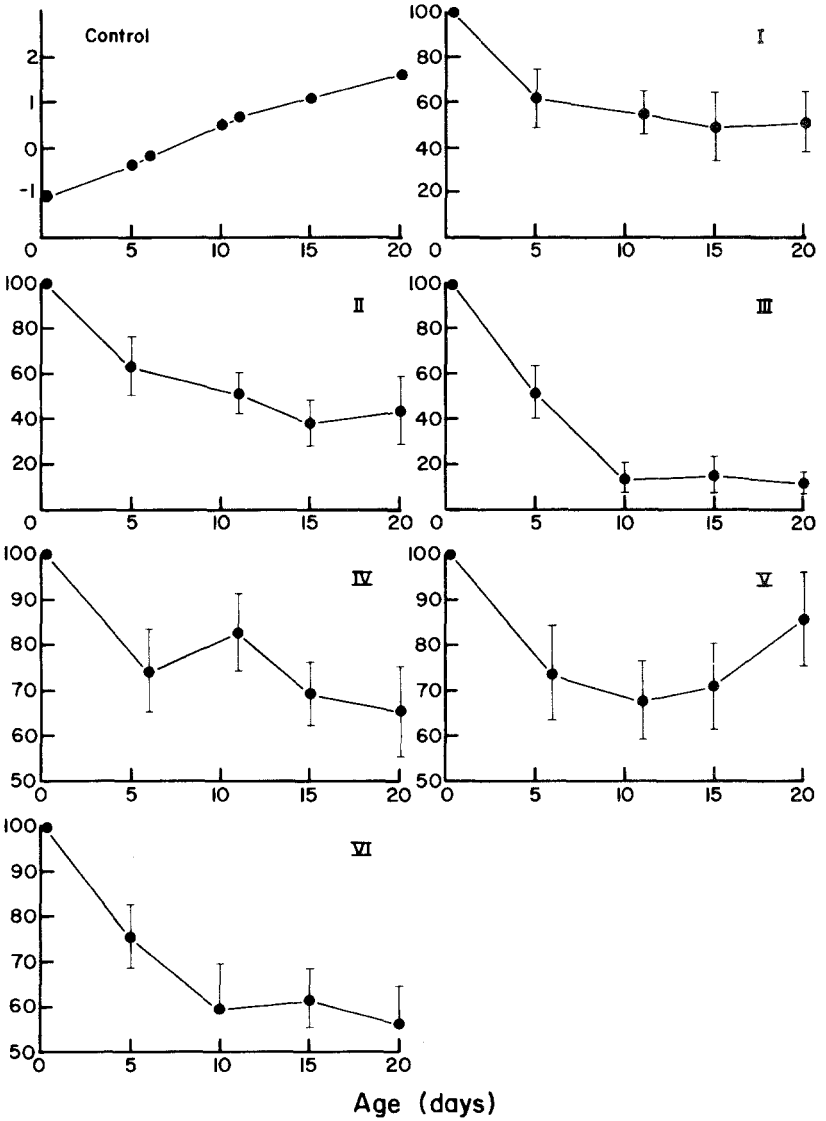

FIG. 5. *Ostrinia nubilalis* growth as percent of control with exposure to diet containing 100 μg/g compounds I–VI. The growth of control larvae, as log wt (mg), is given in the top left figure. Bars represent one standard deviation.

thiophenes suggested that these compounds reduced the feeding activity of these insects. Experiments in which leaf disks of appropriate plants were treated with 100 μg/g of the test compounds without irradiation showed that neonates of *M. sexta* were strongly deterred by α-T (III) and PHT (IV) (Table 1). Supression of feeding was much less in neonates of *E. messoria* and even less in neonate

O. nubilalis with these two compounds. A test of all compounds with *E. messoria* showed that neonates were deterred by all compounds except VII. *O. nubilalis* neonates were only deterred significantly by II, III, and VI.

A choice test with fifth-instar larvae of *E. messoria* (Figure 6) yielded a PC_{50} (a 50% protective concentration) of 6.2 μg/g for α-T ($r = 0.99$) and 119 μg/g for PHT ($t = 0.81$). In this same figure we can see feeding rates that are 80% of control feeding with 100 μg/g α-T in a no-choice situation as compared to 25% in the choice test. Irradiation reduced feeding of *E. messoria* to 60% of controls compared to 80% for nonirradiated insects.

Nutritional Indices. Both thiophenes and polyacetylenes were found to de-

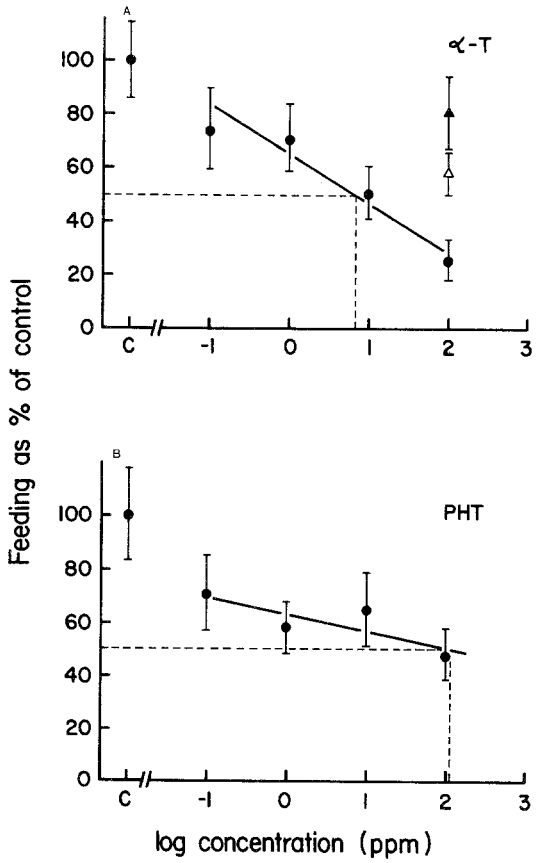

FIG. 6. *Euxoa messoria* feeding rate as percent of control in response to α-T and PHT. (A) Feeding activity with 0-100 μg/g α-T in a choice test. ▲ indicates feeding on diet containing 100 μg/g α-T in a no-choice test without near-UV irradiation, and Δ indicates feeding, in a no-choice test, to a combination of 100 μg/g α-T and 4 W/m^2 near-UV. (B) Feeding activity in a choice test in response to diet containing 0-100 μg/g PHT. In both cases, the dashed line indicates the concentration required to produce a 50% decrease in feeding. Bars represent one standard deviation.

TABLE 2. NUTRITIONAL INDICES FOR HABITUATED *Euxoa messoria* EXPOSED TO DIET CONTAINING 100 μg/g COMPOUNDS II–V WITH AND WITHOUT NEAR-UV IRRADIATION[a]

Compound	Near-UV	ECI	AD	ECD	CI
Control	+	100 a	100 a	100 a	100 a
	−	98.2 a	103.3 a	95.8 a	108.4 a
II	+	49.8 b	89.4 a	51.7 b	92.4 a
	−	39.0 b	86.6 a	37.2 b	87.7 a
III	+	33.7 b	84.4 a	37.2 b	88.9 a
	−	40.3 b	93.5 a	39.0 b	91.5 a
IV	+	23.7 b	103.4 a	21.4 b	102.3 a
	−	38.8 b	96.4 a	37.3 b	98.7 a
V	+	38.8 b	96.4 a	29.9 b	110.3 a
	−	31.8 b	95.9 a	29.9 b	94.1 a

[a] All larvae were exposed to the test compounds for the 18-day period preceding the initiation of the three-day test. Values are given as percent of control; values in a column followed by the same letter are not significantly different (Duncan's multiple-range test, $P \leq 0.05$).

crease the efficiency with which *E. messoria* larvae converted ingested diet into insect biomass. The simplest measure of the ability of an animal to utilize dietary items is the "efficiency of conversion of ingested food" or ECI. Compounds II, III, IV, and V all reduced the ECI to 32–50% of the control in "habituated" larvae that had been exposed to the compounds for 15 days (Table 2). Reduction in the ECI was generally proportional to the decrease in growth rate. Thiophenes (II and III) and polyacetylenes were about equally efficient at reducing the ECI. Among the phototoxic compounds (III and IV), the irradiated larvae had a lower ECI than nonirradiated larvae. The ECI has two components, the "approximate digestibility" (AD) and the "efficiency of conversion of digested food" (ECD). In all cases, the AD was close to control values, demonstrating that the acetylenes and thiophenes did not interfere with the digestibility of the diet. However, the ECD was lowered to 20–60% of control.

Table 3 compares values of ECI for naive fourth-instar larvae and larvae habituated to compounds in their diet for 18 days. Only α-T (III) reduced the ECI in naive larvae significantly ($P \leq 0.05$), while all compounds had a significant effect on habituated larvae.

DISCUSSION

Photosensitizing Activity of Acetylenes and Thiophenes. The present study demonstrates that the interaction of electromagnetic radiation and some acetylenic allelochemicals can lengthen larval development time, increase mortality, reduce growth, decrease ECI and ECD, or decrease feeding of some but not all

TABLE 3. MEAN ECI VALUES FOR NAIVE AND HABITUATED *Euxoa messoria* LARVAE AFTER 24-HR FEEDING ON DIET CONTAINING 100 μg/g COMPOUNDS III, IV, OR V[a]

Compound	Near-UV	Mean ECI at 24 hr	
		Naive larvae	Habituated larvae
Control	+	50.8 a	48.2 a
	−	43.6 a	47.4 a
III	+	23.6 b	16.2 b
	−	23.1 b	19.1 b
IV	+	[b]	11.4 b
	−	46.6 a	16.6 b
V	+	[b]	18.7 b
	−	42.2 a	15.1 b

[a] Naive larvae were not exposed to the test compounds prior to the experiment, whereas habituated larvae had 20 days exposure to treated diets. Values in a column, followed by the same letter, were not significantly different (Duncan's multiple-range test, $P \leq 0.05$).
[b] Not measured.

herbivorous insect species. In previous studies from this and other laboratories (Champagne et al., 1984; Downum et al., 1984), high mortality was observed with insects photosensitized by α-T at relatively high irradiance. The low near-UV levels (0.5–2 W/m^2) used in this study are more representative of levels occurring under leaf canopies. Thus, unadapted insects like *E. messoria* or *M. sexta* feeding on plants containing α-T or PHT at concentrations similar to those used in this study are likely to be photosensitized even in relatively shady microhabitats. Given that *Bidens pilosa* leaves contain 600 μg/g PHT (Bourque, 1984), that above ground parts of *Flaveria trinervis* contain at least 60 μg/g of α-T (Arnason et al., 1983b), and that sunlight may exceed 50 W/m^2 near-UV in temperate regions, it is probable that photosensitization has real ecological significance in plant–pest interactions under natural conditions. Obviously, one insect in the study, *O. nubilalis*, was able to avoid photosensitization by its behavioral adaptations of silk spinning or boring into diet, a behavior that parallels its stem-boring habit in the field. Avoidance of light may be of importance in allowing *O. nubilalis* to feed on host plants rich in phototoxins.

The development of photosensitized lesions in *M. sexta* fed PHT (or to a less extent fed α-T) suggests that the allelochemicals were transported from the gut, through the hemolymph, to tissues adjacent to the hemocoel. The absence of necrotic lesions in *E. messoria* suggests photosensitized targets at the subcuticular level, as has been observed with other cases of photosensitization, i.e., involving furanocoumarins (Berenbaum, 1978; Berenbaum and Feeny, 1981) and various synthetic dye compounds (Lavialle, 1983; Clement et al., 1980; Broome et al., 1975; Yoho et al., 1971, 1973). Polyacetylene photosensitization may be associated with the inhibition of enzymatic processes, as PHT in-

activates membrane-bound enzymes in human erythrocytes (Yamamoto et al., 1979). Synthetic dye sensitizers also inactivated acetylcholinesterase and lactic dehydrogenase in vitro, but in vivo effects were ambigous (Callaham et al., 1975, 1977a,b). Of course, the effect of photosensitizers in insects may be modified by various factors, including the optical density of the cuticle and the ability of the insect to metabolize and excrete compounds, which will affect the amount of phototoxin reaching susceptible target sites. The cuticle of *E. messoria* is more pigmented than that of *M. sexta*, and only transmits about 2% of the incident light at the λ_{max} of α-T, 350 nm.

It is difficult to compare the photoactivity of α-T and PHT to other insecticidal photosensitizers because of differences in experimental designs and test organisms. Xanthotoxin is highly phototoxic to *Spodoptera eridania* larvae at 0.1 and 1.0% concentrations (Berenbaum, 1978). Tests with synthetic dyes have usually involved feeding or injecting test insects with relatively large amounts of compound, then irradiating the insects briefly (1–12 hr) with high levels of visible light. However, it would appear that α-T and PHT, at least, are comparable to the furanocoumarins and dyes in their ability to photosensitize insects.

As many thiophenes, polyacetylenes, and other photosensitizers are highly phototoxic to mosquito and blackfly larvae at low concentrations, these insects have provided a convenient bioassay for photoinsecticidal activity. Arnason et al. (1981c) and Wat et al. (1981) have identified a number of polyacetylenes and thiophenes with marked phototoxic activity to *Aedes aegypti* larvae. Kagan et al. (1983) have produced a number of synthetic 1,3-butadiynes, also toxic to mosquito larvae. α-T has been investigated as a possible commercial mosquito and blackfly larvicide (Philogène et al., 1985) and has been patented for this application (Towers et al., 1984). Three of the compounds utilized in the present study have been tested for mosquito larvicidal activity. Matricaria lactone, compound VII, is inactive against both mosquito larvae and herbivorous insects, whereas α-T and PHT are phototoxic to both. That mosquito larvae are more sensitive to these compounds is likely the result of their route of entry into the insect: in mosquito tests, phototoxins added to the water containing the larvae penetrate the cuticle to reach target tissues, whereas phototoxins ingested by phytophagous insects may be excreted or metabolized before they can translocate through the insect and reach susceptible sites.

Light-Independent Effects on Growth. Without photosensitizing radiation, acetylenes and thiophenes can still act as highly toxic allelochemicals to herbivorous insect species. At 100 μg/g, all of the thiophenes and polyacetylenes examined, except compound VII, reduced the growth of *E. messoria* and *O. nubilalis* larvae in the absence of photosensitizing light. *M. sexta* growth was similarly inhibited by 10 μg/g α-T and 100 μg/g PHT; α-T at 100 μg/g was lethal. In the absence of photosensitization, thiophenes were about ten times as toxic (in terms of both mortality and growth reduction) as polyacetylenes to *M.*

sexta, about twice as effective at reducing the growth of *O. nubilalis*, and the two reduced the growth of *E. messoria* to a similar extent. The increase in thiophene toxicity with increasing number of thiophene rings is a relationship which was also found by McLachlan et al. (1984) in tests with microorganisms.

The ability of thiophenes and polyacetylenes to reduce the growth of phytophagous insect larvae, even in the absence of photosensitizing light, is comparable to well-established allelochemicals. Thiophenes and polyacetylenes effectively impair growth at concentrations below the levels required to reduce growth by 50% with sesquiterpene lactones, methylated flavonoids (Isman and Rodriguez, 1983) and various phenolic compounds (Isman and Duffy, 1982). Effects produced by exposure to polyacetylenes and thiophenes resemble those produced by some alkaloids (Devitt et al., 1980) at concentrations an order of magnitude greater. These compounds would seem to constitute a potentially powerful component of the plant's defense even without access to photochemical reactions.

Allelochemicals may interfere with the growth and survivorship of an insect by disrupting metabolic processes, by interferring with nutrient assimilation, or by deterring feeding. Two components of the light-independent activity of polyacetylenes and thiophenes were discerned in the present study: feeding deterrence and inhibition of nutrient utilization.

Feeding Deterrence. Feeding deterrence is an important compact of the toxicity of many allelochemicals and may, in some cases, largely account for their activity (Isman and Rodriguez, 1983). This activity may explain much of the initial growth response of the three test insects to polyacetylenes and thiophenes.

The high mortality exhibited by neonate *M. sexta* larvae fed α-T may be accounted for by the antifeedant activity of this thiophene as α-T at 100 μg/g abolished feeding and neonates given no diet at all had a similar rate of mortality. PHT also produced a marked initial retardation of growth, due initially to a 95% reduction in feeding. Normal feeding rates were gradually assumed after the initial period of starvation, but growth rates remained below controls, again suggesting another toxic mechanism.

Neonate larvae of *E. messoria* were strongly deterred from feeding on diet containing 100 μg/g of compounds II–VI (Table 1). These compounds all produced an initial decrease in relative weight gain (Figure 3). The two least deterrent compounds, I and VII, produced only a small and transient decline in relative weight, and that only in the absence of near-UV light. In these cases, and possibly with II and V as well, photodegradation of these light-unstable compounds (McLachlan et al., 1984) may have led to a small decrease in the effective concentration of the allelochemical at the surface of the diet cube. This would primarily affect neonate larvae feeding on the surface of the diet, rather than mature larvae which typically ate into the cube to a greater depth than light could be expected to penetrate. In all cases, feeding inhibition was abolished

by 24–72 hr of starvation of neonates. Growth inhibition continued, however, and subsequently became evident in larvae fed compound I as well. This was again consistant with another light-independent toxic activity for polyacetylenes and thiophenes.

The growth of *O. nubilalis* was inhibited by 100 μg/g of compounds I–VI. However, only II, III, and VI inhibited feeding to any extent (Table 1), and this activity was insufficient to account for the observed growth reduction.

α-T was also more deterrent if insects were given a choice between treated and untreated diet (choice test). Choice tests more accurately reflect the situation facing a mobile, polyphagous insect, such as *E. messoria*. If an insect is immobile, monophagous, or occurs in an agricultural monocrop situation, no-choice tests are more likely to indicate its behavior in the field (Schoonhoven, 1982).

Near-UV enhanced the feeding deterrent effect of α-T in a no-choice test with sixth- and seventh-instar *E. messoria*. This may indicate photosensitization of insect chemoreceptors or other sensillae or, alternatively (or perhaps in addition), light-dependent toxicosis may be occurring quickly enough to affect feeding in a 24-hr trial. As an insect feeding in daylight will certainly be exposed to some near-UV, this result suggests that α-T (and perhaps other phototoxins) will be more effective as an antifeedant against diurnally active insect herbivores.

The feeding deterrent activity of polyacetylenes and thiophenes occurred at concentrations well below those found in many Asteraceae, which are likely to deter feeding by all but the highly adapted insects.

Nutrient Utilization. Decreased growth persisted even after larvae assumed normal feeding rates. This led to an examination of the effect of thiophenes and polyacetylenes on some standard measures of the efficiency with which insects (*E. messoria* in this case) assimilate and utilize their diet. All of the compounds tested reduced the gross efficiency with which diet was converted to insect biomass (ECI) (Table 3). This effect did not involve changes in the assimilation (AD) of diet, indicating that the availabilty of diet components was not affected by the test compounds. These compounds do not, then, behave as digestibility reducers. Rather, the conversion of assimilated diet to biomass (ECD) was strongly reduced over 15 days by all of the compounds tested. This is evidence of damage to metabolic processes: nutrients are diverted to the repair of such damage and do not contribute to new growth (Waldbauer, 1968).

Differences in the rate at which thiophenes and polyacetylenes affect the ECI were noted. α-T reduced this index in naive larvae by 48% within 24 hr, whereas polyacetylenes were less effective. Only after long exposure (habituated larvae) were polyacetylenes as effective as thiophenes in reducing ECI. One possible explanation is that thiophenes may attack sites in the midgut and/or hindgut leading to rapid interference in nutrient processing and utilization,

while acetylenes may lower ECI as a response to progressive damage during long-term exposure at the same or another site. An observation consistent with this hypothesis is that fourth- and fifth-instar *M. sexta* fed diet containing α-T frequently produced liquid frass, an indication that the hindgut is failing to readsorb water. This effect was not noted when larvae were fed diet containing PHT. Sublethal doses of some synthetic insecticides (e.g., fenitrothion and aminocarb) increase liquid loss from the digestive tract of insects by disrupting the epithelial membrane of the midgut and by interfering in the function of the rectal glands (Tauton and Khan, 1978).

The effect of thiophenes and polyacetylenes (over the longer term) on the ECI and ECD was similar to that found by Beck and Reese (1976) for a variety of different allelochemicals. For example, thiophene effects were similar to those of highly toxic alkaloids such as L-dopa. The gradual reduction of nutrient utilization by polyacetylenes is similar to the effect of the nonprotein amino acid canavanine on *M. sexta* (Dahlman, 1977). The toxicity of nonprotein amino acids is expressed gradually, as insects accumulate defective proteins.

Insect Host Range and Tolerance to Acetylenes and Thiophenes. A correlation can be made between the response of the neonate larvae to polyacetylenes and thiophenes, and their host plant range. *M. sexta* larvae were the most strongly deterred, as the feeding inhibition due to α-T at 100 μg/g could not be overcome by starvation. As solanaceous host plants of this insect do not usually contain these compounds, the ability to detect and avoid them probably allows *M. sexta* larvae to avoid ingesting potentially toxic nonhost plants. At low concentrations, α-T and PHT had little deterrent effect or were phagostimulatory, even though α-T reduces growth at that level. *E. messoria* larvae were also deterred by all of the test compounds except VII, the only one that did not reduce growth. The host plant range of this polyphagous insect is extensive but apparently includes only those Asteraceae that lack polyacetylenes and thiophenes. Again, avoidance of these compounds may lead the insect to avoid toxic nonhost plants. *O. nubilalis* larvae were only slightly deterred by the thiophenes and PHT and did not respond to the other acetylenes. This insect utilizes at least 25 species of Asteraceae (Caffrey and Worthley, 1927) most of which are phototoxic in screening tests (Towers et al., 1979).

The severely and frequently attacked plants include the highly phototoxic *Bidens* spp. and *Dahlia* spp., which were the source plants for some of the compounds used in this study. First-instar larvae feed on leaves but may be able to avoid resin canals, glands, and other sites with high levels of phototoxins. Such a behavior allows neonate *Heliothis zea* to avoid ingesting gossypol from cotton leaves (Reese et al., 1981). Later instar *O. nubilalis* avoid photosensitization by spinning silk and by boring into stems or, less frequently, fruits. Furthermore, the concentration of polyacetylenes and thiophenes is frequently lower in the stems than in other plant tissues (Bohlmann et al., 1973; Towers

and Wat, 1978). This may be one reason why *O. nubilalis* is able to exploit plants not accessible to *M. sexta* or *E. messoria* or, presumably, many other insects.

The activity of polyacetylenes and thiophenes may be compared to other known feeding deterrents. These compounds are not as active as azadirachtin and warburganal, which are antifeedants of potential commercial significance (Schoonhoven, 1982; Jermy, 1983). However, polyacetylenes and thiophenes are as efficient as several other limonoid and isoflavonoid antifeedants and are considerably more effective than tannins, gossypol, or tomatine. In particular, several of the compounds tested in this study are effective antifeedants at much lower concentrations than are the sesquiterpene lactones, widely considered to constitute a major part of the biochemical defenses of many plants, especially in the Asteraceae (Burnett et al., 1974; Mabry and Gill, 1979).

Evolutionary Considerations. Chemotaxonomic and biosynthetic evidence indicates strongly that the series fatty acid to polyacetylene to thiophene represents an evolutionary elaboration (Bohlmann et al., 1973). The toxicity of the substances appears to be correlated with their position in this evolutionary sequence. Fatty acids, particularly oleic acid, are ubiquitous among vascular plants (Robinson, 1980). Some of these compounds are toxic to herbivorous and nonherbivorous insects; this led to their early use in insecticidal soaps (Puritch, 1975). The C_{10}–C_{12} fatty acids and oleic acid are the most toxic. Binder et al. (1979) and Binder and Chan (1982) examined the toxicity of a series of C_{10}–C_{12} fatty acids to three phytophagous lepidopterans, *Pectinophora gossypiella*, *Heliothis zea*, and *Heliothis virescens*. Concentrations required to reduce larval growth by 50% (ED_{50}) ranged from 0.1 to 0.6%, with some variation between species. Toxicity increased with increasing unsaturation.

Polyacetylenes are derived from the fatty acids (oleic acid) via β-oxidations, and occur in a more limited range of plant families, particularly the Asteraceae and the Apiaceae (Bohlmann et al., 1973). We have determined that several of these compounds markedly reduce the growth of lepidopteran herbivores at a concentration of 0.01%. In addition, matricaria ester, a widely distributed polyacetylene in the Asteraceae, was the most toxic of the compounds examined by Binder et al. (1979), with an EC_{50} of 0.0046–0.017%. Polyacetylenes appear to be an order of magnitude more toxic to insects than their fatty acid precursors.

Thiophenes represent a further evolutionary step: they are derived from polyacetylenes by cyclization with sulfur. These compounds are restricted to advanced tribes of the Asteraceae, including the Veronieae, Eupatorieae, Inuleae, Heliantheae, Helenieae, Anthemideae, Senecioneae, Cynareae, and particularly the Tageteae (Hohlmann et al., 1973). The more toxic bithiophene and terthiophene compounds are restricted to the last three tribes listed. In our study, these compounds were one order of magnitude more toxic than the polyacetylenes to *M. sexta*, about twice as toxic to *O. nubilalis*, and equally toxic to *E. messoria*.

The increasing toxicity of this evolutionary sequence of secondary metabolites suggests that they have been selected for on the basis of their activity against herbivores and other plant enemies. This supports the hypothesis that polyacetylenes and thiophenes function in the defense against herbivores and pathogens.

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INSECT DEFENSES AGAINST PHOTOTOXIC PLANT CHEMICALS

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Abstract—In addition to avoidance strategies, insects may use one or more biochemical defenses against phototoxic plant constituents or the reactive forms of oxygen they generate. These biochemical defenses may include metabolism; excited state quenching; deactivation of singlet oxygen, superoxide, or free radicals; and destruction of reaction products. This article is a survey of the antioxidative enzymes and simpler molecules produced by insects and their possible roles in combating phototoxic chemicals.

Key Words—Phototoxicity, enzymatic, detoxification, Insecta, insect herbivory, furanocoumarins, polyacetylenes, thiophenes, hypericin, oxygen quenching, defense.

INTRODUCTION

Photochemically activated toxicants from higher plants, such as 8-methoxypsoralen (8-MOP) (*1*) and related furanocoumarins, alpha-terthienyl (*2*), and hypericin (*3*), are known to have several biochemical interactions with target organisms. The formation of 2 + 2 cycloadducts between furanocoumarins and DNA bases is well known; however, there are many other possible biologically significant pathways for the generation of cytotoxic species. In addition to direct adduct formation, a phototoxin may also transfer an electron from its excited state to a target biomolecule (or accept one from a biomolecule) or produce an intermediate reactive species by interacting with oxygen.

An insect may deactivate an ingested phototoxin by several routes; it may metabolize the toxin fast enough so that phototoxicity is never manifested; quench, by physical or chemical means, the excited state of the phototoxin;

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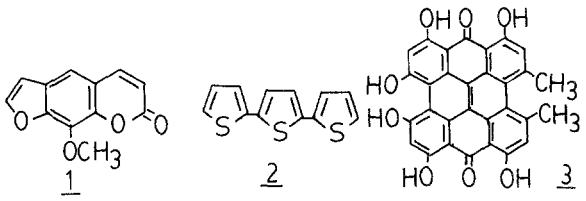


quench or destroy reactive forms of oxygen produced by phototoxin–O_2 interactions; or destroy toxic intracellular reaction products. Very few of these alternatives have been examined in the case of insects. In this paper, I will discuss the existing literature and also speculate on possible routes of detoxification used by insects.

EXCITED OXYGEN SPECIES AND PHOTOTOXICITY

Many of the biological effects of phototoxic agents are due to the formation of reactive oxygen species—either free radicals or other modified forms of oxygen itself, or oxygenated organic compounds produced by the interactions between oxygen and photoexcited organic substrates. Ground-state oxygen, a triplet (diradical) species, is negligibly reactive with nearly all singlet organic molecules having paired spins, but does react very rapidly with many organic free radicals, including those formed photochemically. The product of such reactions, a peroxy radical (ROO·) is usually quite unstable; its fate depends on its surroundings. In the case of lipid peroxidation, peroxy radicals abstract hydrogen atoms from neighboring molecules, often other lipids, leading to lipid hydroperoxides (ROOH) and new radicals which can again react with oxygen by a chain reaction. Some hydroperoxides are acutely toxic, and others may also have mutagenic activity (Yamaguchi and Yamashita, 1980).

Ground-state oxygen can be converted to a more reactive species, singlet oxygen ($^1O_2$), by reaction with certain photochemically excited states of dyes known as photosensitizers. Many synthetic and natural pigments have been shown to have photosensitizing activity (Foote, 1981). Singlet oxygen, as its name suggests, is a spin-paired species and exhibits a different range of reactivity (relative to ground-state O_2) toward organic molecules. It has little tendency to react rapidly with most free radicals but is much more reactive than ground-state oxygen to a few selected organic compounds which include electron-rich phenols, highly substituted olefins, small unsaturated heterocyclic rings, and organic sulfur compounds (Foote, 1981).

Under certain conditions, ground-state oxygen can also be reduced by one-electron transfer to a monoradical form, superoxide radical anion (O_2^-). Among the species which can promote this reaction are photochemically excited organic molecules such as flavins and ketones (via intermediate organic free

radical intermediates). Superoxide, in nonpolar environments, is a very powerful nucleophile, displacing leaving groups such as halogens and cleaving ester linkages at fast rates. In polar environments, its conjugate acid, HOO· is formed; it reacts very rapidly with itself (with a half life about 1 sec at 10^{-5} M) in a spontaneous redox ("dismutation") reaction to afford two nonradical species, oxygen and hydrogen peroxide. Hydrogen peroxide, in turn, may be homolytically cleaved either by transition metal-catalyzed processes or by UV light to an extremely reactive, unselective, and potentially toxic intermediate, the hydroxyl radical (HO·).

Thus, by a number of light-induced pathways, oxygen can be converted to many reactive and potentially damaging species (Figure 1).

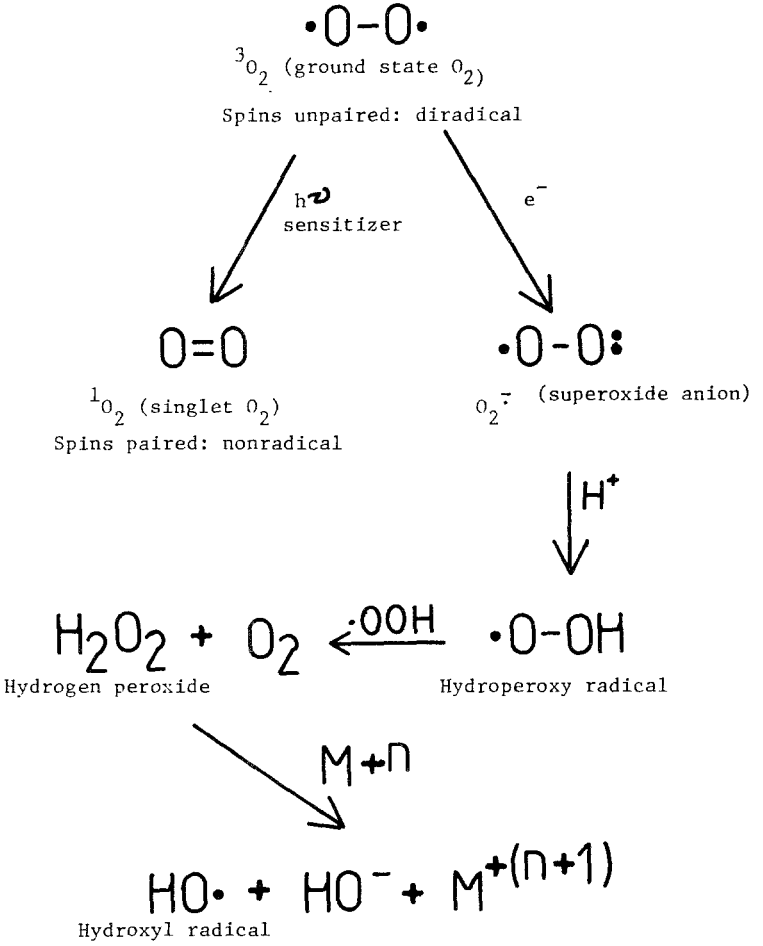


FIG. 1. Reactive oxygen species.

PHOTOBIOCHEMISTRY OF PHOTOTOXIC SUBSTANCES

Furanocoumarins. For many years, furanocoumarins, a group of oxygenated tricyclic aromatic compounds found primarily in the plant families Umbelliferae and Rutaceae, have been known to be phototoxic. The broad phototoxicity of these molecules has stimulated much fundamental research on their photochemical and photophysical properties as well as on their photobiological effects. The lowest excited singlet state of 8-MOP (and perhaps other furanocoumarins) is reached by excitation with UV light around 330 nm. It is a pi-pi* state (Mantulin and Song, 1973), strongly localized in the pyrone ring, which fluoresces more or less efficiently depending on the solvent. The best known photoreactions of furanocoumarins are their cycloadditions with DNA constituents (for a review, see Song and Tapley, 1979). However, there are other photoreactions of furanocoumarins with biologically important molecules. 8-MOP strongly inactivates the *E. coli* enzyme, DNA polymerase I, in the presence of UV light (Granger and Helene, 1983). It binds physically to the protein in an oxygen-independent step and then becomes covalently bound in the presence of light. Continued irradiation of the bound 8-MOP-modified enzyme led to progressive loss of enzymatic activity, which was not significantly retarded by the addition of a $^1O_2$ quencher, 1,8-diazabicyclooctane (DABCO) (see Table 1). In view of this evidence, the authors proposed that 8-MOP sensitized the destruction of amino acids in the neighborhood of its binding sites by a mechanism not involving $^1O_2$.

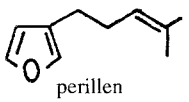
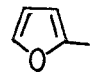
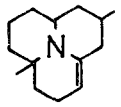
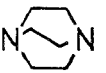
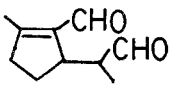
Earlier work had shown that 8-MOP forms photoadducts with tryptophan (Megaw et al., 1980) and that tyrosine, as well as tryptophan, efficiently quenched furanocoumarin triplets (Land and Truscott, 1979).

8-MOP also sensitizes the photooxidation of the naturally occurring phenolic antioxidant, alpha-tocopherol (vitamin E) to unspecified products, apparently without participation of $^1O_2$ (Potapenko et al., 1983).

The photoionization mechanism for furanocoumarin phototoxicity has not received much attention. In this mechanism, an excited state ejects an electron into the surrounding medium and an acceptor takes it up (becomes photoreduced). Joshi and Pathak (1983) showed that many furanocoumarins, when irradiated in aerated aqueous ethanol solution, produce O_2^-; apparently, electron transfer to dissolved O_2 is quite efficient for furanocoumarins having a variety of structural elements.

The universal occurrence of unsaturated cell and membrane lipids among living organisms could also account for some of the broadly biocidal effects of furanocoumarins. Insects, for example, are susceptible to UV-mediated furanocoumarin phototoxicity. Free fatty acids constitute almost 50% of surface components of some insects, and previous evidence suggests epidermal involvement in furanocoumarin toxicity; the epidermal layer appears discolored and damaged after ingestion (Berenbaum, 1978). There are at least two possible mech-

TABLE 1. POTENTIAL $^1O_2$ QUENCHERS IN INSECTS

Insect chemical	Source	Analogue
 perillen	*Lasius fuliginosus* (field ant): Bernardi *et al.*, 1967	 $k_q\ ^1O_2 = 9 \times 10^{7a}$
Beta-carotene and other carotenoids	Widespread in hemolymph, integument, and wings: Needham, 1974	$\beta - C\ k_q = 1.6 \times 10^{10}$
Histamine	*Zygaena lonicerae* and other photopohagous moths (Bisset *et al.*, 1960)	$k_q = 2.8 \times 10^7$
Serotonin	*Automeris illustris* (silkworm moth): Welsh and Batty, 1963	Tryptophan $k_q = 2.5 \times 10^8$
 Propyleine	*Chauliognathus pulchellus* (soldier beetle): Moore and Brown, 1978	 DABCO $k_q = 1.5 \times 10^7$
Benzoquinone	Many families	$k_q = 3.4 \times 10^7$
 Chrysomelidial	*Gastrophysa cyanea* (leaf beetle): Blum *et al.*, 1978	 $k_q = 3.3 \times 10^7$

[a] K_q = quenching rate constant for $^1O_2$ in M^{-1} sec^{-1}.

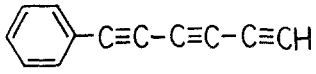
anisms for the interaction of furanocoumarins with unsaturated lipids. In the first, furanocoumarins may generate $^1O_2$ by the interaction of their triplet states with ground-state oxygen (Poppe and Grossweiner, 1975). Singlet oxygen is very reactive toward unsaturated fatty acids; the reactivity increases with the number of double bonds (Doleiden et al., 1974), and the initial products are hydroperoxides. A second possibility is the direct light-induced cycloaddition of one of the double bonds of the furanocoumarin with the double bonds of the lipids. Although no reports of such a reaction have appeared, a similar cycloaddition occurs efficiently between 8-MOP and tetramethylethylene (Shim and Kim, 1983).

Some attempts have been made to correlate the biological activity of furanocoumarins with their relative photoreactivity toward pyrimidine nucleotides (see, for example, Song et al., 1971). For genotoxic damage, the correlation appears good. There are many other factors to consider, however. Salet et al. (1982) pointed out that the relatively hydrophobic furanocoumarin, 4,5,8-tri-

methylpsoralen, was much more active in inducing oxidative damage in rat liver mitochondrial membranes than was the less hydrophobic compound, psoralen. The quantum yield of triplet formation for the two compounds was comparable. The authors suggested that the partition coefficients of the two molecules drove the furanocoumarins to different regions of the cell and that the higher concentration of 4,5,8-trimethylpsoralen within the hydrophobic membranes permitted it to react more rapidly with some membrane constituents or to generate $^1O_2$ which reacted with them.

Polyacetylenes and Thiophenes. Thiophenes such as alpha-terthienyl which are biosynthetically derived from long-chain polyacetylenes, are common constitutents of the Compositae. They display a broad range of phototoxic effects toward a variety of organisms including insect eggs (Kagan et al., 1983) and larvae (Wat et al., 1981; Arnason et al., 1981) and humans (contact dermatitis; Chan et al., 1975). Aromatic polyacetylenes are similarly broadly biocidal; phenylheptatriyne (*4*), for example, is toxic to bacteria, fungi, protozoa, nematodes, insects, fish, and human fibroblast cells (Arnason et al., 1981; Wat et al., 1979).

Light and oxygen appear to play important roles in the toxicity of many polythiophenes and aromatic polyacetylenes. In some cases, $^1O_2$ is apparently involved in the cytotoxic events. In particular, there is good evidence that photoexcited alpha-terthienyl is a photosensitizer (Wat et al., 1980). Downum et al. (1982) suggested that $^1O_2$-mediated attack on membrane proteins was responsible for the membrane photodestruction observed in *E. coli* treated with alpha-terthienyl. The membrane was also the site of damage for several viruses treated with phenylheptatriyne in the presence of light. In this case, however, O_2 was not required for activity (Hudson et al., 1982).



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Many aliphatic polyacetylenes, which occur principally in the Umbelliferae, are also quite toxic but may have a different mode of action. Although some of them absorb solar UV light, the importance of light or even of O_2 for their toxicity has not been conclusively established.

Hypericin. Many plants, especially in the families Polygonaceae and Guttiferae, contain complex phototoxic quinones. Livestock poisoning caused by ingestion of *Hypericum* (St. John's-wort or Klamath weed) material has long been known. The active priniciple, hypericin a dark red, fluorescent pigment, occurs in glands on the leaves, petals, sepals, and stems. It is phototoxic only when irradiated with visible light; near-UV wavelengths are virtually ineffective. Oxygen is required for the toxic effects, which include erythrocyte hemo-

lysis. Light-colored mammals are particularly susceptible (Towers, 1982). Little is known concerning possible toxic effects of hypericin and similar quinones on insects. *Hypericum* has a specifically associated insect, the Klamath beetle (*Chrysolina quadrigemina*) that is not only not affected by hypericin but has a specific receptor for it, apparently using it as a feeding stimulant (Rees, 1969). The Klamath beetle is highly reflective; it may be using this property as a physical method to reduce light intensity inside its body cavity (see next section). There have been few studies of the fundamental photochemical properties of hypericin or related compounds; however, a somewhat similar quinone, cercosporin (a fungal product), has been shown to produce $^1O_2$ (Youngman et al., 1983; Dobrowolski and Foote 1983). Hypericin itself sensitizes the photooxidation of tyramine, by what is apparently a $^1O_2$ process (Seely, 1977).

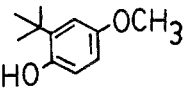
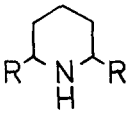
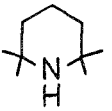
INSECT DEFENSE MECHANISMS

Physical Defenses. Light-avoidance defenses appear to be used by some phytophagous insects. For example, leaf rollers shield themselves for light by wrapping themselves in leaves as they feed. Dark pigmentation or highly reflective surfaces may also be ways of preventing damaging light wavelengths from reaching critical cellular machinery (Pathak and Fitzpatrick, 1974).

Metabolism. Very few data are available on the metabolism of furanocoumarins or other phototoxic molecules by any organisms. Work on their metabolic products in insects is limited to the findings of Ivie's group, which are discussed in detail elsewhere in this issue. In brief, a susceptible species (*Spodoptera frugiperda*) lacked the ability to metabolize 8-MOP, whereas a resistant species (*Papilio polyxenes*) was able to oxidatively cleave the furan ring.

Excited-State Quenching. In order to prevent photochemically excited states from reacting with biomolecules or oxygen, a possible strategy of defense would be to intercept the excited state with another molecule; this interaction would keep the toxin in the presumably harmless ground state. In classical photochemistry, several types of excited state quenching mechanisms have been described (Calvert and Pitts, 1966). In physical or collisional quenching, the electronic energy of the excited state ("donor" molecule) is removed by the "acceptor" (quencher) as some combination of electronic, vibrational, rotational, and kinetic energy. The excited quencher is not chemically changed, that is, no covalent bonds break. Either singlet or triplet excited states of donor molecules may be quenched, but because of the very short lifetime of most singlet states, the concentration of a singlet quencher, even a very efficient one, must be rather high (typically 10^{-3} M or greater). Such molecules as beta-carotene, amines, furans, and a few anions such as I^- and N_3^- have been repeatedly shown to be efficient physical excited state quenchers (Calvert and Pitts, 1966). Carot-

TABLE 2. POTENTIAL FREE RADICAL QUENCHERS IN INSECTS

Insect chemical	Source	Synthetic analogue
Vitamin C	Synthesized by many but required by some phytophagous types (Chippendale, 1978)	
Vitamin E	Essential dietary requirement for some (Needham, 1978)	 "BHA"
Nicotine	Tobacco-feeding insects and *Musca domestica*, *P. americana*, and *B. germanica* (Self *et al.*, 1964)	Nicotine is a patented (US Pat. 1916437, 1933) petroleum antioxidant
	*Solenopsis* (fire ant) spp: Brand *et al.*, 1972	

enoids, as well as some amines and furans, are constitutents of many phytophagous insects (see Tables 1-2).

Physical quenching may involve radiative or nonradiative phenomena. In radiative processes, the acceptor absorbs some of the electromagnetic energy emitted by the donor as it fluoresces. For this process to occur efficiently, the absorption spectrum of the acceptor must be similar to the emission spectrum of the donor. For the special case of furanocoumarins, with maximal fluorescence in the region around 450–550 nm, it would seem reasonable that insect pigments such as carotenoids would be effective quenchers, since their absorption maxima occur in that region. There has been no work reported on this possibility, however. At short ranges (ca. 10 angstroms), one of the most important mechanisms of quenching is exchange energy transfer, a nonradiative process in which the electron clouds of the donor and acceptor molecules overlap and there is electrostatic transfer of energy between two of the overlapping orbitals.

Chemical quenching is the rapid photochemical reaction of an excited state with another molecule to form one or more products. The normal consequence of this process is that both the donor and acceptor molecules are destroyed. A good example of chemical quenching is the 2 + 2 cycloaddition of the singlet state of *trans*-stilbene with electron-rich unsaturated esters (Lewis and DeVoe, 1980).

Oxygen Quenching. Reactive oxygen species, either $^1O_2$ or O_2^- (and related free-radical species whose ultimate source is oxygen) are well-known inter-

mediates in phototoxicity. Deactivation of these substances by enzymes or lower-molecular-weight constitutents would be a possible defense pathway for phytophagous insects.

The quenching of $^1O_2$ has been rather well studied (for a review, see Wilkinson and Brummer, 1981) for synthetic molecules and a few naturally occurring chemicals. Only a few classes of compounds destroy $^1O_2$ rapidly; these include amines, highly substituted olefins (particularly carotenoids), furans, polycyclic aromatic hydrocarbons, and some electron-rich sulfur and oxygen derivatives. The rate constants for these quenchers with $^1O_2$ range from near diffusion control (k_q around 10^{10}/M/sec) to about three orders of magnitude lower. Some of these materials react with $^1O_2$ to form oxygenated derivatives such as peroxides, and others quench it physically.

Certain insect constituents are known fast quenchers of $^1O_2$, and others appear to be close relatives of such quenchers (Table 1). It was recently discovered that several plant alkaloids, closely related to some nitrogen heterocycles found in insects, are efficient $^1O_2$ quenchers (Larson and Marley, 1984; Gorman et al., 1984). Admittedly, some of these chemicals may have other physiological functions for insects, and some appear to be restricted to special glands where contact with ingested phototoxins would seem unlikely. Nevertheless, it is possible that these or related compounds may occur at sufficiently high concentrations in tissue to act as $^1O_2$ quenchers and inhibit photosensitized toxicity to a significant degree. This possibility requires further research.

Free radical inhibitors are widely used in industry to prevent oxidative deterioration of many products such as foods, fuels, plastics, rubber, etc. Phenolic derivatives are very widely used. Mechanistically, synthetic inhibitors act by deactivating peroxy radicals. The well-known antioxidant BHA, for example, converts two moles of peroxy radicals to inactive, nonradical products. Some naturally occurring phenols have the same mechanism of action. Vitamin E, for example, exhibits one of the fastest rates of attack toward peroxy radicals, and has the same 2:1 stoichiometry as BHA (Burton and Ingold, 1981). Vitamin E is an essential dietary requirement for some insects; it and other insect chemicals with possible free radical quenching activity, are listed in Table 2 along with some related industrial antioxidants.

Sohal et al. (1983) demonstrated that adult houseflies (*Musca domestica*) contain chloroform-soluble free-radical antioxidants and that the antioxidant concentration declines with the age of the insect. No individual antioxidants were identified.

Some commercial antioxidants are reducing agents; they act by destroying peroxides that can act as chain carriers in free-radical chain reactions. Their structures incorporate easily oxidized groups like disulfides, P=S linkages, etc. One low-molecular-weight insect chemical that may have similar activity is dimethyl disulfide (Table 3). Its high volatility, however, probably limits its utility.

TABLE 3. POTENTIAL PEROXIDE DESTROYERS IN INSECTS

Insect chemical	Source	Synthetic Analogue
$CH_3-SS-CH_3$	*Paltothyreus tarsatus* (ponerine ant) (Casnati *et al.*, 1967)	Ph-SS-Ph
Glutathione	*Musca domestica* (Sohal *et al.*, 1983) *Aedes aegypti* (Hazelton & Lang, 1982)	—
SOD (superoxide dismutase)	*Apis mellifera* *Drosophila* spp. *Musca domestica*	—
Catalase	*Apis mellifera* *Drosophila* spp. *Brachinus* spp. *Musca domestica*	—

Glutathione, a tripeptide with an easily oxidizable —SH group, can act as an antioxidant either nonenzymatically or in a catalyzed reaction involving glutathione peroxidase. The enzymatic reaction is important in mammals; however, insects appear to lack glutathione peroxidase (Smith and Shrift, 1979). Adult houseflies, however, do contain glutathione; its concentration is maximal at about 10 days of age and declines sharply thereafter (Sohal et al., 1983). A similar decline in glutathione concentration with senescence was reported for the mosquito, *Aedes aegypti* (Hazelton and Lang, 1982).

Other likely peroxide-destroying agents are enzymes; nearly all aerobic organisms that have been tested contain highly active catalysts for the destruction of reactive oxygen species such as O_2^- and H_2O_2. Few insect species have been examined for their content of these enzymes. Sohal et al. (1983) demonstrated that both catalase and superoxide dismutase activity were present in houseflies.

SUMMARY AND CONCLUSIONS

Higher plants have evolved a wide variety of molecules phototoxic to some phytophagous insects. Their photobiological mechanisms of action are diverse; many forms of transfer of photochemical excitation energy are possible and a number of biochemical targets are available. Insects, however, appear to have coevolved several efficient defense mechanisms. A number of other insect chemicals also appear on structural grounds to be possible defensive agents. Very little work has been done in the whole area, and it appears that many

fruitful avenues of interdisciplinary research will be available to groups of entomologists, chemists, and botanists who are willing to work together.

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COMPARATIVE METABOLISM OF [^{3}H]PSORALEN AND [^{3}H]ISOPSORALEN BY BLACK SWALLOWTAIL (*Papilio polyxenes* FABR.) CATERPILLARS

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Abstract—The comparative fate of tritiated preparations of a linear furanocoumarin (psoralen) and an angular furanocoumarin (isopsoralen) was determined in last-instar caterpillars of the black swallowtail butterfly (*Papilio polyxenes* Fabr.). Oral administration of either furanocoumarin at 5 μg/g is followed by rapid metabolism, primarily through oxidative cleavage of the furan ring, and the metabolites are rapidly excreted. Isopsoralen is, however, metabolized at a somewhat slower rate than is psoralen, and levels of unmetabolized isopsoralen in body tissues of the treated caterpillars are about three-fold higher. These data are compatible with the hypothesis that a reduced detoxification rate accounts at least in part for the susceptibility of *P. polyxenes* caterpillars to the deleterious effect of isopsoralens.

Key Words—Psoralen, isopsoralen, furanocoumarin, *Papilio polyxenes*, Lepidoptera, Papilionidae, metabolism, detoxification.

INTRODUCTION

Furanocoumarins occur in nature as components of hundreds of higher plant species. These compounds are of interest from a coevolutionary perspective because many furanocoumarins act as potent photosensitizers and apparently function in the plants that contain them as protective agents against parasites and predators. Furanocoumarins act as phytoalexins against microbial and other stresses in some plants (Beier and Oertli, 1983), as allelopathic agents (Friedman et al., 1982; Shimomura et al., 1982), and as allomones against herbivorous mammals (Ivie, 1978). It is now well known that furanocoumarins are

important factors in the resistance of some plant species to the predatory actions of herbivorous insects (Berenbaum, 1978, 1981; Muckensturm et al., 1981).

There are two major groups of natural furanocoumarins (Figure 1). The potent biological activity of these compounds is thought to arise primarily as a result of light-induced alkylation of DNA (Scott et al., 1976). Psoralens may form both mono- and di-adducts with pyrimidine bases of DNA, but the angular configuration of the isopsoralens permits only mono-adducts to be formed. Some researchers believe that among the naturally occurring furanocoumarins, the psoralens possess greater photosensitizing potential than the isopsoralens (Pathak et al., 1960, 1962; Musajo and Rodighiero, 1962; Scott et al., 1976), although others suggest that such differences may not be significant under natural, multiwavelength light activation circumstances (Potapenko et al., 1984).

Irrespective of the high photosensitizing potential of furanocoumarins, certain insect species, particularly among butterflies of the family Papilionidae, have adapted to feed successfully and preferentially on plants that contain relatively high levels of linear furanocoumarins (Berenbaum and Feeny, 1981). Recent studies in our laboratories have shown that tolerance to these compounds by caterpillars of the black swallowtail butterfly (*Papilio polyxenes* Fabr.) results from rapid and almost quantitative detoxification of psoralens in the midgut and body tissues to nonphotosensitizing metabolites that are rapidly excreted (Ivie et al., 1983; Bull et al., 1984). These reactions are mediated by microsomal mixed-function oxidases present in high titer in both midgut and body tissues (Bull et al., 1986). However, *P. polyxenes* avoids plants that contain appreciable quantities of angular furanocoumarins (isopsoralens) and, in fact, isopsoralens are known to reduce growth and fecundity of this insect species (Berenbaum and Feeny, 1981).

The studies reported here were undertaken to evaluate the possibility that differences in metabolism of psoralens and isopsoralens by caterpillars of *P. polyxenes* might account for the observed differences in toxicity of these compounds.

FIG. 1. Structures of the linear furanocoumarin, psoralen, and of the angular furanocoumarin, isopsoralen (angelicin). The asterisks (*) indicate sites of tritium incorporation in the labeled compounds used in these studies.

METHODS AND MATERIALS

Insects. Caterpillars of wild *P. polyxenes* were collected on *Thamnosma texana* (Gray) (Rutaceae) in Medina County, Texas. They were reared in the laboratory on sprigs of fresh parsley obtained from a local supermarket. Emerging adults were fed a mixture of honey and water, and 2- to 3-day-old females were mated with 1- to 2-day-old males. Parsley was provided for oviposition and successive generations were reared as above.

Chemicals. Samples of tritiated psoralen (4.5 Ci/mM) and isopsoralen (1.2 Ci/mM) were obtained from HRI Associates, Emeryville, California. In each labeled preparation, the label was incorporated into the double bond of both the furan and lactone rings (Figure 1). Unlabeled samples of psoralen and isopsoralen were also obtained from HRI. The radiochemical purity of each labeled sample, as determined by thin-layer chromatography and subsequent liquid scintillation counting (LSC), was approximately 98%. Prior to use, the labeled samples were diluted with unlabeled psoralen or isopsoralen as appropriate to a specific activity of 0.14 μCi/μg.

In Vivo Metabolism Studies. Last-instar caterpillars were weighed, held individually in covered Petri dishes, and starved 2 hr prior to treatment. [^{3}H]Psoralen and [^{3}H]isopsoralen dosages were individually tailored to the prestarvation weights of each caterpillar. To a small sprig of parsley was pipetted (using a microsyringe) an appropriate volume of radiochemical (as a 0.25 μg/μl solution in acetone) so that subsequent consumption by the insect would result in a dosage equivalent to 5 μg of psoralen or isopsoralen/g of body weight. The appropriate solution was spread evenly over the dorsal leaf surface and allowed to air dry for a few minutes, then the caterpillar was placed on the twig. Essentially total consumption of the dosage was usually complete within 1–5 min, and the caterpillar was then transferred to a clean covered Petri dish (with fresh, untreated parsley added), and the residual treated parsley twig (stem) subjected to LSC to determine that small portion of the dosage, if any, not consumed. All subsequent calculations of dosage accountability, etc., were based on actual dpm consumed by each insect. Four replicates were run for each chemical and analysis interval.

Treated caterpillars were held under low-intensity fluorescent lighting and analyzed at 0.75, 1.5, 3, 6, and 12 hr after treatment. For analysis, the caterpillars were pinned in wax, slit open along the dorsal midline, and the entire gut and content removed. This procedure resulted in three fractions: gut and contents, body, and excreta. Each fraction was transferred to a 50-ml-capacity screw-cap glass tube containing 10 ml of distilled water, and the samples were then immediately frozen for later analysis.

Sample Extraction and Analysis. Sample fractions in the glass tubes were thawed, acidified to pH < 2.0 with concentrated aqueous hydrochloric acid, and extracted three times with 20-ml volumes of ethyl acetate. Polytron ho-

mogenization was utilized to facilitate tissue disruption and enhance extraction efficiency, and homogenized samples were centrifuged to separate the organic and aqueous phases. Aliquots of the combined organic, and aqueous–tissue slurry phases were quantitated by LSC. The organic phases were then dried over anhydrous sodium sulfate and concentrated by vacuum distillation and finally by a gentle stream of nitrogen to a final volume of 0.2–0.3 ml for subsequent TLC analysis.

Resolution and Quantification of Metabolites. TLC studies utilized precoated silica gel chromatoplates (Silplate F-22, 20 × 20 cm, 0.25-mm gel thickness, with fluorescent indicator, Merck, Darmstadt, Germany). The concentrated sample extracts were spotted as short bands along one edge of the plates, then the plates were developed in a solvent system consisting of ethyl acetate–acetic acid (99 : 1). Appropriate gel areas containing the radioactive components were subsequently scraped from the plates and quantitative measurements were made by LSC.

Isolation of Psoralen and Isopsoralen Metabolites on a Submilligram Scale. To facilitate metabolite characterization, several last-instar *P. polyxenes* caterpillars were fed parsley twigs that had been dipped in 1.0 mg/ml solutions of unlabeled psoralen or isopsoralen. Excreta collected from these caterpillars was extracted as described above and the extracts subsequently analyzed by TLC. Metabolite isolation was facilitated by the fact that with both psoralen and isopsoralen, two major metabolites were generated (see below) that were readily visualized by viewing the developed plates under long-wavelength ultraviolet light. These products were extracted from the gel with either ethyl acetate or methanol, were subjected to methylation with diazomethane, and were subjected to further TLC cleanup (ethyl acetate–acetic acid, 99 : 1; chloroform–ethyl acetate, 2 : 1; or ethyl acetate–methanol–acetic acid, 150 : 50 : 2). The metabolites were then subjected to mass spectral analysis.

Mass Spectroscopy. Direct insertion probe mass spectral studies were performed as previously described (Bull et al., 1984).

RESULTS

Absorption and Elimination of Psoralen and Isopsoralen. Exposure of last-instar *P. polyxenes* caterpillars to either [^{3}H]psoralen or [^{3}H]isopsoralen as 5.0 μg/g oral doses was followed by rapid disappearance of radiolabel from the gut (Figure 2). Within 1.5 hr after dosing, less than half of the administered [^{3}H]psoralen or [^{3}H]isopsoralen remained within the gut, and by 12 hr post-treatment, $<5\%$ of the dose of either compound remained inside the gut.

Tritium-labeled residues in body tissues (Figure 3) reached levels that were consistently about two-fold higher in caterpillars treated with [^{3}H]isopsoralen than in caterpillars treated with [^{3}H]psoralen. Residues in body tissues appeared to reach their maximum levels 0.75–1.5 hr after treatment, then steadily declined to $\leq 5\%$ of the dose after 12 hr.

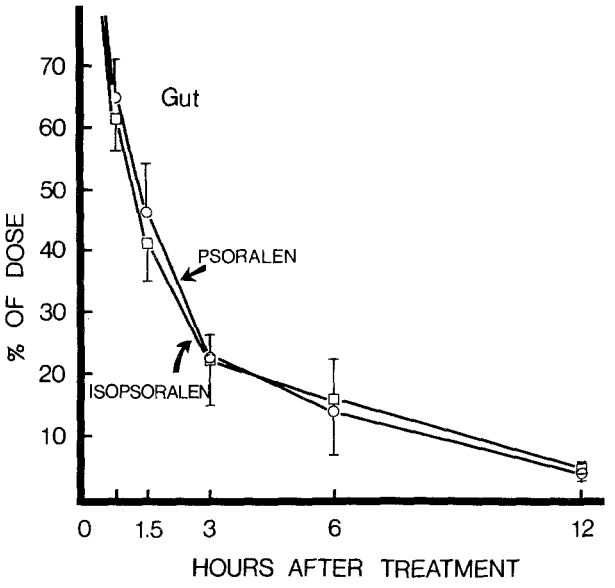

FIG. 2. Disappearance of tritium residues from the gut of last-instar *Papilio polyxenes* caterpillars after oral treatment with either [^{3}H]psoralen or [^{3}H]isopsoralen at dosages equivalent to 5 μg/g. Data points are means $\pm$SD.

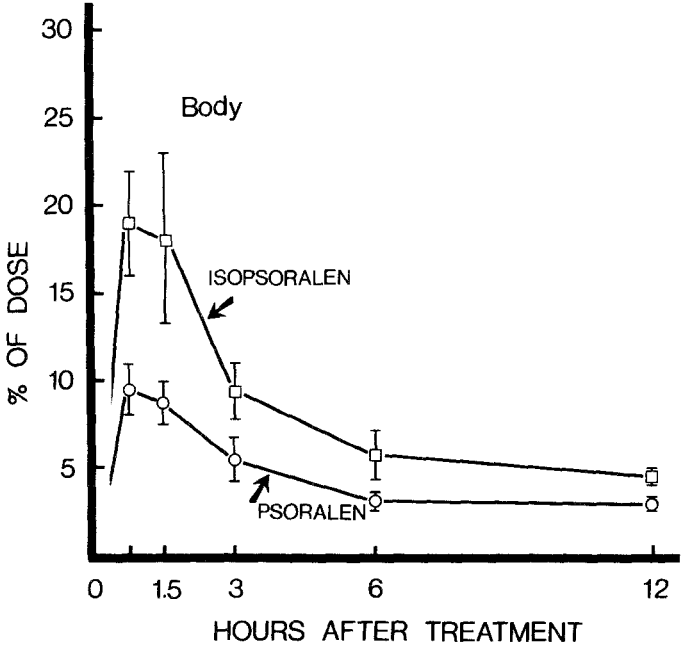

FIG. 3. Tritium residues in body tissues (exclusive of the gut and its contents) of last-instar *Papilio polyxenes* caterpillars after oral treatment with either [^{3}H]psoralen or [^{3}H]isopsoralen at dosages equivalent to 5 μg/g. Data points are means $\pm$SD.

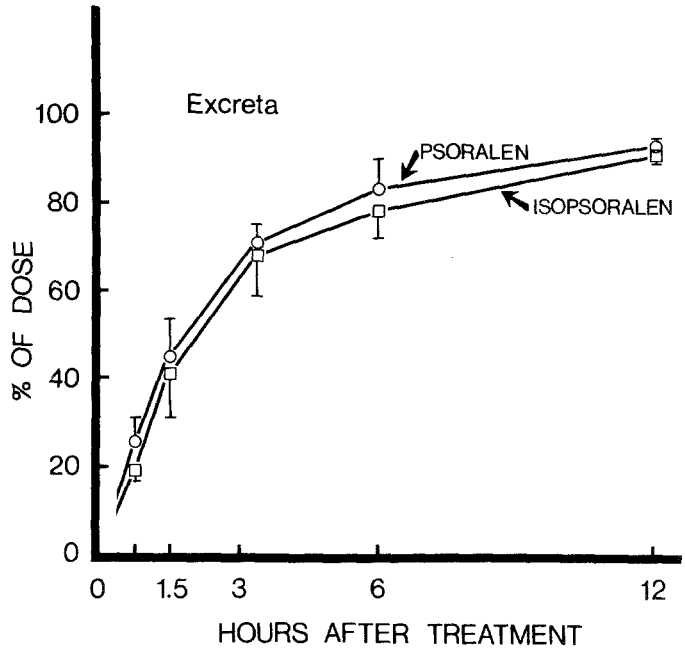

FIG. 4. Elimination of tritium residues in the excreta of last-instar *Papilio polyxenes* caterpillars after oral treatment with either [^{3}H]psoralen or [^{3}H]isopsoralen at dosages equivalent to 5 μg/g. Data points are means $\pm$SD.

Tritium residues from both psoralen and isopsoralen treatments were rapidly eliminated in the excreta (Figure 4). Within 0.75 hr after dosing, 20–25% of the administered dosages were recovered in fecal pellets, and by 3 hr, >70% of the doses had been eliminated. Caterpillars treated with [^{3}H]psoralen exhibited slightly higher excretion levels of tritium than those treated with isopsoralen at all analysis intervals (Figure 4), but these differences are not statistically significant.

Metabolite Distribution. Ethyl acetate extraction of the gut and its contents, body tissues, and excreta resulted in most cases in >90% partitioning of the tritium present into the organic phase (Tables 1–3). Almost all samples, irrespective of the compound, fraction, or analysis interval, contained two major metabolites (designated P1 and P2 for psoralen and IP1 and IP2 for isopsoralen). In the 0.75-hr gut samples from the psoralen-treated caterpillars, metabolites P1 and P2 comprised about 66% of the total ^{3}H residues present, whereas only about 30% of the tritium seen in the 0.75 hr gut samples of isopsoralen-treated caterpillars was as metabolites IP1 and IP2 (Table 1). Similar relative distributions of tritium was observed in body tissues (Table 2). In body tissue extracts from isopsoralen-treated caterpillars, the unmetabolized

TABLE 1. DISTRIBUTION OF TRITHIUM IN GUT (TISSUES AND CONTENTS) OF LAST-INSTAR *Papilio polyxenes* CATERPILLARS AFTER ORAL TREATMENT WITH [^{3}H]PSORALEN OR [^{3}H]ISOPSORALEN AT 5 μg/g

Analysis interval posttreatment (hr)	% of administered ^{3}H as indicated product or fraction in gut ($\bar{X} \pm$ SD)[a]				
	Psoralen[b]	P1[b]	P2[b]	Unidentified[c]	Water soluble/ unextractable[d]
Psoralen					
0.75	12.9 ± 2.7	24.5 ± 3.8	18.4 ± 3.2	1.9 ± 0.2	7.1 ± 1.3
1.5	2.0 ± 0.4	18.8 ± 6.2	17.5 ± 3.6	1.7 ± 0.2	6.3 ± 0.3
3	0.8 ± 0.4	9.3 ± 1.8	7.5 ± 2.2	1.3 ± 0.3	4.4 ± 0.6
6	0.3 ± 0.2	6.8 ± 4.3	3.2 ± 1.7	0.4 ± 0.1	3.1 ± 0.6
12	0.0 ± 0.0	0.8 ± 0.4	0.5 ± 0.3	0.2 ± 0.1	2.6 ± 0.2
	Isopsoralen[b]	IP1[b]	IP2[b]		
Isopsoralen					
0.75	24.8 ± 4.0	10.0 ± 1.3	8.5 ± 3.6	7.6 ± 1.1	11.1 ± 1.4
1.5	10.6 ± 5.6	7.8 ± 2.1	6.7 ± 1.9	7.2 ± 0.6	8.7 ± 0.5
3	3.4 ± 3.0	4.0 ± 1.3	5.2 ± 1.8	3.1 ± 0.6	7.2 ± 1.1
6	1.2 ± 0.7	3.4 ± 1.6	3.9 ± 2.8	2.2 ± 1.0	5.9 ± 1.0
12	0.2 ± 0.1	0.2 ± 0.2	0.5 ± 0.2	0.2 ± 0.1	3.6 ± 0.6

[a] Four replicates run for each analysis interval.
[b] See Figures 1 and 5 for structures of compounds.
[c] Cumulative totals for metabolites extracted into ethyl acetate and resolved by TLC but not chemically characterized.
[d] ^{3}H remaining in the aqueous/residue slurry after extraction with ethyl acetate.

TABLE 2. DISTRIBUTION OF TRITIUM IN BODY TISSUES (WHOLE BODY EXCLUSIVE OF GUT AND CONTENTS) OF LAST-INSTAR *Papilio polyxenes* CATERPILLARS AFTER ORAL TREATMENT WITH [^{3}H]PSORALEN OR [^{3}H]ISOPSORALEN AT 5 μg/g

Analysis interval posttreatment (hr)	% of administered ^{3}H as indicated product or fraction in body ($\bar{X}$ ± SD)[a]				
	Psoralen[b]	P1[b]	P2[b]	Unidentified[c]	Water soluble/ unextractable[d]
Psoralen					
0.75	2.8 ± 1.0	1.1 ± 0.4	1.3 ± 0.4	0.4 ± 0.1	3.7 ± 0.6
1.5	1.5 ± 0.4	1.5 ± 1.0	1.5 ± 1.0	0.4 ± 0.1	4.0 ± 0.5
3	0.9 ± 0.6	0.6 ± 0.3	0.6 ± 0.4	0.2 ± 0.1	3.1 ± 0.4
6	0.4 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	2.2 ± 0.4
12	0.3 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	2.3 ± 0.2
	Isopsoralen[b]	IP1[b]	IP2[b]		
Isopsoralen					
0.75	8.8 ± 2.0	0.9 ± 0.3	1.5 ± 0.2	0.9 ± 0.2	6.9 ± 1.2
1.5	6.0 ± 4.7	0.6 ± 0.4	1.3 ± 0.3	0.9 ± 0.1	9.2 ± 1.1
3	2.4 ± 2.0	0.3 ± 0.1	0.9 ± 0.5	0.4 ± 0.0	5.4 ± 0.2
6	1.0 ± 0.3	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.0	4.2 ± 1.3
12	0.4 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.3 ± 0.1	3.6 ± 0.5

[a] Four replicates run for each analysis interval.
[b] See Figures 1 and 5 for structures of compounds.
[c] Cumulative totals for metabolites extracted into ethyl acetate and resolved by TLC but not chemically characterized.
[d] ^{3}H remaining in the aqueous/residue slurry after extraction with ethyl acetate.

TABLE 3. DISTRIBUTION OF TRITIUM IN EXCRETA OF LAST-INSTAR *Papilio polyxenes* CATERPILLARS AFTER ORAL TREATMENT WITH [^{3}H]PSORALEN OR [^{3}H]ISOPSORALEN AT 5 μg/g

Analysis interval posttreatment (hr)	% of administered ^{3}H as indicated product or fraction in excreta ($\bar{X} \pm$ SD)[a]				
	Psoralen[b]	P1[b]	P2[b]	Unidentified[c]	Water soluble/ unextractable[d]
Psoralen					
0.75	0.7 ± 0.2	11.4 ± 1.8	9.9 ± 3.3	2.4 ± 0.4	1.4 ± 0.7
1.5	1.6 ± 0.3	16.2 ± 3.7	22.8 ± 5.2	1.9 ± 0.7	2.3 ± 0.6
3	2.7 ± 0.5	28.4 ± 6.7	34.4 ± 4.9	2.8 ± 0.6	2.8 ± 0.4
6	3.0 ± 0.4	41.4 ± 1.8	32.4 ± 3.7	2.7 ± 0.4	3.7 ± 0.8
12	4.4 ± 2.2	44.6 ± 7.2	29.9 ± 8.8	8.0 ± 7.4	6.2 ± 3.3
	Isopsoralen[b]	IP1[b]	IP2[b]		
Isopsoralen					
0.75	1.7 ± 0.3	6.4 ± 1.6	5.0 ± 1.2	2.6 ± 0.4	3.8 ± 0.6
1.5	3.6 ± 1.4	9.0 ± 3.4	15.4 ± 10.3	5.6 ± 2.0	7.2 ± 0.8
3	6.9 ± 1.9	12.4 ± 3.2	26.8 ± 10.4	11.0 ± 1.2	10.6 ± 2.0
6	7.5 ± 1.2	18.0 ± 4.0	25.6 ± 4.3	13.2 ± 1.1	13.3 ± 1.7
12	8.4 ± 2.1	15.7 ± 4.2	35.2 ± 5.8	15.0 ± 2.7	16.4 ± 1.9

[a] Four replicates run for each analysis interval.
[b] See Figures 1 and 5 for structures of compounds.
[c] Cumulative totals for metabolites extracted into ethyl acetate and resolved by TLC but not chemically characterized.
[d] ^{3}H remaining in the aqueous/residue slurry after extraction with ethyl acetate.

parent compound was the major radioactive residue, but in similar extracts from psoralen-treated insects, a much greater percentage of the tritium residues was as psoralen metabolites. In the extracts of excreta (Table 3), little of the total tritium recovered was in the form of unmetabolized psoralen or isopsoralen, but it appeared that approximately twice the percentage of isopsoralen was eliminated unchanged in the excreta as was psoralen.

Characterization of Metabolites. Metabolites P1 and IP1 exhibited very similar TLC behavior, and metabolites P2 and IP2 likewise showed almost identical TLC properties. Furthermore, the TLC behavior of P1 and IP1 was quite similar to that of the monohydroxy acid metabolite of xanthotoxin previously identified by us from *P. polyxenes* (Ivie et al., 1983; Bull et al., 1984). The other major metabolite of xanthotoxin in the earlier *P. polyxenes* study was conclusively shown to be a dihydroxy acid, and the metabolites P2 and IP2 from this study closely share its chromatographic properties.

On the basis of our previous observations (Ivie et al., 1983; Bull et al., 1984), the structures in Figure 5 were proposed for metabolites P1, P2, IP1, and IP2. Mass spectral analysis of the small quantities of metabolites available supported these assignments. Treatment of each metabolite with diazomethane produced methylated products consistent with the proposed structures. The monomethyl derivative of P1 gave a molecular ion at *m/z* 234, with diagnostic ions at *m/z* 203 $[M-OCH_3]^+$, 202 (base peak) $[M-OCH_3,H]^+$, 175 $[M-CO_2CH_3]^+$, and 174 $[M-OCH_3,H\ CO]^+$. The dimethyl derivatives of both P1 and IP1 exhibited, as expected, very similar mass spectra, with the molecular ion at *m/z* 248, a $[M-OCH_3,CH_3]^+$ ion at 202, and the base peak at 189 $[M-CO_2CH_3]^+$. The monomethyl derivatives of metabolites P2 and IP2

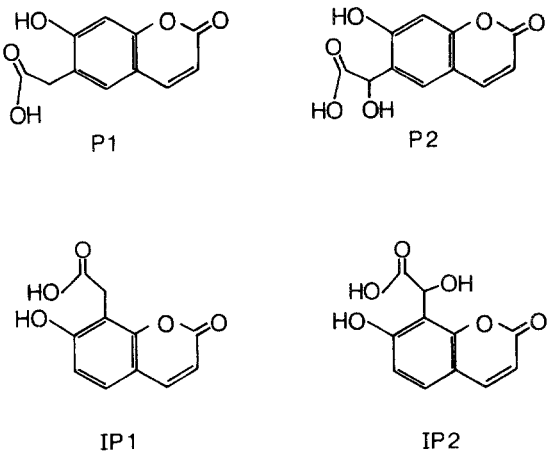

FIG. 5. Proposed structures for the major metabolites of psoralen (P1, P2) and isopsoralen (IP1, IP2) in *Papilio polyxenes* caterpillars.

likewise gave almost identical mass spectral data, with the molecular ion at *m/z* 250, and other diagnostic fragments as follows: *m/z* 202 $[M-OCH_3,OH]^+$, 191 (base peak) $[M-CO_2CH_3]^+$, 190 $[M-OCH_3,H,CO]^+$, and 174 $[M-CO_2CH_3,OH]^+$. The observed molecular ions and fragmentation products of these metabolite derivatives fully support the structural assignments shown in Figure 5; therefore, these structures almost certainly represent the major metabolic products for psoralen (P1, P2) and isopsoralen (IP1, IP2) in *P. polyxenes*. These findings are consistent with our earlier studies of the fate of xanthotoxin (8-methoxypsoralen) in this insect species (Ivie et al., 1983; Bull et al., 1984). It was not possible to conduct proton or [^{13}C]NMR studies with these metabolites due to their availability in only very limited amounts.

The chemical nature of the organic extractable tritium residues listed as "unidentified" in Tables 1–3 was not studied. This radioactivity consisted of several, generally, minor, products in most samples. That portion of each fraction shown in the tables as "water soluble/unextractable" almost certainly consisted, at least in part, of tritium released from the [^{3}H]furanocoumarin molecules as a result of metabolic transformations. Because tritium was incorporated into the double bond of the furan ring (as well as in the lactone ring double bond), the generation of metabolites P1, P2, IP1, and IP2 would certainly have resulted in some loss of label, presumably as tritiated water. The relative extent of labeling at each site of the two furanocoumarins studied is unknown, thus it is not possible to make firm conclusions regarding the extent of label loss to be anticipated as a result of the metabolic transformations observed.

The data in Tables 1–3 are expressed as percent of administered ^{3}H, and indicate 100% accountability of dosage. Calculated accountability of dosage actually ranged between 89 and 101%. This variation probably reflects experimental error. On the basis of these data, it seems clear that volatility or other loss of label did not occur to any major extent.

DISCUSSION

Our studies on the comparative metabolism of a linear furanocoumarin (psoralen) and an angular furanocoumarin (isopsoralen) by *Papilio polyxenes* caterpillars were designed to test the hypothesis that differences in the rate of metabolic detoxification might explain why *P. polyxenes* prefers psoralen-rich host plants rather than potential hosts that contain isopsoralens (Berenbaum and Feeny, 1981). We deliberately selected for study the parent unsubstituted compounds of each series to avoid possible complications in data interpretation that might arise as a result of substituent effects on metabolic phenomena.

Data obtained in the present studies on the metabolism of [^{3}H]psoralen by *P. polyxenes* are fully consistent, both qualitatively and quantitatively, with data gathered earlier by us in studies of the fate of a substituted psoralen, 8-

TABLE 4. UNMETABOLIZED PSORALEN OR ISOPSORALEN IN LAST-INSTAR *Papilio polyxenes* CATERPILLARS AFTER ORAL TREATMENT WITH EITHER COMPOUND AT 5 μg/g

Analysis interval posttreatment (hr)	% of administered ^3H as unmetabolized parent compound in indicated fraction ($\bar{X} \pm$ SD)			
	Body[a]	Gut[b]	Excreta	Total
Psoralen				
0.75	2.8 ± 1.0	12.9 ± 2.7	0.7 ± 0.2	16.4 ± 1.7
1.5	1.5 ± 0.4	2.0 ± 0.4	1.6 ± 0.3	5.1 ± 1.0
3	0.9 ± 0.6	0.8 ± 0.4	2.7 ± 0.5	4.4 ± 0.8
6	0.4 ± 0.1	0.3 ± 0.2	3.0 ± 0.4	3.7 ± 0.3
12	0.3 ± 0.1	0.0 ± 0.0	4.4 ± 2.2	4.7 ± 2.4
Isopsoralen				
0.75	8.8 ± 2.0	24.8 ± 4.0	1.7 ± 0.3	35.3 ± 5.9
1.5	6.0 ± 4.7	10.6 ± 5.6	3.6 ± 1.4	20.2 ± 11.5
3	2.4 ± 2.0	3.4 ± 3.0	6.9 ± 1.9	12.7 ± 6.1
6	1.0 ± 0.3	1.2 ± 0.7	7.5 ± 1.2	9.7 ± 1.2
12	0.4 ± 0.1	0.2 ± 0.1	8.4 ± 2.1	9.0 ± 2.2

[a] Whole body exclusive of gut and contents.
[b] Gut tissue and contents.

methoxypsoralen (xanthotoxin) in *P. polyxenes* caterpillars (Ivie et al., 1983; Bull et al., 1984). The present studies extend the data base on furanocoumarin interactions with *P. polyxenes* to show that isopsoralens are degraded in this insect by metabolic reactions analogous to those observed with psoralens. The isopsoralen detoxification and excretion rates documented here must be considered as very rapid by any standard, although it is clear that *P. polyxenes* caterpillars do metabolize isopsoralen at a slower rate than is observed with an identical psoralen dose. Table 4, which represents a partial summarization of data from Tables 1–3, indicates a consistent two- to threefold higher concentration of unmetabolized isopsoralen, as compared to psoralen, in almost all fractions. In body tissues in particular, the ratio of unmetabolized isopsoralen/psoralen appears to be at least 3.0 in samples analyzed within 1.5 hr after treatment. On the basis of these data, it seems reasonable to expect that under comparable conditions of constant dietary exposure, levels of intact isopsoralens within the body tissues (where toxicity would be expressed) would be at similarly higher levels (relative to psoralens) were the insects foraging on isopsoralen-containing host plants.

Berenbaum and Feeny (1981) have cited evidence that the biosynthesis of isopsoralens in plants has evolved more recently than that of psoralens. These workers have proposed that the biosynthesis of isopsoralens may be an evolutionary response, by plants that already produce psoralens, to the selective pressures of certain highly specialized insect herbivores such as *P. polyxenes* that are tolerant to the otherwise toxic effect of the linear compounds.

On the basis of data from earlier, limited studies on the interactions of unlabeled psoralen and isopsoralen with *P. polyxenes* caterpillars, we concluded that a reduced rate of metabolic detoxification of isopsoralens probably was not a factor in the relative sensitivity of these insects to isopsoralens (Bull et al., 1984). Considering the much more definitive data presented here, we now believe that our earlier conclusions were premature. Our present data do, in fact, appear to be compatible with the hypothesis that a reduced detoxification rate accounts at least in part for the susceptibility of *P. polyxenes* caterpillars to the deleterious effects of isopsoralens. If such be the case, however, our studies suggest that the "competitive edge" possessed by plants that synthesize isopsoralens as insect host plant resistance agents is a very slim one indeed, at least as regards herbivory by the black swallowtail.

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IN VITRO METABOLISM OF A LINEAR FURANOCOUMARIN (8-METHOXYPSORALEN, XANTHOTOXIN) BY MIXED-FUNCTION OXIDASES OF LARVAE OF BLACK SWALLOWTAIL BUTTERFLY AND FALL ARMYWORM

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Abstract—Studies were made of the comparative in vitro metabolism of [^{14}C]xanthotoxin and [^{14}C]aldrin by homogenate preparations of midguts and bodies (carcass minus digestive tract and head) of last-stage larvae of the black swallowtail butterfly (*Papilio polyxenes* Fabr.) and the fall armyworm [*Spodoptera frugiperda* (J. E. Smith)]. The two substrates were metabolized by 10,000*g* supernatant microsomal preparations from both species. Evidence gained through the use of a specific inhibitor and cofactor indicated that mixed-function microsomal oxidases were major factors in the metabolism and that the specific activity of this enzyme system was considerably higher in midgut preparations from *P. polyxenes* than in similar preparations from *S. frugiperda*. Aldrin was metabolized 3–4 times faster by *P. polyxenes*, and xanthotoxin 6–6.5 times faster.

Key Words—Aldrin, xanthotoxin, mixed-function oxidases, black swallowtail butterfly, fall armyworm, *Papilio polyxenes*, *Spodoptera frugiperda*, Lepidoptera, Papilionidae, Noctuidae.

INTRODUCTION

The results of recent studies of the in vivo metabolism of a linear furanocoumarin (xanthotoxin, 8-methoxypsoralen) by last-instar larvae of the black swallowtail butterfly (*Papilio polyxenes* Fabr.) and the fall armyworm (*Spodoptera frugiperda* J.E. Smith) gave convincing indirect evidence that the plant-derived

phototoxin was detoxified primarily via oxidative routes in both species (Ivie et al., 1983; Bull et al., 1984). The two predominant metabolites of xanthotoxin were both formed through cleavage of the furan ring moiety of the molecule; based upon mass spectrometry and nuclear magnetic resonance analyses these products were identified as monohydroxy and dihydroxy derivatives of xanthotoxin. More recent comparative studies by Ivie et al. (1985) demonstrated that analogous acidic products were formed during the in vivo metabolism of another related linear furanocoumarin (psoralen) and its angular analog (isopsoralen) by *P. polyxenes* larvae. In the case of xanthotoxin, metabolic formation of mono- and dihydroxy derivatives occurred in vivo following oral administration to *P. polyxenes* or *S. frugiperda* larvae, as well as in vitro when the compound was incubated with slices of midgut or body (carcass less digestive tract and head) tissues (Ivie et al., 1983; Bull et al., 1984). These studies also indicated that a profound difference in the rate of the in vivo metabolic degradation of xanthotoxin was most likely the major factor contributing to the tolerance of *P. polyxenes* and susceptibility of the *S. frugiperda* to the phototoxin.

A logical metabolic route to the formation of mono- and dihydroxy metabolites of furanocoumarins would include formation of a 2,3 epoxide at the furan ring moiety of the molecule as a result of oxidation by mixed-function microsomal oxidases (MFO) (Kolis et al., 1979) followed by ring cleavage to form the hydroxy derivatives. The purpose of the research described herein was to determine through in vitro techniques whether MFO enzymes were indeed responsible for the metabolic transformations of xanthotoxin that we previously observed in insects.

METHODS AND MATERIALS

Insects. Last-stage larvae of both species were obtained from colonies maintained in the laboratory at 27 ± 2°C and 60 ± 5% relative humidity under a photoperiod regimen of 14:10 hr light–dark. The *P. polyxenes* colony was reared on parsley (*Petroselinum sativum*) as described by Ivie et al. (1985). *Spodoptera frugiperda* larvae were from a colony maintained on a semidefined diet (Shaver and Raulston, 1971). In studies requiring parsley-reared *S. frugiperda*, third-stage larvae were selected from the stock colony and held individually in Petri dishes where they were allowed to feed on fresh parsley until they reached the desired stage of development.

Actively feeding larvae from groups of the same chronological age were selected on the basis of apparent similarity in physiological development. They were dissected in chilled (4°C) phosphate buffer (0.1 M, pH 7.5) with aid of a binocular microscope. Midguts were removed and emptied of their contents. These tissues and the remainder of the body (minus the head, foregut, and hindgut) were rinsed separately in 1.15% KCl and then returned to chilled phos-

phate buffer. During dissection, larvae were subjected to further selection for uniformity based upon the physical appearance of the midgut and fat bodies. Those with empty guts or that apparently were not actively processing ingested food were discarded. Dissected gut and body tissues were brought into brief contact with absorbent toweling to remove excess water and were then weighed and returned to cold buffer for further processing.

To obtain approximately equal amounts of tissue (wet weight) in samples of each species, we used three larval equivalents of *S. frugiperda* midguts or bodies for each one equivalent from *P. polyxenes*. These tissues were homogenized briefly in cold buffer (1.5 m and 0.5/ml for *S. frugiperda* and *P. polyxenes*, respectively) using a motor-driven Teflon pestle and glass tube apparatus, and the crude homogenate was filtered through cheesecloth. The filtrate was then centrifuged (Beckman L5-50 centrifuge) at $10{,}000g_{max}$ and 4°C for 15 min. Precipitated solids were discarded, and the supernatant was then filtered through glass wool and centrifuged again for 1 hr at $105{,}000g_{max}$ and 4°C. Immediately after centrifugation, the supernatant was decanted, the pellet was resuspended in the same volume of phosphate buffer containing 30% glycerol, and then preparations were used for different analyses. Protein content of different homogenate preparations was determined with the method of Bradford (1976).

Chemicals. Xanthotoxin radiolabeled with ^{14}C (specific activity of 9.86 mCi/mM) at the 8-methoxy position was prepared as described by Ivie et al. (1983). [^{14}C]Aldrin (2.6 mCi/mM) was purchased from California Bionuclear Corp., Sun Valley, California. Both radiolabeled compounds were >99% pure based upon thin-layer chromatography (TLC) analyses with methods described below. Radioactive samples of xanthotoxin or aldrin were diluted with pure unlabeled compounds so that each incubation mixture would contain a total of ca. 100,000 dpm of radioactivity. All other chemicals were obtained from commercial sources.

Enzyme Assays. Microsomal oxidase activity was determined with methods similar to those described by Yu (1982). Aldrin was included in all enzyme assays as a model to confirm that MFO activity was present or absent in different preparations.

Incubation mixtures were contained in standard 20-ml glass scintillation vials, and the incubations were carried out for 30 min in air in a water bath shaker maintained at 35°C. Each incubation mixture contained 2 ml of tissue preparation; a NADPH-generating system that included 1.8 μmol of NADP, 18 μmol of glucose-6-phosphate, and 1 unit of glucose-6-phosphate dehydrogenase; either 25 μg of [^{14}C]xanthotoxin or 100 μg of [^{14}C]aldrin; and sufficient buffer for a final volume of 5 ml. In all assays, an equal number of samples lacking the NADPH-generating system were included. Acetone solutions of [^{14}C]xanthotoxin were added first to the incubation vials and allowed to evaporate to form a thin film on the bottom surface. Preliminary tests indicated that

this method of addition resulted in greater metabolism of xanthotoxin than when it was added as a solution. The [^{14}C]aldrin was added first to the incubation vials as a solution (1 μg/μl) in ethylene glycol monomethyl ether. Each assay also included control samples containing all the above components except for tissue.

At the conclusion of the incubation period, the reaction was stopped by the addition of 0.5 ml of 6 N HCl and immediate partition (two times) with methylene chloride. Aqueous and organic fractions were radioassayed, the organic fraction was dried by filtration through anhydrous sodium sulfate, and then the sample volume was reduced under vacuum to ca. 0.5 ml and analyzed by thin-layer chromatography (TLC) as described below. This extraction procedure resulted in essentially complete recovery of radioactive materials associated with both substrates. All incubations were run in duplicate and replicated two or more times.

Analyses. Extracts containing radiocarbon were assayed by conventional liquid scintillation (LSC) procedures using a standard fluor (Permablend II®; Packard Instrument Co., Inc., Downers Grove, Illinois) in a solvent mixture of 2 : 1 (v/v) toluene and ethylene glycol monomethyl ether. Corrections were made as needed for quench and instrument efficiency.

TLC analyses of samples containing [^{14}C]xanthotoxin and its metabolities were done with precoated plates (Silplate F-254, 20 × 20 cm, 0.25-mm gel thickness, with fluorescent indicator; Brinkmann Instruments Inc., Westbury, New York) and a solvent mixture of 75 : 25 : 1 (v/v) ethyl acetate, methanol, and acetic acid [see Ivie et al. (1983) for R_f values of xanthotoxin and its metabolites]. Samples containing [^{14}C]aldrin and its metabolic products were analyzed with the same kind of TLC plates using a 1 : 1 (v/v) mixture of *n*-hexane and chloroform. This system provided good resolution of aldrin, its oxidative metabolite dieldrin, and minor amounts of polar radioactive material that were retained at the baseline.

Radioactive zones on TLC plates were located by autoradiography with X-ray film, and quantitated by LSC.

RESULTS AND DISCUSSION

Preliminary investigations indicated that the 105,000*g* supernatant was inactive against either [^{14}C]xanthotoxin or [^{14}C]aldrin, whether fortified or not with NADPH. Furthermore, there was no activity against either substrate in 10,000*g* supernatant or microsomal preparations that were boiled prior to addition to incubation mixtures.

The results of comparative studies (Table 1) indicated there was definite activity against both substrates in different homogenate preparations of the two species. TLC analysis demonstrated that the array of xanthotoxin metabolites

TABLE 1. IN VITRO METABOLISM OF [^{14}C]ALDRIN AND [^{14}C]XANTHOTOXIN BY SUBCELLULAR FRACTIONS OF HOMOGENATES OF LAST-STAGE *P. polyxenes* AND *S. frugiperda* LARVAE[a]

Homogenate preparation	NADPH	Metabolized (pmol/min/mg protein ± SD)		
		Aldrin	Xanthotoxin	Protein (mg)/sample
P. polyxenes (parsley-reared)				
Gut, 10,000*g* sup.	+	961 (±53)	317 (±83)	4.8 (±0.9)
Gut, 10,000*g* sup.	−	131 (±0)	22 (±18)	
Body, 10,000*g* sup.	+	3 (±0)	2 (±1)	15.3 (±1.7)
Body, 10,000*g* sup.	−	0	2 (±1)	
Gut, 105,000*g* ppt	+	1577 (±285)	1017 (±839)	0.8 (±0.0)
Gut, 105,000*g* ppt	−	3 (±3)	15 (±1)	
S. frugiperda (parsley-reared)				
Gut, 10,000*g* sup.	+	251 (±69)	49 (±10)	3.7 (±1.0)
Gut, 10,000*g* sup.	−	61 (±26)	25 (±10)	
Body, 10,000*g* sup.	+	217 (±66)	96 (±19)	8.5 (±1.7)
Body, 10,000*g* sup.	−	132 (±60)	3 (±1)	
Gut, 105,000*g* ppt	+	601 (±28)	166 (±90)	0.7 (±0.2)
Gut, 105,000*g* ppt	−	0	9 (±0)	
S. frugiperda (diet reared)				
Gut, 10,000*g* sup.	+	147 (±30)	—	3.2 (±0.5)
Gut, 10,000*g* sup.	−	69 (±20)	—	
Body, 10,000*g* sup.	+	5 (±0)	—	10.4 (±1.3)
Body, 10,000*g* sup.	−	1 (±0)	—	

[a] Incubation mixture was 5 ml of phosphate buffer (0.1 M, pH 7.5) containing one *P. polyxenes* or three *S. frugiperda* equivalents, 25 μg of xanthotoxin or 100 μg of aldrin, 1.8 μM NADP, 18 μM glucose-6-phosphate, and (±) one unit of glucose-6-phosphate dehydrogenase.

produced in vitro was the same as that found with our previous in vivo studies (Ivie et al., 1983; Bull et al., 1984). Dieldrin was the only significant product of aldrin metabolism, although traces of radiocarbon (<0.1% of dose) were occasionally retained at the baselines of TLC plates.

That enzyme activity was appreciably greater in most tissue samples fortified with NADPH, especially the microsomal preparations, is good evidence that MFO enzymes were involved, However, there was essentially no activity in 10,000*g* supernatant preparations of *P. polyxenes* bodies against either substrate. This was somewhat surprising in view of our previous demonstration that incubation of xanthotoxin with preparations containing slices of body tissues resulted in substantial (>60%) degradation (Bull et al., 1984). The absence of activity in homogenates of *P. polyxenes* body tissue most likely can be attributed to the presence of endogenous inhibitors comparable to those that have been implicated by others in the unexplainable absence of MFO activity in certain insect tissue preparations (Nakatsugawa and Morelli, 1976).

Specific activities of NADPH-fortified 10,000*g* supernatant and microsomal (105,000*g* precipitate) preparations of *P. polyxenes* midguts were clearly higher against both substrates than those of *S. frugiperda* (i.e., 3.8 and 2.6× for aldrin and 6.5 and 6.1× for xanthotoxin, respectively). Concentrations of total protein in midgut preparations were only slightly higher in those from *P. polyxenes*. We also found (Table 1) that the specific activities of 10,000*g* supernatant preparations of either the midgut or body homogenates of *S. frugiperda* against aldrin were substantially higher in larvae reared on parsley than in those reared on artificial diets (ca. 2 and 43×, respectively, in NADPH fortified samples). At first appraisal, our data from tests with parsley-reared *S. frugiperda* seem to contradict a recent report by Yu (1984) that *S. frugiperda* larvae fed for two days on artificial diets fortified with xanthotoxin had enhanced levels of some MFO enzymes, but not aldrin epoxidase, which instead was diminished. Parsley does contain xanthotoxin and at least seven other linear furanocoumarins; together these phototoxins have a concentration of ca. 100–150 ppm based upon wet weight (R.C. Beier, unpublished information). In our study however, the *S. frugiperda* larvae were fed on parsley for a longer period of time (ca. 10 days) and, too, other chemicals in the plant may have been responsible for the observed induced activity of aldrin epoxidase.

Inclusion of the MFO inhibitor piperonyl butoxide in incubation mixtures containing 10,000*g* supernatant preparations fortified with the NADPH-generating system resulted in a definite reduction in the in vitro metabolism of aldrin and xanthotoxin by *P. polyxenes*, and of aldrin by *S. frugiperda* larvae (Table 2). This reduction in activity was directly proportional to the concentration of inhibitor. Our results, especially with aldrin, are very similar to those reported

TABLE 2. EFFECT OF PIPERONYL BUTOXIDE ON IN VITRO METABOLISM OF [^{14}C]ALDRIN AND [^{14}C]XANTHOTOXIN BY HOMOGENATES OF LAST INSTAR *P. polyxenes* AND *S. frugiperda* LARVAE[a]

Piperonyl butoxide concentration (M)	Enzyme activity (% of control ± SD)		
	P. polyxenes		*S. frugiperda*
	Aldrin	Xanthotoxin	Aldrin[b]
0	100.0	100.0	100
10^{-6}	99.6 (±3.0)	78.6 (±8.8)	78
10^{-5}	82.6 (±4.3)	51.3 (±0.6)	41
10^{-4}	38.4 (±8.3)	29.8 (±3.2)	22
10^{-3}	3.5 (±1.8)	18.1 (±1.2)	5

[a] Except for the addition of piperonyl butoxide in 50 μl of ethylene glycol monomethyl ether, incubation conditions were the same as noted in Table 1.
[b] Data for *S. frugiperda*/aldrin are from one replicate with duplicate samples per concentration.

by Yu et al. (1979) for the variegated cutworm (*Peridroma saucia* Hübner) and by Yu (1982) for *S. frugiperda*. These authors considered that such a response to piperonyl butoxide was good evidence that the enzyme inhibited was a typical microsomal monooxygenase.

The results of these in vitro studies substantiate our previous speculation (Ivie et al., 1983; Bull et al., 1984) that the extensive in vivo metabolism of ingested xanthotoxin that we found in *P. polyxenes* larvae was due largely to the action of MFO enzymes associated with midgut tissues. Moreover, the large differences between *P. polyxenes* and *S. frugiperda* larvae in the in vivo detoxification of xanthotoxin in the aforementioned studies can probably be attributed for the most part to the substantially greater levels of MFO activity found in *P. polyxenes*.

In nature, certain specialist members of the genus *Papilio* feed successfully and preferentially on plants from the families Umbelliferae and/or Rutaceae that are rich in linear furanocoumarins (Berenbaum, 1981a,b). Not only are linear furanocoumarins nontoxic to *Papilio* larvae, there is also evidence that they might stimulate feeding and enhance growth (Berenbaum, 1981a). In contrast, generalist feeders such as *Spodoptera* spp. tend to avoid plants that contain linear furanocoumarins; when forced to consume furanocoumarins such as xanthotoxins, both growth and survival are negatively affected (Berenbaum, 1978).

It is believed that a primary function of MFO enzymes in herbivorous insects is to detoxify naturally occurring chemicals that are repellent or toxic to the insect and that the capability for such inactivation of plant defensive chemicals can have a profound influence on the feeding strategies of different insect groups (Krieger et al., 1971; Brattsten et al., 1977). Our collective studies (Ivie et al., 1983, 1985; Bull et al., 1984; and the present work) therefore suggest that the evolutionary adaptation of *P. polyxenes* that allows that species to feed and thrive on furanocoumarin-rich plant groups is directly related to the development of a highly efficient MFO system in the digestive tract.

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EFFICACY OF THE PLANT PHOTOTOXIN α-TERTHIENYL AGAINST *Aedes intrudens* AND EFFECTS ON NONTARGET ORGANISMS

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Abstract—The botanical phototoxin, α-terthienyl (α-T) was spray applied to natural or artificial pools containing mosquito (*Aedes intrudens*) larvae and nontarget invertebrates (caddisfly, damselfly, midge, shrimp, *Daphnia*, snail) and one vertebrate (trout) at concentrations varying from 0.01 to 1 kg/hectare, under field and laboratory conditions. All field-treated nontarget invertebrates survived α-T treatment better than *A. intrudens* which can be controlled at doses as low as 0.01 kg/hectare within one week. Under laboratory conditions, snails and trout survived α-T and UV treatments up to 10 kg/hectare. These results compare favorably with organophosphates and pyrethroid insecticides currently used for mosquito control. The results confirm that α-T is a highly effective larvicide with acceptable nontarget effects.

Key Words—α-Terthienyl, *Aedes intrudens*, Diptera, Culicidae, caddisfly, Trichoptera, Limnephidae, damselfly, Odonata, Libellulidae, midge, Chaoboridae, shrimp, *Daphnia*, snail, Physa, trout, Salmo, thiophene.

INTRODUCTION

The thiophene α-terthienyl (α-T) was recently shown to have exceptional larvicidal activity to mosquitoes and blackflies in laboratory and field trials (Wat et al., 1981, Arnason et al., 1981, Philogène et al., 1985). The value of this naturally occurring compound from the tribe *Tagetae* (Asteraceae) as a commercially acceptable insecticide depends upon its efficacy towards target species and its toxicity level towards nontarget organisms. In the present paper, we

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report on further field and laboratory experiments with the mosquito *Aedes intrudens* and some nontarget invertebrates and vertebrates and demonstrate that α-T is an efficient mosquito larvicide that is relatively nontoxic to nontarget species.

METHODS AND MATERIALS

Field Trials. Spray applications of α-T formulated in ethanol to large (20–40 m^2) natural breeding pools (temporary snow-melt ponds) were undertaken in the same way as described earlier (Philogène et al., 1985). Water temperature was in the range 10–14°C during the testing period.

The following organisms were field tested: *Aedes intrudens* (Diptera, Culicidae); caddisfly larvae (Trichoptera, Limnephidae), damselfly larvae (Odonata, Libellulidae), midge larvae (Diptera, Chaoboridae), the freshwater shrimp, *Chirocephalopsis bundyi*, and an unidentified ostracod (Crustacea).

Two tests were undertaken: one in early May with third- and fourth-instar *A. intrudens* larvae present and a second in mid-May with fourth-instar larvae and pupae. In the first test, eight pools were treated in applications of 0, 0.01, 0.1, and 1.0 kg/hectare, in duplicate. Intermediate application rates were used in the second test: 0.05, 0.1, 0.5 kg/hectare. Larval counts were made in 25 dips, and the percent reduction calculated according to the formula of Mulla et al. (1971).

To achieve a more precise evaluation of effective levels of control, applications were also made to 25 fourth-instar larvae (the most resistant instar) of *A. intrudens* in bioassay cages placed in 1-m-diameter wading pools containing pond water. The bioassay cages consisted of a cylindrical cage (12.5 × 15 cm) constructed of diamond-stamped aluminum surrounded by muslin held in place by rubber bands.

The pools were placed in a hardwood forest in partial shade, as in the preceding field trials, with 3 replicates for each application. Nontarget organisms (caddisfly, damselfly, and midge larvae) were also treated in the same way, but the pools were moved out of the canopy into full sunlight, adjacent to a permanent pond from which the nontarget organisms were taken.

EC_{50} and EC_{90} values were calculated on the basis of three concentrations giving intermediate survival of larvae.

The numbers of ostracods and freshwater shrimps present in temporary snow-melt pools did not permit observations in contained enclosures. These ecosystems were, however, treated with α-T in the same way as the bioassay cages.

Laboratory Tests. The water flea, *Daphnia magna* (Ostracoda), was obtained from Carolina Biological Supply and the snail, *Physa* sp. (Gastropoda), purchased from a local supplier. These organisms were treated at 23°C in Pyrex

crystallizing dishes (28 cm^2) containing 200 ml of water. Following α-T application, the preparation was maintained for 30 min in the dark and then treated with near-UV (5 W/m^2) from four 20-W Westinghouse blacklight blue tubes. UV treatment was 1 hr for snails and 15 min for *Daphnia* (the maximum near-UV exposure with limited effects on controls).

Toxicity tests with trout were based on guidelines outlined by Sprague (1973). Rainbow trout (*Salmo gairdneri*) were obtained from the Val des Bois Hatchery (Quebec) and held for two weeks in the lab prior to testing. Ten 5- to 8-cm fish were placed in each of six 10-gal aquarium tanks maintained at 14°C and equipped with an aeration tube, freshwater supply, and syphon. After 48 hr, air and water flow were arrested and α-T was applied. One hour later the aquaria were exposed to near-UV (5 W/m^2) from six 24-in. 20-W blacklight blue fluorescents for 1 hr. Following UV treatment, air and water supplies were resumed. Fish were held in aquaria for a further 72 hr or more.

RESULTS AND DISCUSSION

All the organisms treated in the field or in the lab under controlled conditions survived α-T and light exposure much better than the target mosquito *A. intrudens* (Table 1). Complete control of the latter was achieved within two days at 1 kg/hectare and within one week at 0.1 and 0.01 kg/hectare in the first field test (Figure 1). In the second test, where intermediate application rates were used, larvae were controlled in three days at all application rates, while pupae were only partially controlled at 0.05 kg/hectare, with substantial reductions in numbers at 0.1 and 0.5 kg/hectare (Figure 2). In the bioassay cages, at 72 hr after application time, an EC_{50} of 0.046 kg/hectare and an EC_{90} 0.104

TABLE 1. TOXICITY DATA FOR TARGET AND NONTARGET ORGANISMS[a]

Type of organism	Scientific name	Common name	EC_{50}	EC_{90}	Remarks
Insects					
Diptera	*Aedes intrudens*	Mosquito larvae	0.046	0.104	Outdoors/sunlight
	Chaoborus sp.	Midge larvae	0.178	0.280	Outdoors/sunlight
Trichoptera	*Limnephidae*	Caddisfly larvae	1.32	21.0	Outdoors/sunlight
Odonata	*Lestes* sp.	Damselfly larvae	0.38	1.52	Outdoors/sunlight
Crustacean	*Daphnia magna*	Water Flea	0.044	1.50	Indoors/near UV
Mollusc	*Physa* sp.	Snail	≥ 10	≥ 10	Indoors/near UV
Fish	*Salmo gairdneri*	Rainbow trout (5-8 cm)	≥ 10	≥ 10	Indoors/near UV

[a] EC_{50} and EC_{90} values are based on a probit analysis of two to three application rates with intermediate survival.

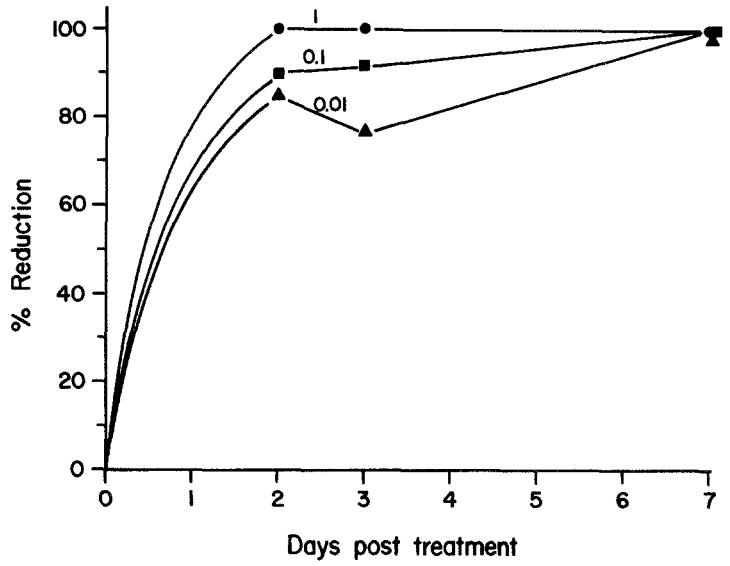

FIG. 1. Percent reduction in third- and fourth-instar larval counts following α-T treatment of natural breeding ponds on May 3, 1984. Application rates in kg/hectare. Results are average of duplicate trials.

kg/hectare were calculated, values that agree well with the results in natural pools.

Toxicity data for nontarget organisms under field conditions show that only midge larvae, which are close relatives of mosquitoes, would be adversely affected by the phototoxic properties of α-T, at application rates required to control mosquito larvae. The EC_{50} and EC_{90} values for caddisfly and damselfly larvae indicate that they would survive α-T treatment at mosquito larvicidal levels.

Of the other invertebrates observed in the field, the ostracod was unaffected at the highest application rate (1 kg/hectare), while the freshwater shrimp survived the 0.1 kg/hectare application but suffered 100% mortality at 1 kg/hectare.

Under laboratory conditions *Daphnia*, snails, and trout survived α-T and UV treatment without any significant or visible signs of intoxication.

The efficacy of α-T in the field is comparable to the organophosphate temephos which gave 100% control of *Psorophora* larvae at 18 g/hectare (Kottkamp et at., 1981) or pirimphos methyl, which gave 100% control of *Culex* larvae at 50 g/hectare (Mulla et al., 1973). Stream macroinvertebrates including amphipods, caddisfly larvae, and mayfly larvae show adverse reactions, including detachment and mortality to these two insecticides (Muirhead-Thompson, 1978).

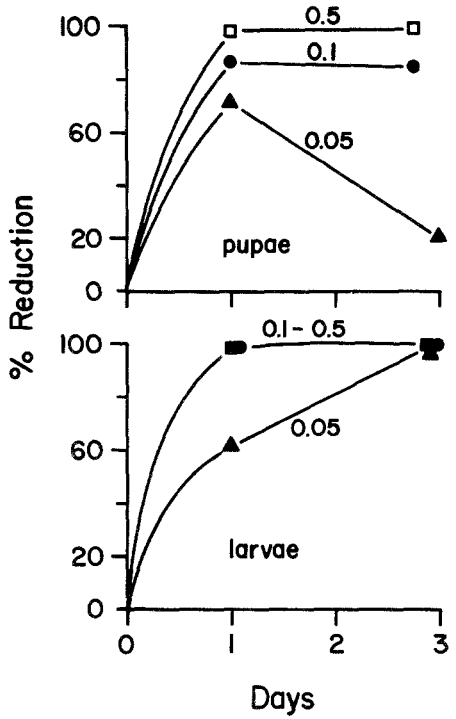

FIG. 2. Percent reduction of fourth instars and pupae in natural breeding pools treated on May 7, 1984.

Some pyrethroids are, however, more active than α-T or organophosphates. For example, cypermethrin controlled *Culex* larvae at 3–5 g/hectare (Mulla et al., 1982) and permethrin at 11 g/hectare in trials against *Psorophora* larvae (Thompson and Meisch, 1977). However, these materials reduced mayfly larvae to very low levels at mosquito larvicidal applications (Mulla et al., 1982). α-T has good efficacy and acceptable nontarget effects, which may therefore provide a new alternative to organophosphates and pyrethroids.

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DELAYED PHOTOTOXIC EFFECTS OF 8-METHOXYPSORALEN, KHELLIN, AND SPHONDIN IN *Aedes aegypti*

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Abstract—At concentrations up to 6.7 ppm, 8-methoxypsoralen, sphondin, and khellin are not toxic to first-instar larvae of the mosquito *Aedes aegypti*. The irradiation of sensitized larvae with long-wavelength ultraviolet light did not always produce any immediate toxicity enhancement, but delayed effects were clearly visible. These were observed over the development of the organisms from first-instar larvae to adults. No adverse effects were noted when larvae were irradiated in the absence of sensitizers, or when they were placed in solutions of sensitizers which had been previously irradiated with the same light sources. 8-Methoxypsoralen was slightly more phototoxic than its isomer sphondin. Khellin, recently reported to undergo photoinduced cyclization with DNA components, showed minimal phototoxicity in the concentration range used.

Key Words—Phototoxicity, 8-methoxypsoralen, sphondin, khellin, *Aedes aegypti*, Diptera, Culicidae.

INTRODUCTION

8-Methoxypsoralen (8-MOP) is a natural product primarily found in plants belonging to the families Rutaceae, Umbelliferae, Leguminosae, and Moraceae. Its phototoxic properties have long been recognized, particularly in relation to

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the treatment of dermatological disorders in man, notably vitiligo and psoriasis (Anderson and Voorhees, 1980).

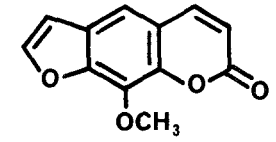
Numerous mechanistic studies have been performed, demonstrating the importance of cycloaddition reactions to pyrimidine bases of nucleic acids produced in the presence of long-wavelength ultraviolet light (Shim et al., 1984, and references cited). Either one or two double bonds in the furanocoumarin ring system can participate in the cycloaddition reactions. Less spectacular, but perhaps of comparable biological importance, the generation of singlet oxygen by 8-MOP has also been demonstrated (Joshi and Pathak, 1983; Kagan et al., 1984, and references cited).

The question of how insects interacting with plants producing 8-MOP deal with this phototoxic molecule has been raised. Camm et al. (1976) showed that linear furanocoumarins lacked toxicity to the aphids *Aphis heraclella* and *Cavariella pastinacae* feeding exclusively on Umbelliferae. Berenbaum (1978) discovered that the phototoxicity of 8-MOP was greater than its toxicity in the larvae of the Southern armyworm *Spodoptera eridania*, a generalist insect herbivore. Ivie et al. (1983) demonstrated a rapid detoxification of 8-MOP in larvae of the black swallowtail butterfly *Papilio polyxenes*. In contrast, the larvae of the psoralen-sensitive fall armyworm *Spodoptera frugiperda* metabolized 8-MOP at a much slower rate.

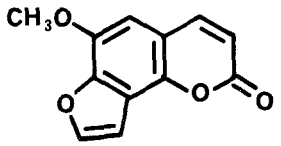
The phototoxicity of 8-MOP in insects whose diet is completely unrelated to plants producing this compound has apparently not been demonstrated, with the exception of the photoovicidal activity in *Drosophila melanogaster* (Kagan and Chan, 1983). However, its lack of photoinduced activity has been recorded in mosquito larvae (*Aedes aegypti*) and blackfly larvae (*Simulium vittatum*) by Wat et al. (1981), and in the larvae of *A. aegypti* and *Drosophila melanogaster* by Kagan et al. (1983).

The mosquito *Aedes aegypti* is a convenient organism for toxicity work in the laboratory. It is particularly valuable for phototoxicity studies since it can grow from egg to adult in the complete absence of light. The results of any light treatments on this organism are therefore easily determined by comparison with dark controls. A cursory screening had revealed the lack of acute phototoxicity of 8-MOP in second- and third-instar larvae of *A. aegypti* at a concentration of 0.78 ppm (Kagan et al., 1983) and 6.7 ppm. Prior studies of phototoxic effects in mosquito larvae have generally used late instars because their larger sizes made them more convenient to handle. However, we have frequently observed these later instars to be less sensitive to treatments with other sensitizers, and Arnason et al. (1981) also reported increased photosensitivity of first instars compared to older larvae. As illustrated in this report, delayed phototoxic effects can be readily demonstrated.

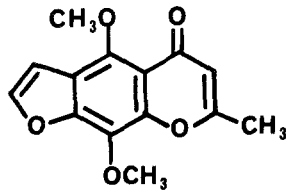
The phototoxicity of 8-MOP is compared to that of two natural products, sphondin and khellin (Figure 1). Sphondin is an isomer of 8-MOP characteristic of the genus *Heracleum*, where it has been detected in 30 species, usually in



8-METHOXYPSORALEN



SPHONDIN



KHELLIN

FIG. 1. Structures of 8-MOP, sphondin, and khellin.

the roots. There have been scattered reports of its occurrence in 10 other genera of the family Umbelliferae, and only one finding outside of this family, in the fruits of *Ruta pinnata* (Rutaceae), by Estevez Reyes and Gonzalez Gonzalez (1963). Sphondin is not commercially available but has recently become more readily accessible through chemical synthesis (Wulff and McCallum, manuscript in preparation). It is highly inhibitory to the growth of hypocotyl cuttings of cucumber seedlings, while accelerating their root formation (Shimomura et al., 1982). It also has mitotic activity in wheat-seedling roots (Zhamba et al., 1970) and inhibits seed germination and root growth in radishes (Komissarenko et al., 1971). Finally, sphondin has shown phototoxicity to the fungus *Penicillium expansum* (Van der Sluis et al., 1981), to *Candida albicans* (Kavli et al., 1983a), and has generated weak photoepicutaneous response (Kavli et al., 1983b).

Khellin is a furanobenzopyrone well known for its vasodilator properties (Hassan and Zubair, 1980), which is also of value in controlling atherosclerosis (Schurr and Day, 1981). It was originally isolated from the seeds of *Ammi visnaga*, family Umbelliferae (Fantl and Salem, 1930), and it has been shown to be phototoxic to three gram-positive organisms (*Bacillus subtilis*, *Staphylococcus aureus*, and *S. faecalis*) in a classic paper by Fowlks et al. (1958). It induced skin pigmentation in rabbits in the presence of sunlight (Kabilov, 1962) and was phototoxic to viruses (Cassuto et al., 1977). A more recent paper con-

firmed the phototoxicity of khellin against *B. subtilis* and *S. aureus*, but not against *S. faecalis*, and established its phototoxicity against *S. albus*, *Candida albicans*, and *Saccharomyces cerevisiae* (Abeysekera et al., 1983). These authors demonstrated that khellin and ultraviolet light induced 2 + 2 photoaddition to thymine and chromosomal changes in Chinese hamster ovary cells. In our earlier screenings, however, khellin induced no acute phototoxic effects in third- and fourth-instar *A. aegypti* larvae (Kagan et al., 1983).

A number of furanocoumarins, including 8-MOP, and related furanoquinolines and furanochromones, including khellin, display feeding inhibitory activity against the tobacco cutworm (*Spodoptera litura*), cockroaches (*Periplaneta americana*, *Stylopyga rhombifolia*, and *Blattela germanica*), and the housefly *Musca domestica* (Yajima and Munakata, 1979). Whether or not the magnitude of the effect depends on the presence of ultraviolet light is unknown. Unfortunately, this activity was not investigated in sphondin.

METHODS AND MATERIALS

Khellin and 8-MOP were purchased from Sigma. Sphondin (mp 191°C) was synthesized in the laboratory in four steps from furan via 2-furyl(methoxy)methylene pentacarbonyl chromium(0). The details of the synthesis will be reported separately. All three sensitizers showed a single peak on HPLC analysis. Sixfold serial dilutions from a stock solution containing 1 mg/ml of sensitizer in dimethyl sulfoxide provided the samples which were assayed. Prof. G. Craig, Jr., University of Notre Dame, provided the original eggs of *A. aegypti* (strain Rock) from which the laboratory colony was established. Eggs were placed in aged tap water in the morning, and first-instar larvae were collected in the afternoon. For each experiment, 10 larvae were transferred into a 6-ml vial, the excess water was pipetted out, and 3 ml of the appropriate sensitizer solution were added under very dim incandescent light. Between 8 and 12 replicates were usually prepared at each sensitizer concentration (Table 1). All samples were incubated overnight in the dark. The next morning all the larvae were still surviving.

One set of vials was then irradiated for 30 min, receiving 13.5 W/m^2, under a bank of eight light sources emitting above 320 nm, with a maximum at 350 nm (RPR-3500A from the Southern New England Ultraviolet Corp., Hamden, Connecticut). The contents of these vials and of an equivalent set continuously kept in the dark were transferred into Petri dishes containing 40 ml of water, to which 0.5 ml of a 16 g/liter liver powder suspension (ICN Pharmaceuticals, Cleveland, Ohio) was added. This was done in the dark room dimly lit through an amber Kodak OC Safelight filter. Control and irradiated larvae were then kept in a darkroom throughout the experiments. The surviving or-

TABLE 1. SUMMARY OF EXPERIMENTS, EACH UTILIZING 10 FIRST-INSTAR LARVAE OF *A. aegypti*, PERFORMED WITH EACH SENSITIZER AT EACH CONCENTRATION

Compound	Concentration (ppm)	Number of replicates		Cumulative % adults day 11, $\bar{X}$ (SEM)	
		Light	Dark	Light	Dark
8-MOP	6.7	11	12	0 (0)	80.3 (4)
	1.1	13	6	2.7 (1)	77.1 (9)
	0.2	13	6	64.9 (7)	85.1 (6)
	0.03	13	6	77.2 (5)	82.4 (9)
Sphondin	6.7	5	6	0 (0)	90.3 (4)
	1.1	7	6	15.9 (7)	81.8 (9)
	0.2	7	6	85.4 (3)	80.9 (9)
	0.03	7	6	77.1 (9)	91.7 (4)
Khellin	6.7	8	9	74.4 (9)	88.9 (2)
	1.1	9	9	80.6 (11)	81.6 (5)
	0.2	9	9	83.3 (11)	86.4 (4)
	0.03	8	8	57.5 (14)	92.5 (3)
Irradiated 8-MOP	6.7		8		76.9 (6)
Irrad. sphondin	6.7		4		92.2 (5)
Irrad. khellin	6.7		7		89.8 (3)
None		18	18	80.9 (5)	82.7 (3)
8-MOP (1 hr incubation)	6.7	6	6	0	83 (5)
	1.1	6		4.2 (3)	
	0.2	6		69.7 (6)	
	0.03	3		83 (3)	

ganisms were counted every day afterwards, and they were fed in the same manner three and six days after the irradiation.

In other control experiments, 3-ml samples of 6.7 ppm solutions of each sensitizer were photolyzed for 30 min and were then seeded with the first-instar larvae. These organisms, continuously kept in the dark, were transferred into Petri dishes the next day and handled as above to check for toxicity of photoproducts which might have been formed from the original sensitizers.

Table 1 summarizes the number of experiments performed with each sensitizer, as well as the mean cumulative adult emergence on day 11. Every day, the experimental points shown on Figures 1 to 4 were determined by comparing the total number of surviving organisms at each developmental stage in all the runs to the total number of original larvae.

The experiments with 8-MOP, in which the incubation time was only 1 hr, were performed identically. All the results discussed in the text were obtained from the average of six experiments, both in the dark and in light.

Besides the actual irradiations with UV light, the organisms had to be ex-

posed to light once a day for a few minutes while the survivors were counted. This was done in the dark room, about 20 cm from a 15-W incandescent bulb.

Statistical analyses were performed using SAS82.4 programs. Adult emergence on day 11 for irradiated samples and dark controls was compared by a two-tailed Mann-Whitney-Wilcoxon test for each concentration. LC_{50} values were determined by linear regression analysis.

RESULTS AND DISCUSSION

The highest concentration used in this work with the three sensitizers was 6.7 ppm. At this level, the irradiations for 30 min did not have any immediate lethal effect. Notable differences in behavior and survival between the dark controls and the organisms which had been irradiated began to appear later on, and these effects were also clearly dependent upon the concentration of sensitizer used in the experiment.

A dynamic picture of the development of the *A. aegypti* in our experiments was obtained by plotting on a single graph the percentage of surviving larvae, pupae, and adults recorded each day following the irradiation. Petri dishes are very convenient for housing sets of irradiated organisms, since they allow a count of the adults to be made very rapidly without providing them an opportunity to escape, and they minimize the space requirements for storing the large number of experiments in progress. One of the earlier studies on the toxicity of photodynamic compounds in *A. aegypti* was conducted in Petri dishes (Barbosa and Peters, 1970). The drawback of the technique is that the adults die rapidly, because flight space is limited, and it is difficult to feed them without allowing their escape. For these reasons the present work did not investigate the behavior of the adults and their reproductive ability, which will be the subject of a later study.

The effects of irradiation in the absence of any sensitizers are shown in Figure 2. The survival curves for the larvae, pupae, and adults corresponding to the fate of the irradiation controls (open squares) and the dark controls (solid circles) are practically superimposable. This proves that irradiation for 30 min with UV light alone caused no short-term developmental abnormalities. In these experiments the first pupae were visible on day 6 following the irradiations, they were at their peak on day 8, and less than 10% remained at the end of the experiments, on day 11. The percent [$\bar{X}$ (SEM)] of adults present in dark and light controls were 82.7 (3) and 80.9% (5), respectively.

Phototoxicity of 8-MOP. The experiments in which the sensitizer was irradiated prior to the introduction of the larvae were necessitated by the fact that 8-MOP is known to have a rich photochemistry at the wavelengths used in the experiments. For example, in addition to the photocycloadditions to pyrimidines already mentioned, 8-MOP can generate singlet oxygen, and this reagent

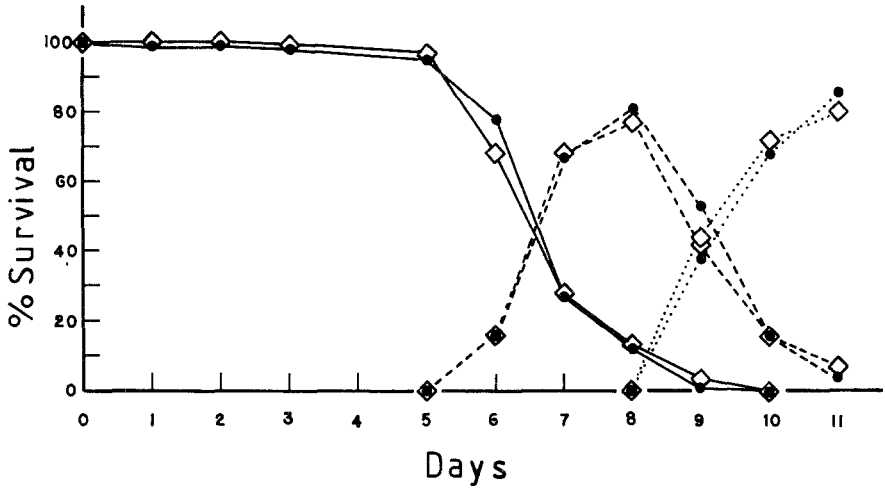


FIG. 2. Larvae (solid lines), pupae (broken lines), and adults (dotted lines) obtained from first-instar *A. aegypti*, as a function of time. The fate of the irradiated organisms (open squares) is compared to the dark controls (closed circles). See text for details.

has been shown by Logani et al. (1982) and by Wasserman and Berdahl (1982) to react with 8-MOP itself, resulting in oxidation products in which the furan ring had been opened, in a manner reminiscent of the enzymatic detoxification reported by Ivie et al. (1983). The biological activity of these products was unknown.

At a concentration of 6.7 ppm, 8-MOP in the dark had practically no effects on the development of *A. aegypti*, compared to the controls. The only noteworthy difference may have been that at the end of the experiments a few organisms had still not left the larvae stage. However, the peak concentration of pupae and the number of adults present at the end were essentially the same as in the controls. These results are presented in Figure 3A, which summarizes three different experiments performed at that same concentration. One shows the survival in the dark (solid circles), which is indistinguishable from the survival of organisms placed in a 6.7 ppm solution of 8-MOP which had been previously irradiated for 30 min (triangles). The third curve (open squares) shows the survival of the organisms which had been irradiated for only 30 min. While no acute toxicity could be detected the day of the irradiation, only 26% of the irradiated, sensitizer-exposed larvae were still alive the next day, and all were dead three days after the irradiation. The high phototoxicity of 8-MOP, which could not be detected on the day of the irradiation, was now clearly demonstrated. While consistent with DNA interference, this observation could not be interpreted unequivocally as a proof for it in the absence of supporting biochemical and genetic results.

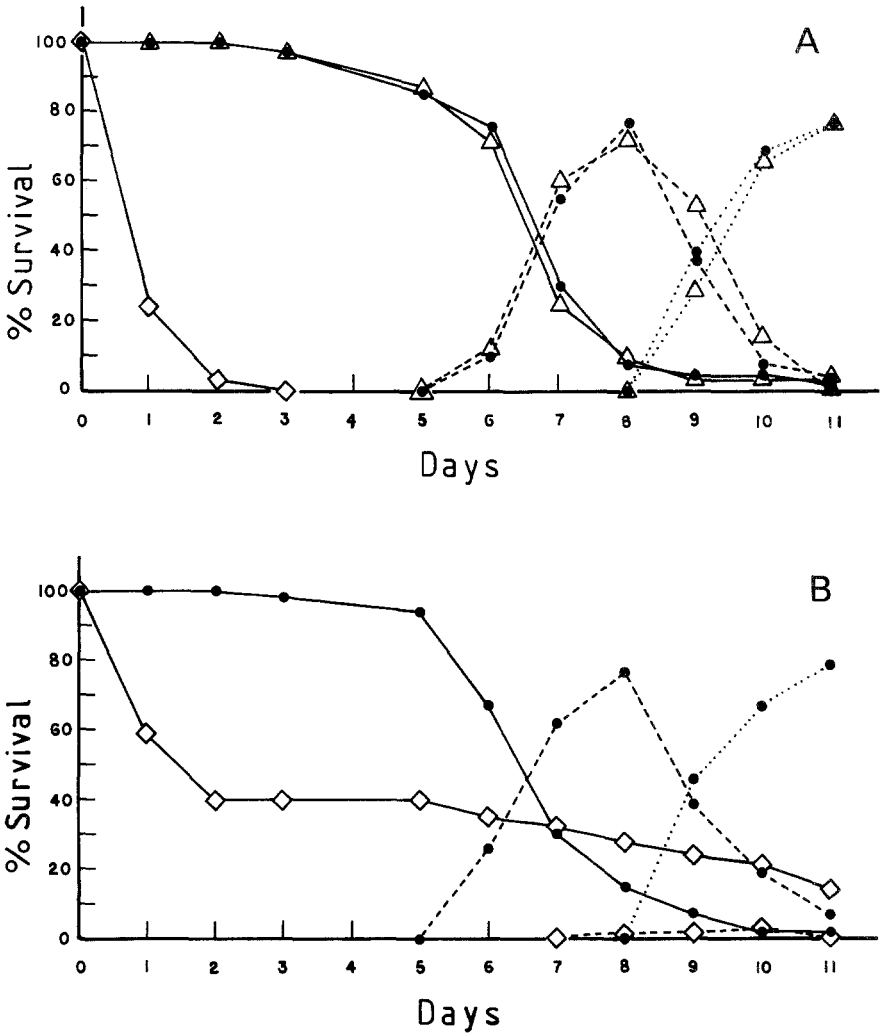


FIG. 3. Experiments with 8-MOP: larvae (solid lines), pupae (broken lines), and adults (dotted lines) obtained from first-instar *A. aegypti*, as a function of time. The sensitizer concentration was 6.7 ppm in A, 1.1 ppm in B and D, and 0.2 ppm in C. The survival of the dark controls is shown with solid circles, that of the irradiated organisms with open squares. The open triangles show the fate of the organisms kept in the dark in an identical solution which had been irradiated separately for the same duration. The irradiations took place after overnight incubation in the presence of the sensitizer, except in the case of D where only 1 hr of incubation was used. The appearance of 1% of adults from days 10 to 11 for the irradiated organisms in B is not visible on this graph. See text for details.

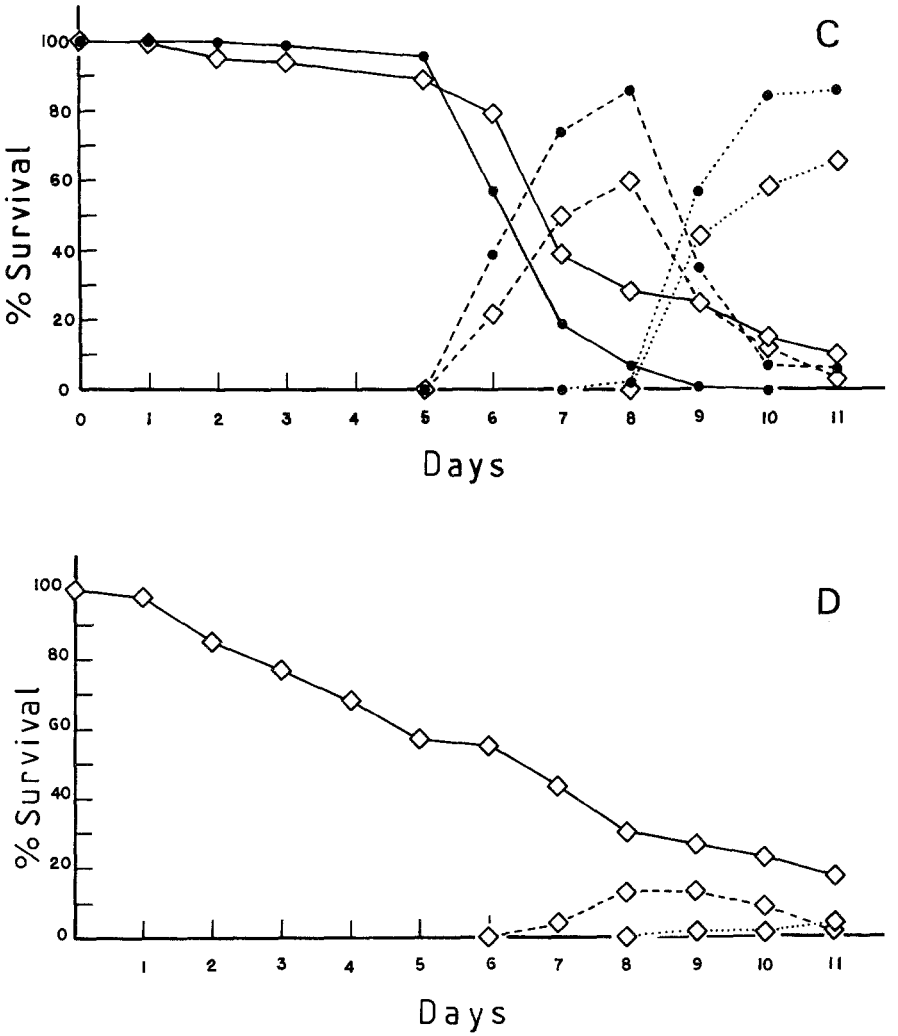


FIG. 3. Continued.

A sixfold dilution of the sensitizer solution (1.1 ppm) led to different results. As shown on Figure 3B, a precipitous drop in the larval survivorship occurred during the two days after irradiation, and most of the surviving organisms then failed to produce pupae and adults. Even though 57% of the larvae had survived on day 2, gradually decreasing to 40% over the next four days, only about 7% of the original pool yielded pupae. At the end of the experiment, only 2.7% of the original sample had turned into adults, while 12.2% were still

larvae. These had not gone beyond the second instar and died over the next days rather than develop normally. The adult emergence for irradiated larvae [2.7% (1)] was significantly lower than for dark controls (77.7% adults) ($Z = 3.7$; $P = .002$).

At this concentration (1.1 ppm), the dark controls showed a development practically identical to that of the controls which had not been treated with either light or chemical (see Table 1).

A further sixfold dilution of the sensitizer solution, to 0.2 ppm, resulted in diminished phototoxic effects (Figure 3C). Some larvae still failed to pupate. Consequently, there was a smaller proportion of pupae than in the controls, but most of those pupae turned into adults. At the end of the experiment, only 65% (7) of the original larvae had turned into adults, compared to 85% (6) in the dark controls ($Z = 1.5$; $P = 0.15$).

Finally, another sixfold dilution of the sensitizer solution, to 0.03 ppm, yielded survival curves identical to the controls, with a 77% conversion of the larvae into adults (not shown).

A linear regression analysis of the adult survival versus sensitizer concentration gave an LC_{50} of 0.41 ppm.

It is interesting to observe the diversity of the delayed phototoxic effects: death of all the larvae at 6.7 ppm; 40% survival on day 6 at 1.1 ppm, but inability for most of them to pupate; reduced ability to pupate at 0.2 ppm, but most pupae viable; and no detectable effects at 0.03 ppm. The etiology of these developmental irregularities is completely unknown.

The incubation time prior to irradiation is expected to be a significant parameter in photosensitizing experiments, although it may be difficult to predict how this parameter will affect the overall transformation of insect larvae into adults. One argument is that a shorter incubation time should result in more limited incorporation of a toxicant into the organisms, and their survival should therefore be greater. An equally plausible argument is that longer incubation times should allow more extensive metabolic inactivation of the sensitizers prior to photolysis. The target organisms should therefore be more seriously affected by the irradiations with shorter incubation times.

In order to help decide between these opposite viewpoints, a series of experiments was conducted with 8-MOP, in which the initial incubation time was reduced to 1 hr, the photolysis time being kept at 30 min. This experiment introduced at least two additional changes, namely that the organisms were younger and perhaps more sensitive at the time of the irradiations and that they were fed earlier than in the other experiments. However, the results were almost the same as those obtained with the overnight incubation time. At 6.7 ppm, all the larvae were dead two days after the irradiation. At 1.1 ppm, the survival curve (Figure 3D) for the larvae declined less steeply than shown on Figure 3B, and it was almost linear over the duration of the experiment. Two days after irradiation, for example, 71% of the larvae were still alive, compared to 57%

earlier, but on day 8 their survival was the same in both cases, about 30%. The mean proportion of adults observed at the end was not very different, 4.2% compared to 2.7% earlier. We must conclude, therefore, that *A. aegypti* larvae do not possess an efficient mechanism for the metabolic inactivation of 8-MOP.

Phototoxicity of Sphondin. Sphondin, an isomer of 8-MOP, was studied in the same manner. Figure 4A summarizes the behavior of the larvae in the presence of 6.7 ppm of sensitizer in the dark (closed circles), and in the presence of a solution of the sensitizer at the same concentration which had been first irradiated for 30 min (triangles). Both series of experiments yielded very similar results, demonstrating the absence of toxicity for the chemical and proving that no toxic products were formed when it was irradiated. The results of the irradiation of the larvae in the presence of sphondin at 6.7 ppm are also shown in Figure 4A (open squares). An immediate effect was detected, with only 40% of the larvae surviving the irradiation and 4% alive one day later. However, no further changes were found during the rest of the experiment. The development of the few surviving larvae was arrested, and no pupae were obtained.

At 1.1 ppm, the survival one day after exposure (61%) was greater than at 6.7 ppm (Figure 4B), but many of the surviving larvae failed to pupate. On day 11, when all the experiments were terminated, 16% of the original population had turned into adults, 6% were pupae, and 30% were still larvae. Dark controls had 82% adults, 12% pupae, and 0% larvae (adult emergence significantly different, $Z = 2.8$; $P = 0.02$).

At 0.2 ppm, the results of the experiments in the dark [81% (9) adult formation] and in the light [85% (3) adult formation], were no longer different ($Z = -0.15$, $P = 0.89$). A linear regression analysis on the data for survival to the adult stage gave $LC_{50} = 0.68$ ppm.

Phototoxicity of Khellin. Although khellin had been reported to be phototoxic, as summarized earlier, we did not observe any short-term acute phototoxic response at concentrations up to 6.7 ppm, using mosquito larvae, *Daphnia magna*, or the eggs of *Drosophila melanogaster* (unpublished results).

Figure 5 summarizes the studies with 6.7 ppm of khellin, and confirms our earlier observations. The results in the dark (closed circles) and in the light (open squares) are very similar. The curve for the irradiated pupae appears to be broader and to have a lower maximum than in the dark experiments, but the ultimate proportion of adults [$\bar{X} = 74$ (9)] is within the normal range observed in untreated larvae.

At 1.1 ppm, the results of the dark and light experiments [$\bar{X}$% (SEM) = 82 (5) and 81 (11), respectively] and that of the untreated controls are all within the normal range (not shown). Our results, showing that 8-MOP was more active then khellin, are in accord with those of Abeysekera et al. (1983) in a different system. They also conform with the results of experiments with the fungus *Penicillium expansum*, which showed that 8-MOP was very phototoxic, sphondin weakly phototoxic, and khellin not phototoxic (Van der Sluis et al.,

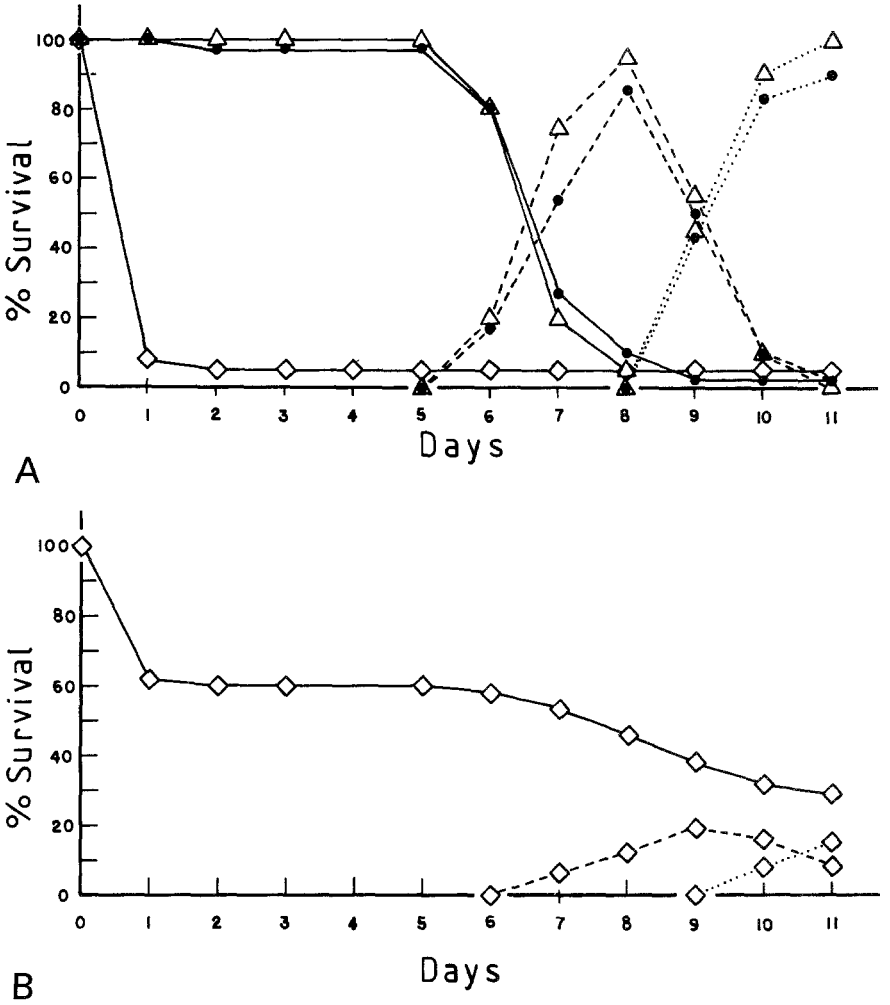


FIG. 4. Experiments with sphondin: larvae (solid lines), pupae (broken lines), and adults (dotted lines) obtained from first-instar *A. aegypti*, as a function of time. The sensitizer concentration was 6.7 ppm in A, 1.1 ppm in B. The survival of the dark controls is represented with solid circles, that of the irradiated organisms with open squares. The open triangles are for the organisms kept in the dark in an identical solution which had been irradiated separately for the same duration. The dark controls in B, identical to the results shown in A, have been omitted. See text for details.

1981). The same relationship was also observed in photosensitizing experiments on the skin of guinea pigs and rabbits (Khadzhai and Kuznetsova, 1965).

The diversity of the phototoxic responses described for khellin and, particularly, its phototoxicity observed in the earlier studies with microorganisms

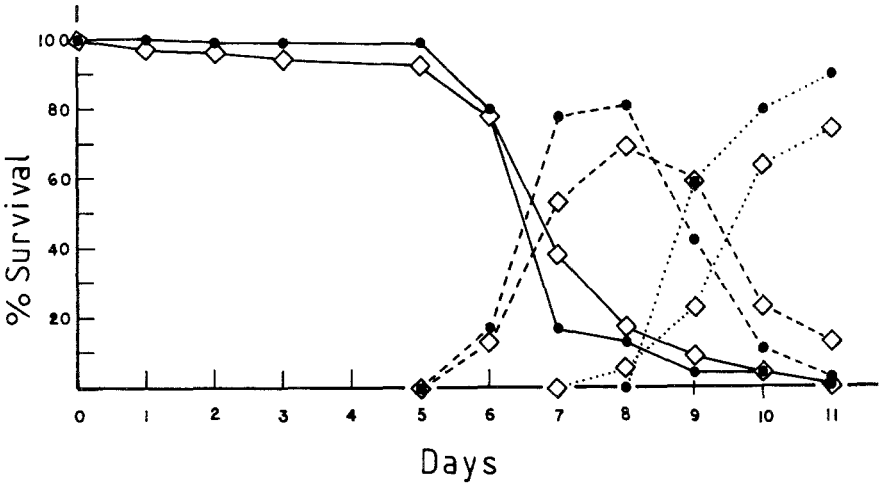


FIG. 5. Experiments with 6.7 ppm of khellin: larvae (solid lines), pupae (broken lines), and adults (dotted lines) obtained from first-instar *A. aegypti*, as a function of time. The survival of the dark controls (solid circles) was identical to that of the organisms in a similar solution which had been previously irradiated (not shown). The irradiated organisms are represented with the open squares. See text for details.

and viruses, could be the results of one or more of the following circumstances: (1) presence of phototoxic impurities in the samples, (2) strong dependence on the wavelength distribution of the irradiating source, and (3) strong dependence on the intensity of the source. With respect to the first hypothesis, it may be relevant to note that the seeds of *A. visnaga*, which contain khellin, and those of *A. majus*, which contain 8-MOP, cannot be unequivocally distinguished by their morphological characters. Only by germinating the seeds can their source be confirmed. While we do not have enough information for providing a specific conclusion, one must note that khellin appears to show a specificity in its photobiochemistry which is, to our knowledge, without precedent.

CONCLUSIONS

This paper illustrates a simple and convenient technique for observing phototoxic effects which are not immediately detectable. By following the development of the mosquito larvae from the first instar, rather than from later stages as was customary, one increases the opportunities either for seeing abnormalities or for gaining confidence that no phototoxic reactions are induced. The quantitative comparison of the phototoxicity for different compounds is greatly facilitated. For example, neither 8-MOP nor sphondin showed any immediate phototoxicity at concentrations of 6.7 ppm and below. The measurement of the

24-hr survival data for 6.7 ppm already produced different results (26% with 8-MOP vs. 4.2% with sphondin), but observations over a longer span (three days or more) clearly showed a greater survival of the larvae treated with sphondin than with 8-MOP. On the other hand, since no adults were produced from either experiments, both sensitizers could also have been viewed as having the same phototoxicity.

Toxicity assay of these compounds at 1.1 ppm shows equipotency at the time of the irradiations (100% survival), and possibly 24 hr later (72% and 61% survival for 8-MOP and sphondin, respectively). It is only afterwards that notable differences are detected, showing that only 3% of the organisms treated with 8-MOP are converted into adults, compared to 16% of those treated with sphondin. However, since low-level incandescent light was briefly used during the course of the experiments, the survival in both the dark controls and the irradiated organisms might have been slightly affected. We believe that the difference would have been insignificant, because of the short duration of the exposures and the low intensity of the lamps utilized.

It is interesting to note that the knowledge that a molecule can undergo photocycloaddition to DNA components is not sufficient for predicting that it will be an efficient photosensitizer in a complex biological organism, as witnessed here by the lack of photosensitization with khellin. While the mechanisms of the phototoxic reactions described in this article are completely unknown, the observation of fairly rapid results with 8-MOP and sphondin is consistent with processes which are not based on modifications of the genetic material. Oxygen-mediated reactions modifying membranes, proteins, or hormone systems via reactions with singlet oxygen, superoxide ion, or hydroxyl radicals are alternatives which deserve serious consideration. The slower results observed at lower concentrations would also be consistent with modifications of the genetic material, and 8-MOP is certainly known for exhibiting this duality of mechanisms, as reviewed in the Introduction. Perhaps oxygen-independent modifications of nucleic acids as well as oxygen-dependent biochemical changes occur in all cases. The latter, being lethal at higher concentrations, prevent the expression of the former ones, which would therefore be observable only at lower sensitizer concentrations.

Note Added in Proof: After presentation of these results on Dec. 12, 1984 at the Sympsoium on Insects and Photoactive Substances in Plants, during the National Meeting of the Entomological Society of America, other participants published a paper describing the effects of 8-methoxypsoralen and khellin on *A. atropalpus* (Philogene et al., 1985). Their results with khellin cannot be fully evaluated on the basis of the published data, but they appear to be quite different from those herein reported.

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Rösler, University of Maryland, called our attention to the problems associated with the identification of the seeds of *Ammi visnaga* and *A. majus*. The financial support by the Research Board of the University of Illinois at Chicago is gratefully acknowledged.

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DETECTION OF FUROCOUMARINS IN PLANTS AND PLANT PRODUCTS WITH AN ULTRASENSITIVE BIOLOGICAL PHOTOASSAY EMPLOYING A DNA-REPAIR-DEFICIENT BACTERIUM

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Abstract—The application of an ultrasensitive photobiological assay which detects photosensitizing furocoumarins with sensitivities as high as 1×10^{-11} g is discussed in relation to these molecules as phytoalexins. Examples of the utilization of this technique, verified by both HPLC and TLC, are the analyses of healthy and diseased celery and carrots, dry seeds, plant extracts and oils, and whole plants and leaves. The usefulness of this method in following the metabolic detoxification of furocoumarins is also illustrated. The extreme sensitivity of the test has permitted the detection, for the first time, of both 5-methoxypsoralen and 8-methoxypsoralen in fresh carrot roots.

Key Words—Furocoumarins, psoralen, 5-methoxypsoralen, 8-methoxypsoralen, phototoxicity, photobiological assay, celery, carrot, analysis, umbelliferous seeds.

INTRODUCTION

The relationship between photosensitizing furocoumarins in a number of plant families and their properties as phytoalexins has been of considerable interest and comment (Scheel et al., 1963; Hahlbrock et al., 1981; Beier et al., 1983, Beier and Oertli, 1983). It is among members of the Rutaceae and Umbelliferae that the greatest variety and highest concentrations of furocoumarins have been reported, although these chemicals do occur in other families.

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The occurrence and distribution of furocoumarins have been used in the chemotaxonomy of plants (Crowden et al., 1969; Heywood, 1971), although often only the presence or absence of these compounds has been used for classification. The absence, for example, of furocoumarins in carrot roots, although present in leaf wax (Stadler and Buser, 1984) has always seemed surprising. However, using new ultrasensitive biological photoassay, backed by HPLC analysis, we have since confirmed the presence of furocoumarins in carrots (Ceska, 1986). Furocoumarins increase in plants when they are subjected to stress. These stresses can take many forms, including exposure to UV radiation, changes in temperature, increases in metal ion content, and infection with bacteria or fungi (Beier et al., 1983). In parsley tissue culture cells the responses to stress are very fast, and the available evidence suggests that gene activation, followed by the synthesis of specific mRNA, precedes increases in enzymes associated with the synthesis of psoralen from umbelliferone (Hahlbrock et al., 1981). Psoralen, which can also be considered the representative furocoumarin molecule, is the precursor of the linear furocoumarins, 5-methoxypsoralen (5-MOP) and 8-methoxypsoralen (8-MOP). The structural formulas of key furocoumarins are illustrated in Figure 1.

The linear furocoumarins can form, in the presence of near UV (320–380 nm), both monoadducts with DNA and DNA interstrand cross-links; the angular furocoumarins such as angelicin can form only DNA monoadducts under normal circumstances (Ashwood-Smith and Grant, 1976). The consequences of these photoadditions to DNA are cell death and, in surviving cells, mutation, chromosome aberrations, and carcinogenicity in both animals and man (Ashwood-Smith et al., 1982; IARC, 1982). Berenbaum and Feeny (1981) have discussed the relative actions of angelicin and xanthotoxin in terms of toxicity to insects which feed on furocoumarin-containing plants. Ivie et al. (1983) and Ashwood-Smith et al. (1984) have both described insect larvae, the resistance of which to furocoumarins appears to be based on enzymatic detoxification.

In this paper we shall demonstrate how the application of an ultrasensitive and rapid photobiological assay for photosensitizing compounds can resolve problems of the occurrence, distribution, and metabolism of furocoumarins.

METHODS AND MATERIALS

The furocoumarins used in this study were carefully checked for purity by TLC, HPLC, and photobiological assay and were recrystallized from 95% ethanol before use. Stock solutions in methanol or ethanol were kept at 4°C in the dark. The sources for the furocoumarin standards have been described previously (Chaudhary et al., 1985). Conditions for both normal and reverse-phase HPLC are indicated in the figure legends together with details of tissue separation and preparation. TLC analysis was carried out on Merck K 60 silica

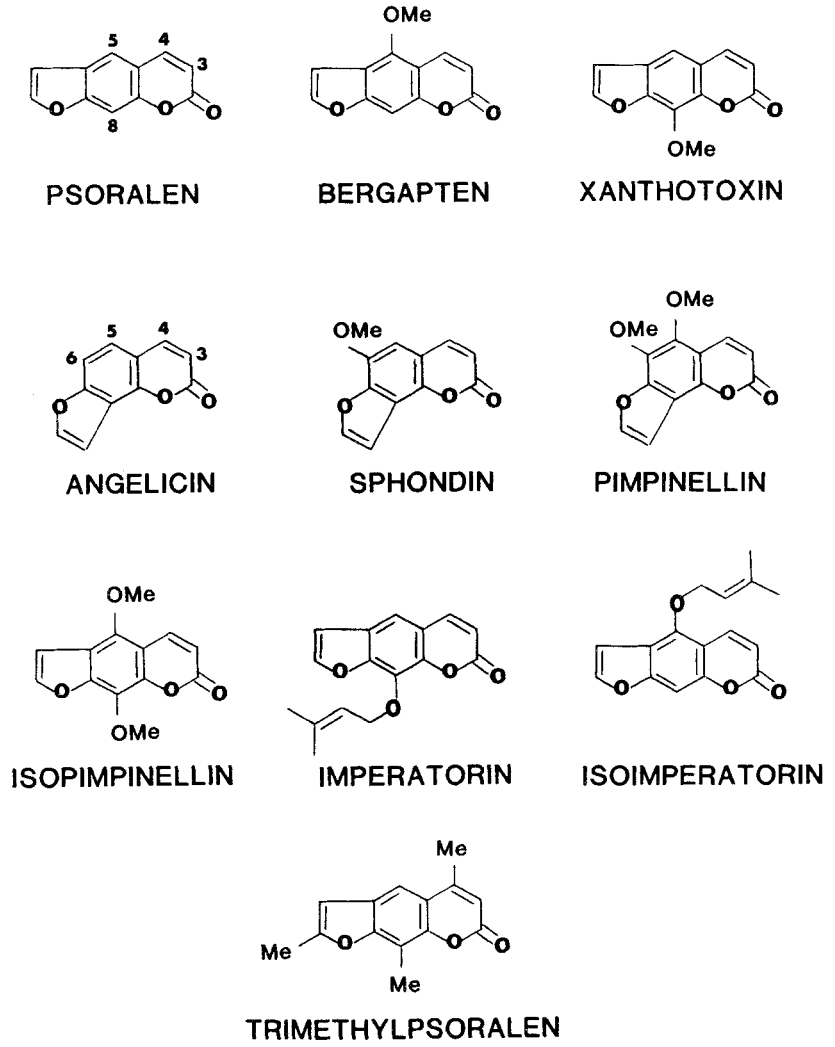

FIG. 1. Structure of furocoumarins; bergapten = 5-MOP; xanthotoxin = 8-MOP.

plates (without fluorescence indicator) and compounds, after development, were visualized with black light (300–380 nm).

Plant Materials. All vegetables and seeds were bought locally. Oil of bergamot was purchased from Sigma (St. Louis, Missouri), and the suntan preparation, Bergasol, specially formulated for the U.K. market and containing no 5-MOP was purchased in England.

Photobiological Assays. The detection of very small amounts of photosensitizing substances in plants, plant extracts, or plant products was carried out utilizing a photobiological assay introduced by Ashwood-Smith et al. (1983). The DNA-repair-deficient mutant *E. coli* B_{s-1} (rec^+, exr^-, hrc^-), which is extremely sensitive to ultraviolet radiation and to chemical alkylating agents (Igali et al., 1970) was grown to about 10^8/ml at 37°C in brain–heart infusion broth. This culture was used in the soft agar overlay which was prepared by dissolving 10 g of bacto-tryptone, 6 g bacto-agar, and 5 g of KCl in 1000 ml of distilled water. Square Petri dishes contained, as a base, commercial brain–heart infusion agar. Dried and developed TLCs were imprinted for 30 min onto the surface of the Petri dishes prior to the addition of the soft agar overlay (3.5 ml + 0.1 ml of the overnight bacterial culture). The seeds and plants themselves were added to the surface of the overlay after it had been poured onto the base agar. The complete details of these procedures are given by Ashwood-Smith et al. (1983).

TLC and HPLC Analysis. Merck precoated silica gel K 60 sheets were used (without fluorescence indicator), and two dimensional chromatograms were developed first with chloroform and second with hexane–pentane–ethyl acetate (35:35:30). Visualization was performed with near UV (300–380 nm). Samples for HPLC were processed as described by Chaudhary et al. (1985) after preliminary separation first through C18 Sep-Pak and then through Silica Sep-Pak cartridges. Normal phase analysis followed, in essence, the technique described by Berenbaum et al. (1984). Further experimental details are contained in the appropriate figure legends.

Enzymatic Detoxification of Furocoumarins. Both 5-MOP and trimethylpsoralen dissolved in methanol were added at a concentration of 5 μg/ml to a reaction mixture containing rat liver microsomes, NADP, and glucose-6-phosphate (Ashwood-Smith et al., 1984). The reaction was stopped after various incubation times at 37°C by the addition of an equal volume of ethyl acetate. After shaking, the ethyl acetate was spotted onto silica plates before one-dimensional TLC in hexane–pentane–ethyl acetate (35:35:30). After the TLC was dry, the normal imprinting procedure was followed by photobiological assay. Part of the top of the TLC was cut away before imprinting in order to fit into the square Petri dish (no active molecules were associated with this portion). The R_f for trimethylpsoralen was 0.58 and for 5-MOP, 0.43.

RESULTS AND DISCUSSION

Photosensitivity of E. coli B_s. It is important to check the sensitivity of the repair-deficient bacterium every week or so; otherwise contamination with a resistant wild-type microorganism could occur and decrease the inherent sensitivity of the assay procedures. The effect of irradiating two cultures streaked

onto the surface of a nutrient agar Petri dish is illustrated in Figure 2. Approximately 150 J/m^2 of UV (Mineralight UV S · 12 253.7 nm distributed by Fisher Scientific Ltd.) resulted in a complete inhibition of growth of the indicator bacterium and little apparent effect on the wild-type *E. coli B/r* (Ashwood-Smith et al., 1983).

Although no experiments are reported in this paper concerning the photosensitizing actions of non-DNA damaging agents such as polyacetylenes and α-terthienyl, the use of these two *E. coli* strains separately in photobiological assays can be very revealing. If the sensitivities with both bacterial strains is the same for any unknown chemical, then it may be inferred that the biological action is some target other than DNA, perhaps proteins or lipids. If there is a large difference, in that *E. coli* B_s is much the more sensitive of the two indicator strains, perhaps by a factor as large as 10,000, then it may be concluded that DNA is the primary target (Ashwood-Smith et al., 1983).

Analysis of Furocoumarins in Celery and Carrot Plants. The results of analyzing fresh, nondiseased celery plants by both normal and reverse-phase

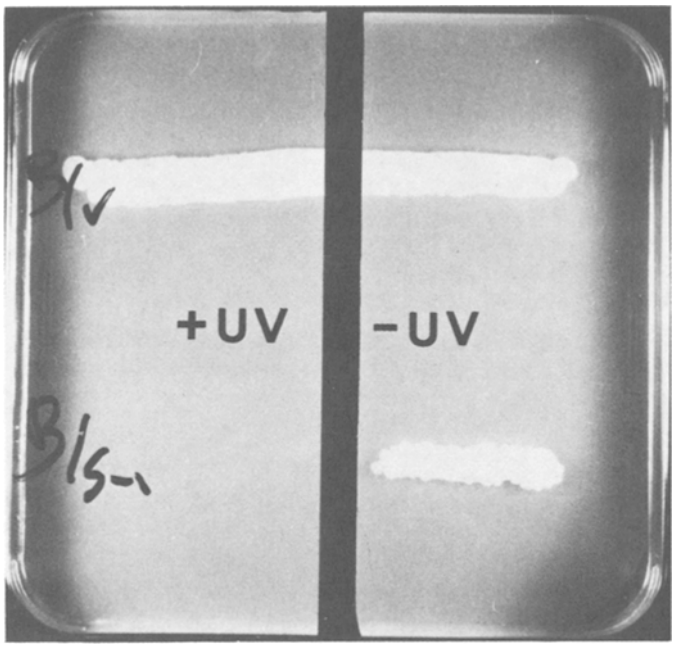

FIG. 2. Photosensitivity of *E. coli* B_s and *E. coli B/r*. Logarithmically growing cultures of the two bacteria (at 37°C in brain–heart infusion broth) were streaked onto the surface of nutrient agar plates and incubated for 24 hr at 37°C after a brief exposure to germicidal ultraviolet radiation (253.7 nm; 150 J/m^2). Half the plate was protected from the radiation.

HPLC are shown in Figure 3. The separation of psoralen, 5-MOP, and 8-MOP is good and was sufficient to demonstrate (Figure 4) the phytoalexin response of diseased celery (Ashwood-Smith et al., 1985). Results after photobiological assay of fresh and diseased celery are illustrated in Figure 5. The increase in furocoumarins, shown in diseased plants, of about 30-fold (Ashwood-Smith et

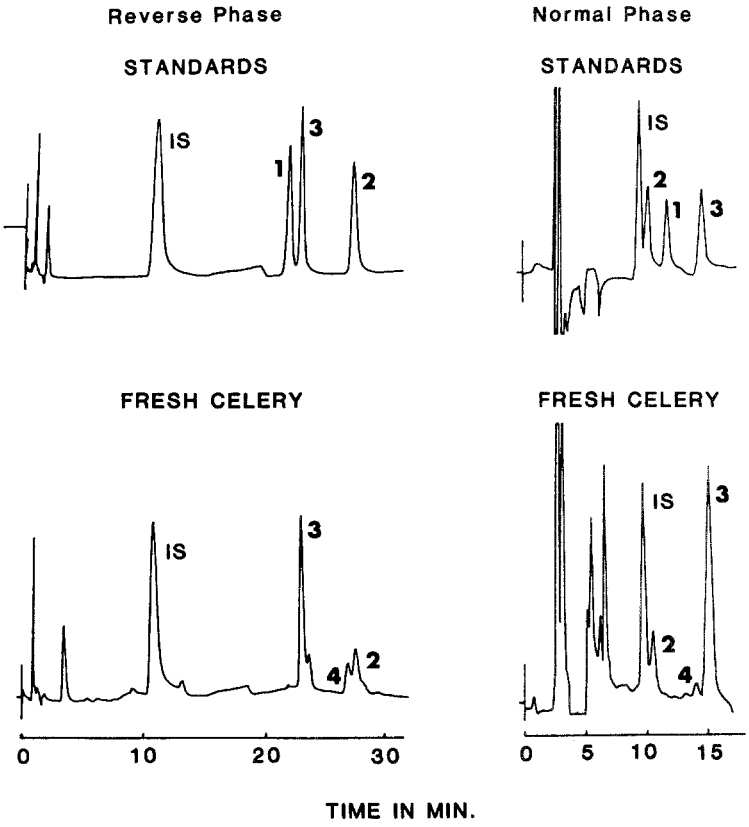

FIG. 3. Normal and reverse-phase HPLC analysis of healthy celery plants. Reverse-phase analysis was performed with a Varian MCH-10 column (4 mm × 30 cm) employing acetonitrile–water as a solvent commencing at 25:75 and remaining constant for 15 min before changing over a period of 5 min to 35:65 and remaining thus for 10 min. The flow rate was 2 ml/min. For normal phase analysis, a Varian Si-5 column (4 mm × 30 cm) was used with a solvent consisting of cyclohexane–isopropylether–*n*-amyl alcohol (15:4:0.5) with a flow rate of 1 ml/min. Detection for both methods was at 254 nm, and quantitation was done by peak area integration with a Hewlett-Packard 3390A integrator. All samples contained coumarin as an internal standard (IS) (30 μg/ml) and were injected as samples of 10 μl. The furocoumarin standards were used at 5 μg/ml. 1 = psoralen, 2 = 5-MOP, 3 = 8-MOP, 4 = isopimpinellin.

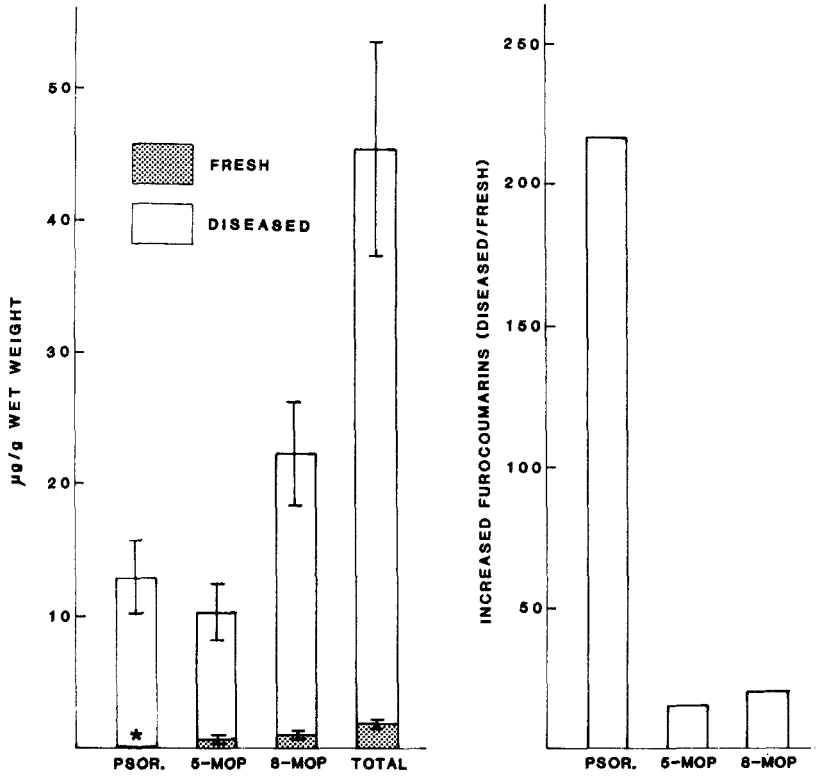

FIG. 4. Total and individual furocoumarin content of fresh and diseased celery. Samples were extracted with ethyl acetate and worked up according to the procedures recently published by Beier et al. (1983) and Chaudhary et al. (1985). Concentrations of furocoumarins are given ±SEM in µg/g wet weight. The control values for psoralen (*) were in a number of cases below reasonable detection limits. The analyses were carried out on 22 fresh samples and 12 diseased samples (in most instances the celery was infected with the fungus, *Sclerotinia sclerotiorum*). Tests for probability (*P*) by the Student's *t* test gave values of *P* greater than 0.001 for all comparisons. The right-hand graph illustrates increases, as ratios of diseased to fresh.

al., 1985) was sufficient to produce a very large area of bacterial kill in the indicator lawn (Figure 5B).

When fresh carrot plants were analyzed, 5-MOP and 8-MOP were, for the first time, shown to be present as constituents of the root. There has been one report (Stadler and Buser, 1984) of 5-MOP and 8-MOP being found in the outer wax of the carrot leaves. Analysis of diseased carrot (the fungal pathogen was not identified) which had been stored for several weeks in a dark, cool basement indicated that psoralen was present at a concentration of 0.87 µg/g, and 8-MOP

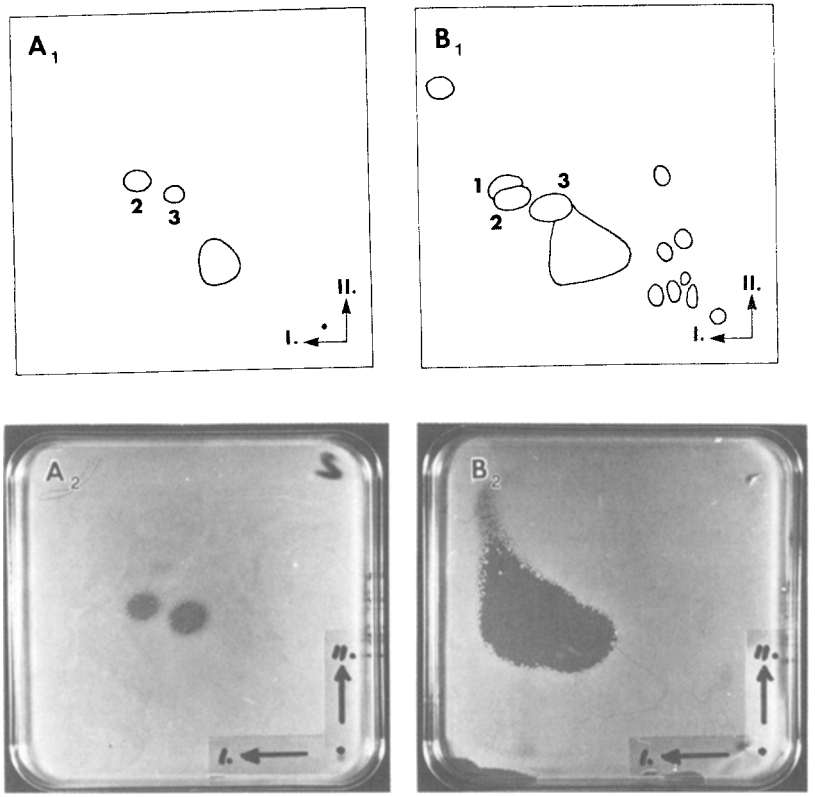

FIG. 5. Photobiological assay of fresh and diseased celery. Top left and top right illustrate the spots revealed by UV radiation (300–380 nm) after two-dimensional TLC of fresh and diseased celery, respectively. Bottom left and bottom right are imprints of the developed TLCs assayed photobiologically. The position of the photoactive spots is seen by the dark areas which, under the lighting conditions used, represent zones of no bacterial growth in an otherwise virgin lawn. Both samples are equivalent to 100 mg of celery (wet weight). 1 = psoralen, 2 = 5-MOP, 3 = 8-MOP.

at 1.55 μg/g; values are for wet weight. 5-MOP levels were also elevated, but HPLC analysis was hindered, even after purification by Sep-Pak, by the presence of other substances eluted from the diseased carrots in the same time interval. The levels of furocoumarins in fresh carrots were below the detectable limits of our HPLC methodology but were clearly seen in the photobiological assay (Figure 6). Only approximations were appropriate for estimation, by comparisons with standards and a knowledge of the sensitivity of *E. coli* B_s: values of about 1×10^{-8} g for both 5- and 8-MOP were obtained (Ashwood-Smith et al., 1983).

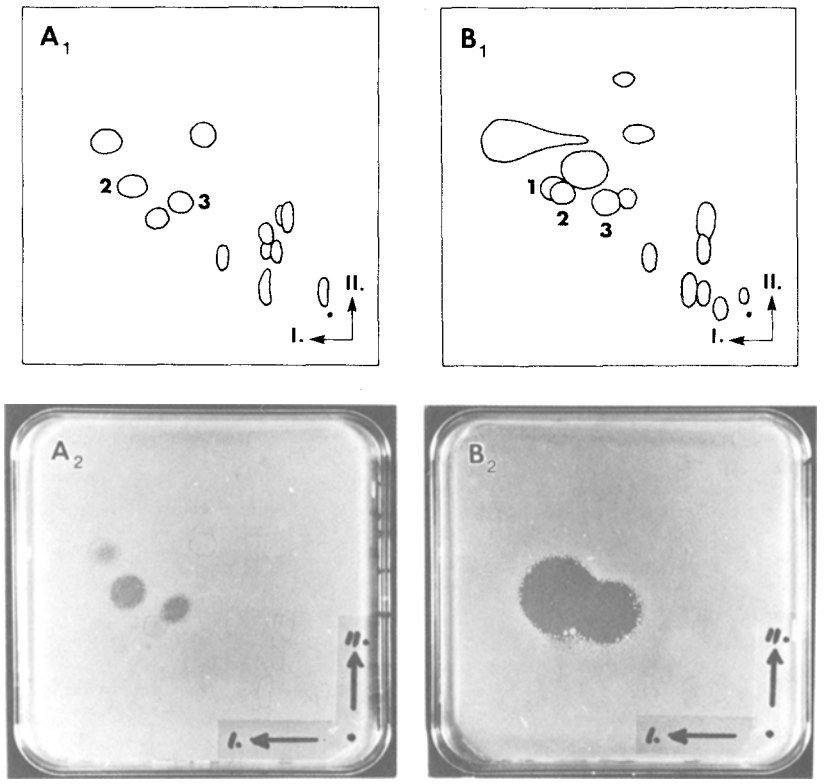

FIG. 6. Photobiological assay of fresh and diseased carrot. Details as for legend to Figure 5. Top left and bottom left are from fresh, healthy carrot; top right and bottom right from diseased carrot. Sample A, fresh carrot, represents 510 mg (wet weight) and sample B, disease carrot, 130 mg. 1 = psoralen, 2 = 5-MOP, 3 = 8-MOP.

Furocoumarin Content in Various Seeds of Umbelliferae. A number of vegetable and herb seeds were analyzed directly by photobiological assay and typical results are shown in Figure 7. Photosensitizing activity was seen with the seeds of *Angelica archangelica* L. (Figures 7, A5 and D6), two varieties of parsnip, *Pastinaca sativa* L. (Figures 7, C3 and C4), and slight activity with two different varieties of celeriac, *Apium graveolens* L. var. *rapaceum* (Miller) D.C. (Figures 7, D4 and D5). Seeds of four varieties of carrot, *Daucus carota* L. (Figures 7, A1–4), coriander, *Coriandrum sativum* L. (Figure 7, C1), anise, *Pimpinella anisum* L. (Figure 7, C2), vegetable fennel, *Foeniculum vulgare*

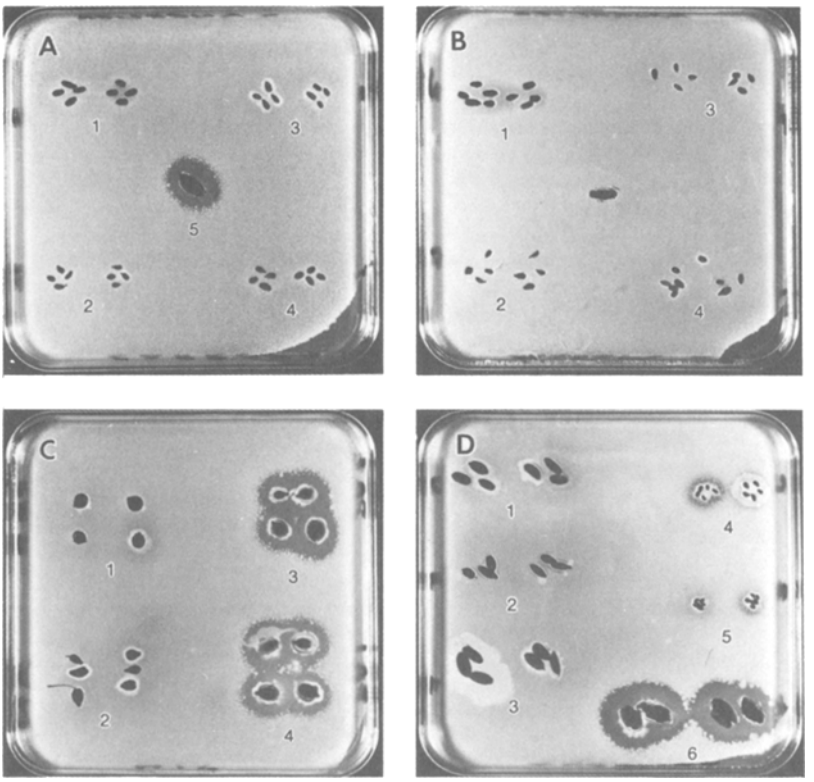

FIG. 7. Photobiological assay of various vegetable and herb seeds of the Umbelliferae. (A) Four varieties of carrot seed 1–4, exposed to near UV, showing no photobiological activity. The positive activity of one seed of *Angelica archangelica* is seen in the center. (B) As for (A) but without exposure to near UV; this plate is the negative control. (C) Seeds of coriander, 1; anise, 2; and two varieties of parsnip, 3 and 4; exposed to near UV. (D) Seeds of vegetable fennel, 1; coumin, 2; green fennel, 3; celeriac, 4 and 5; and angelica, 6; exposed to near UV. Negative controls were done in all experiments but are shown only in (B).

Miller var. *azoricum* (Miller) Thell. (Figure 7, D1), herb fennel, *F. vulgare* var. *vulgare* (Figure 7, D3) and cumin, *Cuminum cyminum* L. (Figure 7, D2) were negative. All seeds were also analyzed in the absence of near-UV radiation and showed no activity with the exception of a very small zone of growth inhibition with one variety of carrot seed (Figure 7, B1). However, for the sake of brevity, only one control plate is illustrated (Figure 7B).

The concentration of furocoumarins per seed was in some instances too low to be measured directly, and seed extracts had to be prepared. In Figure 8 the presence of photobiological activity in extracts from five varieties of parsley seeds, *Petroselinum crispum* (Mill.) A.W. Hill is shown (Figures 8, A1–5). Four seeds that gave negative results when analyzed directly gave weak positive results when extracts were used. These included cumin (Figure 8, B1), green fennel (Figure 8, B2), coriander (Figure 8, B3) and vegetable fennel (Figure 8, B4), together with the strong reaction to the seeds of *A. archangelica* (Figure 8, B5). Carrot seeds sometimes gave positive results but were equivocal. All analyses performed in the dark were negative.

The furocoumarin patterns of celery, *Apium graveolens* L. var. *dulce* (Miller) D.C., and parsnip seed extracts are illustrated in Figure 9A and 9B, respectively. Celery seed had a much lower furocoumarin content than parsnip seed and thus 9A represents 50 mg dry weight and 9B (parsnip) 10 μg weight.

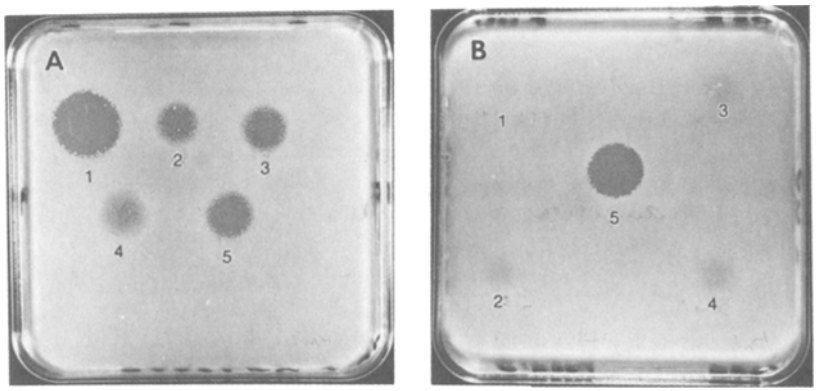

FIG. 8. Photobiological assay of seed extracts. (A) 1–5 are various varieties of parsley equivalent to 50 mg. (B) 1, cumin, 50 mg; 2, green fennel, 50 mg; 3, coriander, 50 mg; 4, vegetable fennel, 50 mg; 5, *A. archangelica*, 2 mg. All samples are tested as spots on silica TLC plates.

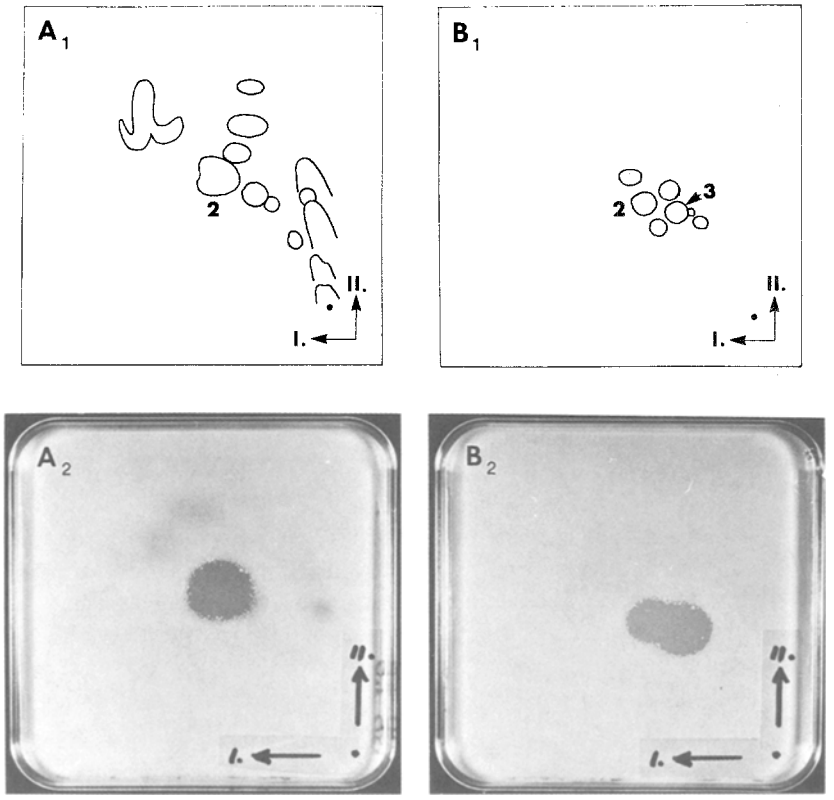

FIG. 9. Photobiological assay of celery and parsnip seed extracts. (A) celery (50 mg); (B) parsnip (10 μg). 2 = 5-MOP, 3 = 8-MOP.

Both seeds contained 5-MOP, but 8-MOP was only seen in parsnips. In addition, three and perhaps four other active molecules were present in celery seeds. Imperatorin was detected in parsnips by HPLC and TLC (compared with a standard). It is known to be phototoxic systemically (Ljunggren, 1977; Ljunggren and Möller, 1978) but not topically (Musajo, 1955); it was photoactive in our bioassay. However, as equivalent amounts of these two seeds were not used, the absence of a particular molecule could not be firmly established.

Application of Photobiological Assay to Detection of Photosensitizing Substances. Illustrated in Figure 10 are the results of the bioassay of extracts made from two rutaceous plants, *Thamnosma montana* Torr. et Frem. and *Skimmia japonica* Thumb. *Thamnosma montana* and *Skimmia laureola* Sieb. et Zucc. ex Walp, are known to cause skin photosensitivity upon contact (Michell and Rook, 1979). The presence of 5-MOP and 8-MOP plus two other unidentified compounds is clearly seen in *T. montana* extracts (Figure 10A, dried stem). The

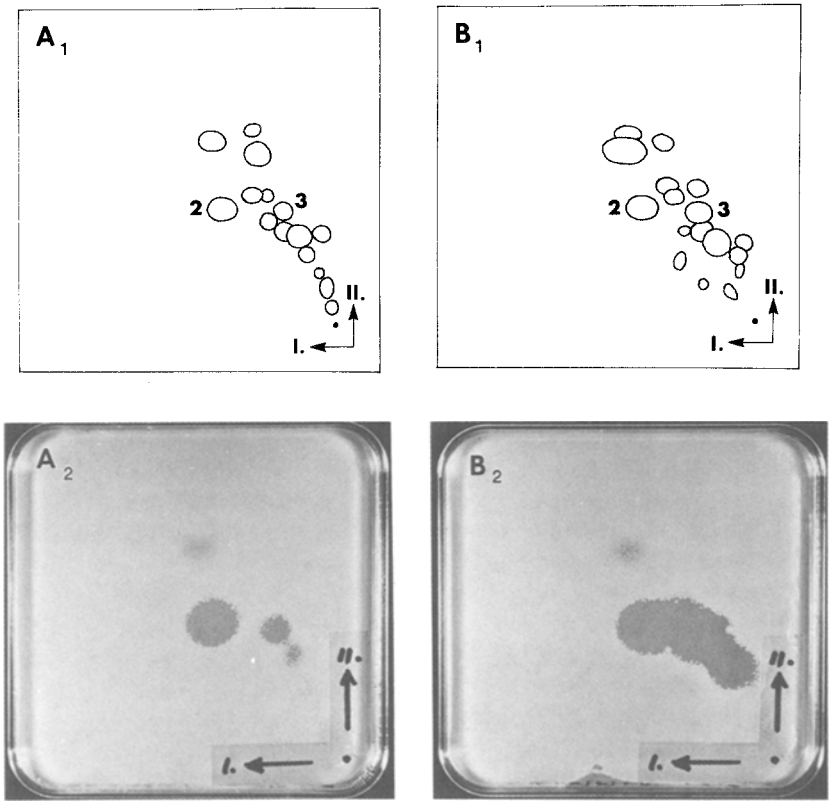

FIG. 10. Photobiological assay of crude plant extracts. (A) Chloroform extract of *Skimmia japonica* fresh leaf representing 1.35 mg wet weight. (B) Chloroform extract of *Thamnosma montana*. This was a two-year-old herbarium specimen representing 0.85 mg dry weight. The chromatographic procedures were as indicated in the legend for Figure 5 and the text. 2 = 5-MOP, 3 = 8-MOP.

fresh leaves of *S. japonica* (Figure 10B) contain 5- and 8-MOP plus four other unidentified photosensitizing molecules.

It is of some interest that the analysis of *T. montana* was performed on a two-year-old dry herbarium sample. The retrospective investigation of furocoumarin content is thus possible provided that herbarium samples have not been treated with chemicals to prevent decomposition and which may, therefore, interfere with the photobiological detection by killing the indicator bacterium.

Several plant oils and one cosmetic product were analyzed photobiologically after two dimensional TLC, and these results are given in Figure 11. Oil of bergamot before and after steam distillation is illustrated in Figure 11A and 11B, respectively, and shows the large reduction in 5-MOP content; dime-

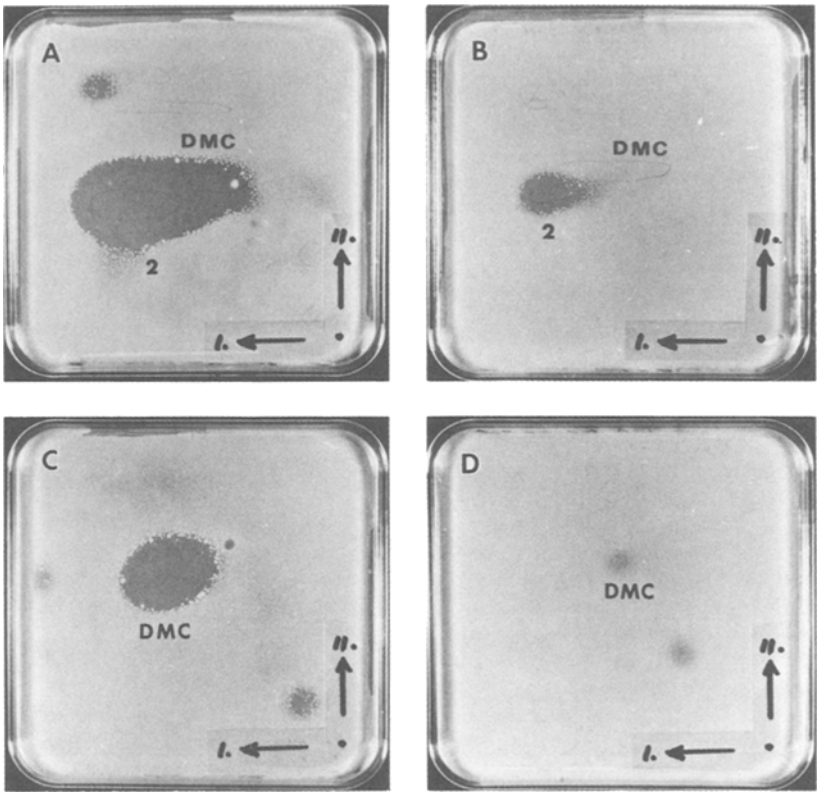

FIG. 11. Photobiological assay of plant oils and suntan lotion. (A) Oil of bergamot (crude); 5 μl. (B) Oil of bergamot (after steam distillation); 5 μl. (C) Refined oil of lemon; 10 μl. (D) Bergasol suntan oil, U.K., 1984 preparation made with citrus essence; 10 μl. The two dimensional TLCs were standard. The inhibition zones in oil of bergamot are due to 5-MOP and dimethoxycoumarin (DMC). Steam-distilled bergamot oil has a low 5-MOP content. Lemon oil contains large quantities of DMC plus two other unidentified components. Bergasol contains DMC plus one other photoactive chemical. There is no 5-MOP, a photocarcinogen found in this suntan oil, prior to changes in the U.K. formulation in 1982. 2 = 5-MOP.

thoxycoumarin is still detectable. An unidentified compound was removed by the steam distillation process. Analysis of lemon oil (10 μl) showed two unknown compounds plus a large amount of dimethoxycoumarin. The usefulness of this assay procedure to investigate cosmetics is demonstrated in Figure 11D in which the results of analyzing a commercial suntan preparation are shown. In this instance 10 μl of Bergasol (UK formulation 1984 with added lemon oil

and minus oil of bergamot) revealed the presence of dimethoxycoumarin and one other active substance but no 5-MOP.

Detection of Photosensitizing Compounds in Plants. The ease with which the presence or absence and, to some extent, the amount and spatial distribution of photosensitizing compounds can be detected in living plants is illustrated in two examples shown in Figures 12 and 13. Two 12- to 13-day-old parsnip seedlings were gently applied to the top surface of the soft agar and analyzed. Figure 12A is the result of the standard exposure to near-UV radiation and 12B the control which received no near-UV radiation. The diffusion of furocoumarins from all parts of the seedlings is obvious. The biological effect of furocoumarins as phytoalexins is well illustrated in the second example, in which the leaves of *Skimmia japonica*, cut in several places (indicated in Figure 13 by

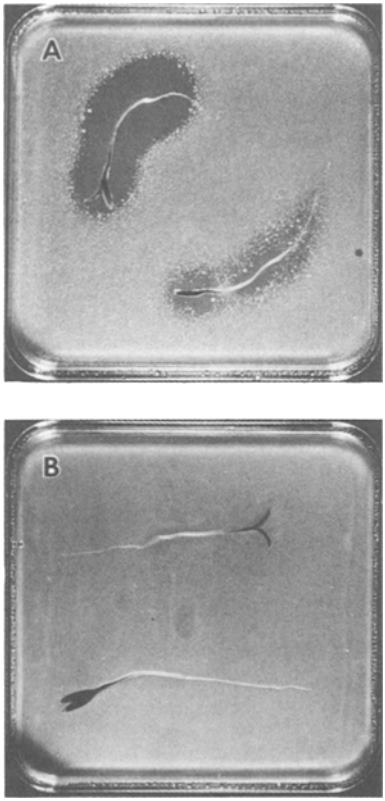

FIG. 12. Photobiological assay of parsnip seedlings. Parsnip seedlings (12–13 days old) assayed for furocoumarin activity (after surface sterilization with chlorine bleach) with (A) and without (B) exposure to near-UV radiation.

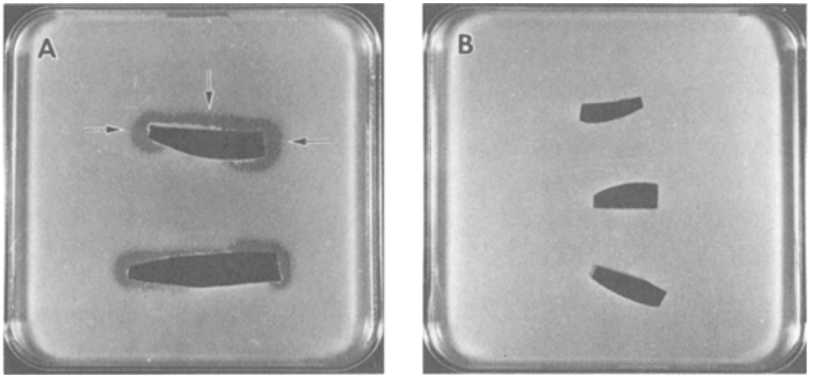

FIG. 13. Photobiological assay of a fresh leaf of *Skimmia japonica* showing effect of deliberate injury (cutting). The inhibition of bacterial growth associated with the cut edges (arrows) is clearly seen indicating the leakage of photoactive chemicals. Left photograph illustrates leaf after exposure to near-UV; right photograph without exposure to near-UV.

arrows), are leaking furocoumarins into the surrounding agar and thus the greatest area of bacterial inhibition is associated with the cut surfaces.

Detection of Metabolites following Enzymatic Detoxification. Microsomal enzymes (mixed-function cytochrome oxidases, P-450 and P-448) from mammalian liver S-9 mix (Maron and Ames, 1983) rapidly inactivate furocoumarins when a suitable source of electrons (NADPH) is present. The changes in photoactivity can easily be followed by one-dimensional TLC of the reaction mixture as a function of time. Illustrated in Figure 14 is the metabolic detoxification

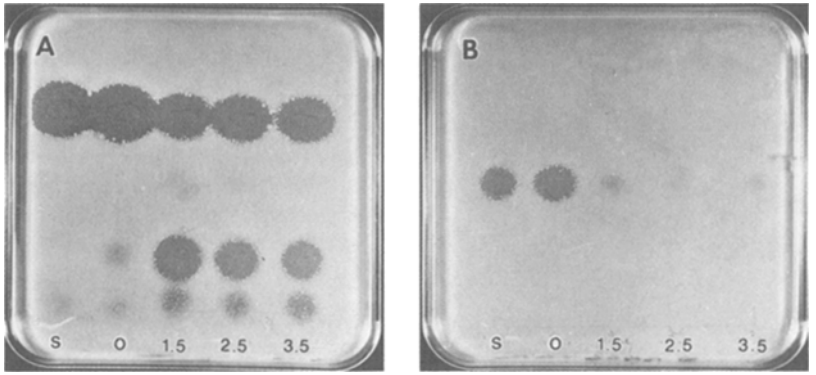

FIG. 14. Enzymatic detoxification of trimethylpsoralen and 5-MOP followed by photobiological assay. (A) Breakdown of trimethylpsoralen into two slower moving but active molecules. (B) Detoxification of 5-MOP and the disappearance of photosensitizing activity. Time is in hours of incubation.

of trimethylpsoralen (Figure 14A) and 5-MOP (Figure 14B). It will be noted that trimethylpsoralen is broken down into two other photoactive compounds in the course of its metabolism, while 5-MOP is detoxified without the formation of photoactive molecules. Other evidence (Ashwood-Smith, unpublished observations) indicates that psoralen, 8-MOP, and angelicin are also detoxified without the formation of active intermediates. The application of this methodology in studying the manner in which insects detoxify furocoumarins is apposite (Ashwood-Smith et al., 1984; Ivie et al., 1983).

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INACTIVATION AND MUTAGENESIS BY PHOTOTOXINS USING *Escherichia coli* STRAINS DIFFERING IN SENSITIVITY TO NEAR- AND FAR-ULTRAVIOLET LIGHT

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Abstract—Four *Escherichia coli* strains carrying all the possible combinations of genes controlling sensitivity to near-UV (NUV; *nur* versus *nur*$^+$) and far-UV (FUV; *uvrA6* versus *uvrA*$^+$) were inactivated with broad-spectrum NUV together with specific phototoxins. The inactivation kinetics of the four strains are consistent with the previous reports that psoralen and angelicin inactivation is based on the formation of DNA adducts, while xanthotoxin (8-MOP) inactivation is based on the combined effects of DNA adduct formation and oxygen-dependent photodynamic action. At sufficiently high NUV fluences, xanthotoxol (8-HOP) induces lethal DNA lesions in an excision-deficient (*uvrA6*) strain. Inactivation by alpha-terthienyl plus NUV involves strictly membrane damage since the genes controlling the sensitivity to either NUV or FUV have no effect on inactivation kinetics. Using mutation to histidine independence (*his-4*$^+$) in the presence of NUV as a measure of mutagenicity by phototoxins, psoralen and xanthotoxin are mutagenic, angelicin is less mutagenic, and xanthotoxol and alpha-terthienyl are not mutagenic. None of the phototoxins tested in the presence of NUV were as mutagenic as FUV. Imperatorin and berberine were neither phototoxic nor mutagenic in this assay system. This assay thus provides a rapid qualitative screening procedure to identify the mode of action and mutagenicity of plant phototoxins with potential insecticidal properties.

Key Words—*Escherichia coli*, alpha-terthienyl, angelicin, xanthotoxin, xanthotoxol, psoralen, histidine independence, mutagenesis.

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INTRODUCTION

Over the past 20 years cellular systems in *Escherichia coli* have been identified for the repair of DNA damage whether it is induced by far-UV (FUV; 200–290 nm), ionizing radiation, or chemicals (for reviews see Witkin, 1976; Walker, 1984). The systems which have been most thoroughly characterized are the excision and recombination repair systems, both of which are inducible (Radman, 1974; Kenyon and Walker, 1981). Mutants defective in DNA repair have been identified principally based upon their hypersensitivity to inactivation by radiation or chemicals. If a strain carrying a mutation in a repair pathway (e.g., *recA*, recombinational repair defective, or *uvrA*, excision repair defective) is sensitive to inactivation by a particular treatment, it can be assumed that the damage induced by the treatment is at the DNA level and is reparable in corresponding nonmutant strains (for review see Scott et al., 1976).

In addition to DNA repair by the recombination and excision systems, there exists in *E. coli* cells another repair system specific for oxidative damage (Demple and Halbrook, 1983) resulting from treatment of cells with H_2O_2 or near-UV (NUV). However, it remains controversial as to whether this repair system is inducible (Sammartano and Tuveson, 1985). As is the case for the excision and recombinational repair systems, mutants have been identified representing defects in the pathway for the repair of oxidative damage. *E. coli* strains with *xthA* mutations (exonuclease III defective) are sensitive to inactivation by H_2O_2 (Demple et al., 1983) and broad-spectrum NUV (Sammartano and Tuveson, 1983). The fact that inactivation by NUV is strongly oxygen-dependent (Peak et al., 1983) suggests that the damage induced by these wavelengths is oxidative and probably involves the generation of H_2O_2.

Another gene (*nur*; NUV resistance) sensitizes cells specifically to inactivation by NUV (Tuveson and Jonas, 1979), photodynamic action (acridine orange plus visible light) and sunlight (Tuveson and March, 1980). Significantly, the *nur* mutation sensitizes *E. coli* to inactivation by NUV, photodynamic action (presumably oxidative), or sunlight, independent of whether the strain carries mutations in either the recombinational (*recA1* or *recA13*) or excision repair systems (*uvrA6*; Tuveson and Jonas, 1979; Tuveson, 1980). Strains with the *nur* mutation are sensitive to inactivation by H_2O_2 (Sammartano, Tuveson and Davenport, in preparation), suggesting that the mutation determines sensitivity to oxidative damage. The identification of mutations which specifically sensitize *E. coli* cells to oxidative damage allowed for the construction of a set of strains responding differentially to DNA damage which is oxidative (presumably DNA base damage; Massie et al., 1972) and nonoxidative DNA damage (such as photoadducts). In this paper, we show that these *E. coli* strains exhibited the inactivation kinetics expected for phototoxins whose mechanisms of action have been deduced previously. These strains may thus be useful in determining the inactivating mechanisms which underlie previously uncharacter-

ized phototoxins. The possible mutagenicity of such phototoxins can be assessed since this set of strains carries a mutable auxotrophic marker as well (*his-4*).

METHODS AND MATERIALS

Bacterial Strains. The strains RT7h, RT8h, RT9h, and RT10h were derived from strains RT7, RT8, RT9, and RT10 (Tuveson, 1980) by P1 transduction of a Tn10 insertion located next to the *his-4* allele in strain AB1157 (Leonardo et al., 1984). The four strains have the following properties in common: F^-, *argA21, lysA22, malA1, str 104,* $\lambda^r\lambda^-$, *supE44, zee::Tn10, his-4.* The strains differ genetically as follows: RT7h, *thi-1, nur, uvrA6;* RT8h, *th-1*$^+$, *nur, uvrA*$^+$; RT9h, *th-1, nur*$^+$ *uvrA6*; and RT10h, *thi-1*$^+$, *nur*$^+$, *uvrA*$^+$.

Media. The complex medium used was Luria-Bertani broth (LB; Miller, 1972). The semienriched minimal medium (SEM) used to assess survival consisted of appropriately supplemented minimal A medium (Miller, 1972) plus casamino acids (Difco, 0.4 ml of a 10% solution per liter; Kato et al., 1977) solidified with 1.5% Bacto-agar (Difco). To detect histidine independent (*his-4*$^+$) mutants, cells were spread on each of three SEM plates lacking histidine. To assess plate mutants (Webb, 1978), unirradiated cells were spread on minimal A medium lacking histidine (but containing arginine and lysine) and SEM lacking histidine. For the calculation of *his-4*$^+$ mutants per survivor, net *his-4*$^+$ mutants in a cell population were assessed as the total *his-4*$^+$ mutants minus the *his-4*$^+$ plate mutants.

Chemicals. Berberine, xanthotoxin (8-methoxypsoralen, 8-MOP), and psoralen were purchased from the Sigma Chemical Company, St. Louis, Missouri. Alpha-terthienyl (α-T) was a gift from J.T. Arnason, Biology Department, Ottawa University, Ottawa, Ontario, Canada. Imperatorin and angelicin were obtained from Roth-Chemie (Nahrstedt, Germany; now Atomergic, Plainview, New York) and HRI Associates (Emeryville, California), respectively. Xanthotoxol (8-hydroxypsoralen; 8-HOP) was synthesized by E. Heininger and J. Sternberg using a modification of the procedure of Schönberg and Sina (1950).

Broad-Spectrum NUV-Fluence Response Curves in Presence of Presumptive Phototoxins. Cells were grown at 37°C with shaking in side-arm flasks (Belco) containing 50 ml of LB broth (Miller, 1972). Growth was monitored by measuring the change in absorbance with a Klett-Summerson colorimeter equipped with a red filter. A 5-ml aliquot of stationary-phase cells (2.5 hr after entering the transition from exponential to stationary growth phase) was removed, washed three times with saline, and diluted to approximately 5.0×10^8 cells/ml in cold saline (ice bath temperature) and placed in a 16 × 160-mm tube with a magnetic flea at the bottom. Appropriately diluted presumptive phototoxin in 10 ml of 95% ethanol was added to the cell suspension following

which a 1-ml aliquot was withdrawn and held in the dark as a check on light-independent toxicity. Viability of the cells in the aliquot held in the dark was assessed after all manipulations had been completed with the NUV-treated suspension.

The broad-spectrum NUV source is identical to that described previously (Tuveson and Jonas, 1979). Briefly, the source consisted of a parallel array of four lamps (General Electric, 40 W BLB, integral filter) which emit radiation between 313 and 425 nm, with a maximum emission at 350 nm. The fluence rate was approximately 30 W/m^2 as estimated using a UVX Digital Radiometer (UVP, Inc., San Gabriel, California). This fluence rate is three times the estimate previously published which had been made with a Blak-Ray UV intensity meter (model J-227; Ultraviolet Products, Inc., San Gabriel, California) equipped with a J221 long-wave UV sensor (Tuveson and Jonas, 1979). The long-wave UV sensor proved to be defective.

Bioassay for Phototoxins. Ashwood-Smith et al. (1983) have described a bioassay procedure for the detection of furocoumarins and other photosensitizers. We have used essentially the same procedure described by Ashwood-Smith et al. (1983) for carrying out the bioassay procedures with our strains. Two thin-layer chromatographic (TLC) plates (silica gel 0.25 mm, glass backed, Merck, Darmstadt, Germany) were spotted with 20 μl of psoralen (1 mg/ml) and 20 μl of α-T (1 mg/ml). The plates were placed in contact with LB agar in each of four Petri dishes for 2 min. Essentially, the phototoxins were replicated onto each of four plates of LB agar. The individual plates in the set of four were overlayed with 4 ml of LB soft agar (0.8%) containing about 10^5 cells/ml of one of the four *E. coli* strains (RT7h–RT10h). One set of four plates was placed in the incubator immediately, while the second set of four was exposed to 43.7 kJ of broad-spectrum NUV before being placed in the incubator.

RESULTS AND DISCUSSION

The four strains (RT7h, RT8h, RT9h, and RT10h) which carry all four possible combinations of genes controlling excision proficiency (*uvrA6* versus *uvrA*$^+$) and sensitivity to oxidative (photodynamic) damage (*nur* versus *nur*$^+$) can be used to distinguish between effects of phototoxins which result from oxygen-independent DNA photoadduct formation and photodynamic damage which is oxygen dependent. To establish that this is the case, it was necessary to inactivate the four strains with phototoxins for which the mechanism of lethal action has been established in other ways (Scott et al., 1976). As expected, the strains carrying the *uvrA6* mutation (RT7h and RT8h) were sensitive to inactivation by psoralen plus NUV, supporting the established fact that psoralen phototoxicity is based on cycloadditions to DNA (Figure 1). When these same strains were tested with xanthotoxin (8-MOP), three of the four strains proved to be sensitive to inactivation (RT7h, RT8h, and RT9h; Figure 2). This is the

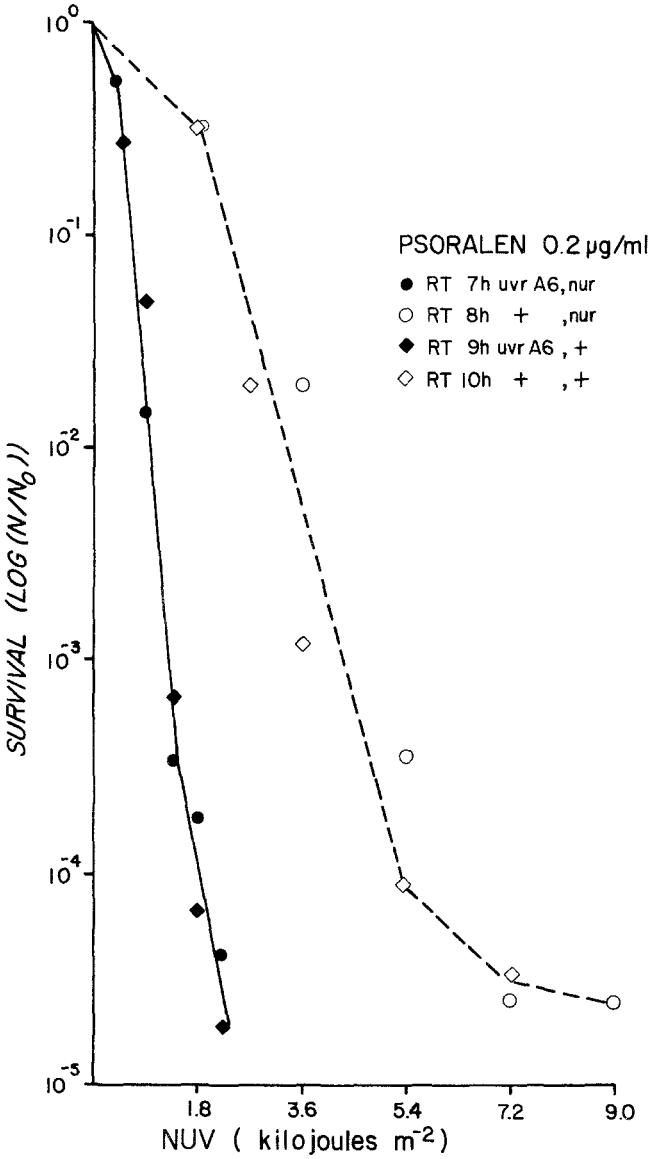


FIG. 1. Fluence–response curves for four *E. coli* strains treated with broad-spectrum NUV in the presence of psoralen.

result to be expected if xanthotoxin can act both as a classical photosensitizer and by DNA cycloadditions as has been suggested by Vedaldi et al. (1983). It should be noted that, although both psoralen and xanthotoxin were used at the rate of 0.2 µg/ml, the fluence of NUV required to obtain equivalent inactivation

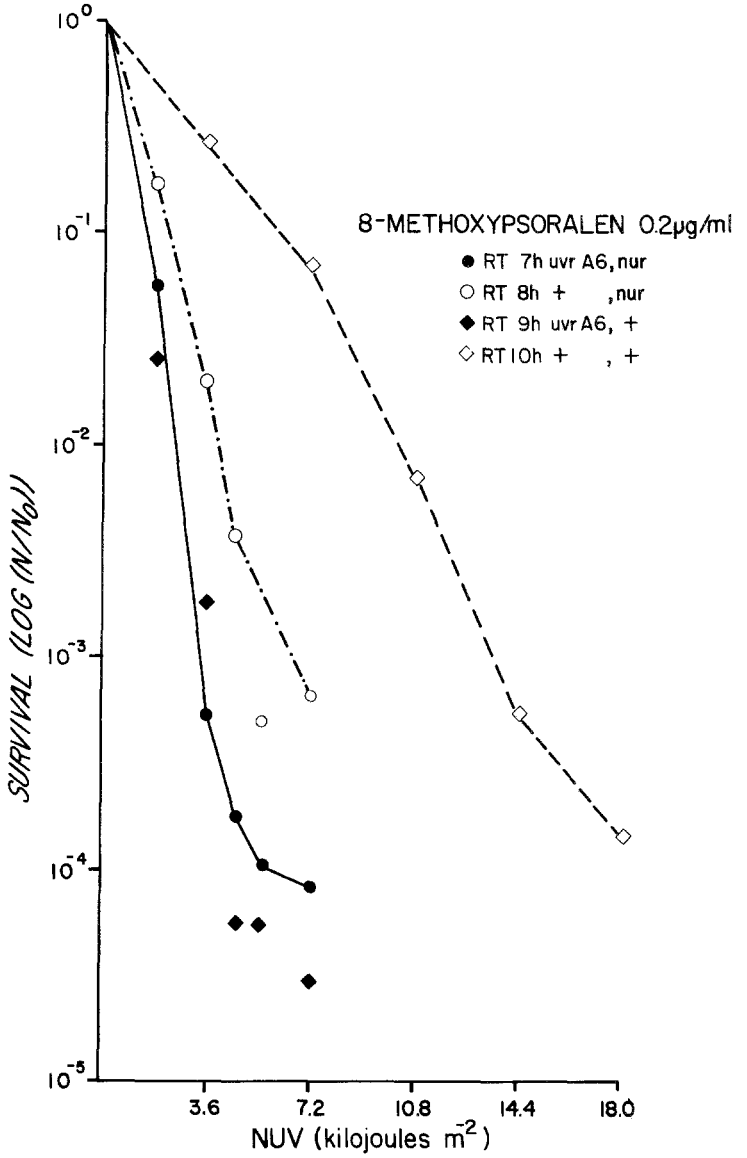


FIG. 2. Fluence–response curves for four *E. coli* strains treated with broad-spectrum NUV in the presence of 8-methoxypsoralen (xanthotoxin).

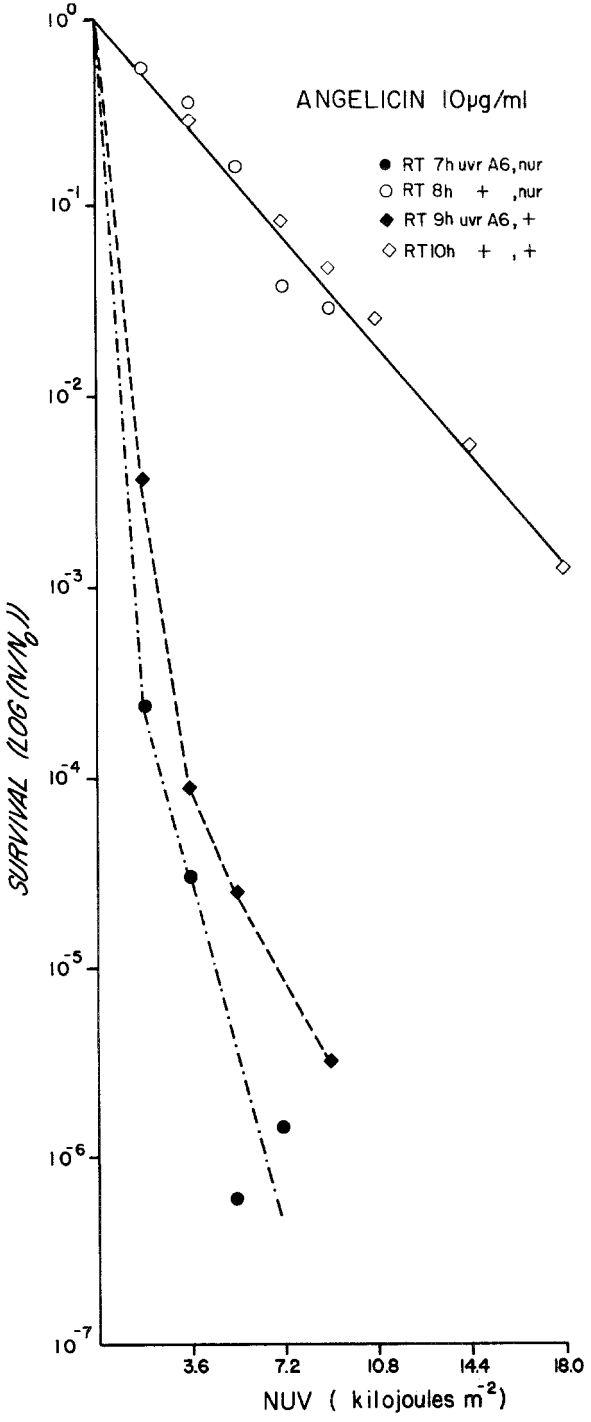
with xanthotoxin using the repair-proficient strain RT10h was greater than twice that required with psoralen. The reason for this difference is not apparent from these experiments since both chemicals produce cycloadditions to DNA.

When angelicin was used, the strains behaved as expected if its mode of

lethal action were based on monoadduct formation in DNA; the strains carrying the *uvrA6* mutation (RT7h, RT9h) were sensitive to inactivation by angelicin and NUV (Figure 3) (Scott et al., 1976). Consistent with the formation of monoadducts in DNA, the concentration of angelicin used in these experiments was five times that used with psoralen or xanthotoxin for equivalent toxicity, although the NUV fluences used were about the same with all three phototoxins. These results support the hypothesis that angelicin inactivates exclusively by monoadduct formation in DNA and does not act as a classical oxygen-dependent photosensitizer. The fact that angelicin inactivates by monoadduct formation in DNA is thought to reflect its angular configuration (Scott et al., 1976).

The addition of a hydroxyl group to the parent psoralen molecule at the 8 position (xanthotoxol or 8-hydroxyporalen) results in the loss of phototoxicity. We tested xanthotoxol as a phototoxin for the four strains used in our assay (RT7h–RT10h, Figure 4). The inactivation observed for three strains is that expected with NUV alone (Leonardo et al., 1984; Sammartano and Tuveson, 1984). The sensitivity of RT9h might be interpreted to mean that xanthotoxol at high NUV fluences (>18 kJ) induces damage which is reparable by the excision repair system (*uvrA*$^+$). However, in the RT7h strain, which also carries *uvrA6* mutation leading to excision deficiency, the NUV fluence tested was not sufficiently high to induce xanthotoxol excision-reparable lesions. The NUV sensitivity of the RT7h and RT8h is controlled by a gene (*nur*) which has been shown to be independent of either *recA* or *uvrA* mutations which sensitize cells to FUV (Tuveson and Jonas, 1979; Tuveson, 1980). We can speculate that, although RT7h carries the *uvrA6* mutation, it is not sensitive to xanthotoxol because the NUV fluences employed were insufficient to induce xanthotoxol lethal lesions but were more than sufficient to induce xanthotoxin lethal lesions (compare Figures 2 and 4).

When the four strains were treated with α-terthienyl (α-T) and NUV, the kinetics of inactivation for all the strains were indistinguishable (Figure 5). There is much evidence available suggesting that α-T acts as an oxygen-dependent (photodynamic) photosensitizer (Arnason et al., 1981), the principal lethal target for which is the membrane (Downum et al., 1982). Photochemical experiments with hydrophobic thiophenes, including α-T, suggest that these compounds, when irradiated with NUV, produce $^1O_2$ which might be expected to interact with membrane components (proteins and unsaturated fatty acids) to form peroxides and hydroperoxides that alter the hydrophobic character of the membrane, leading to leakage of cellular components and cell death (Reyftmann et al., 1985). The inactivation kinetics observed with our four strains are consistent with the membrane being the principal lethal target when *E. coli* cells are treated with α-T and NUV, since the mutations controlling NUV (*nur* versus *nur*$^+$) or FUV (*uvrA6* versus *uvrA*$^+$) sensitivity do not influence the inactivation kinetics. It has been shown that the *nur* gene sensitizes *E. coli* to photodynamic action when cells are treated with acridine orange plus visible light (Tuveson


ANGELICIN 10µg/ml
RT 7h uvr A6, nur
RT 8h + , nur
RT 9h uvr A6, +
RT 10h + , +
SURVIVAL (LOG(N/N0))
NUV (kilojoules m-2)
3.6
7.2
10.8
14.4
18.0

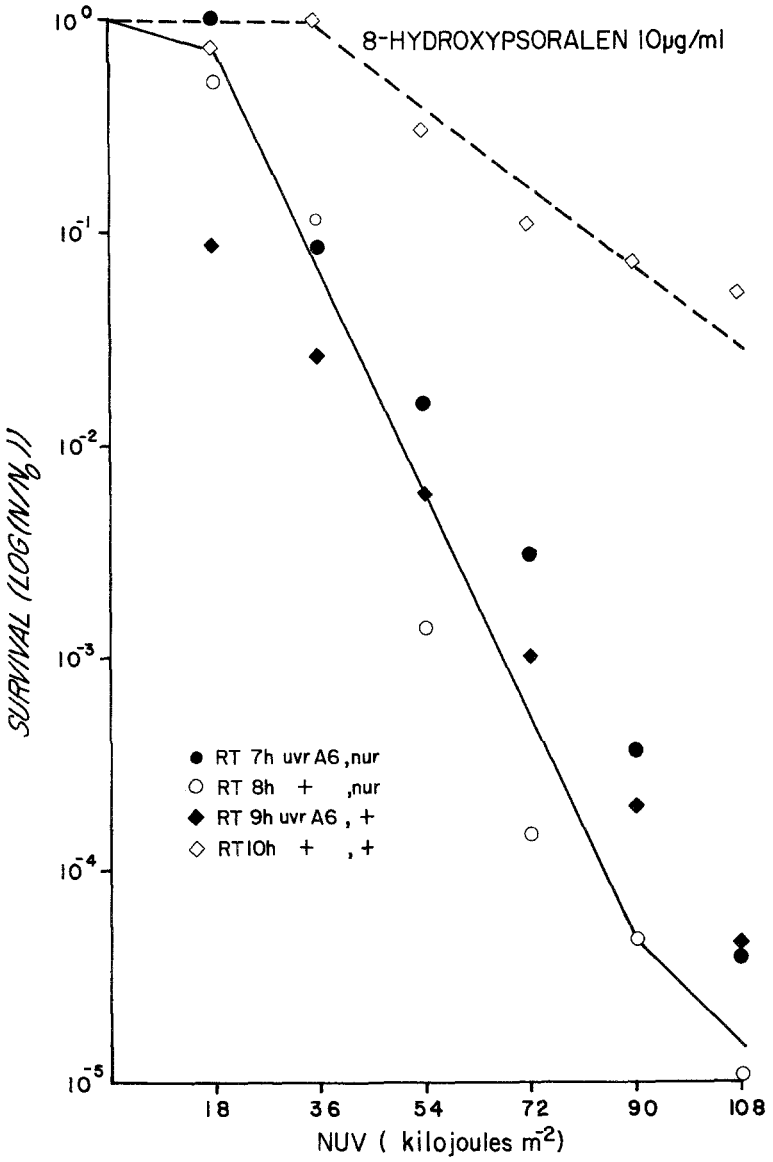


FIG. 4. Fluence–response curves for four *E. coli* strains treated with broad-spectrum NUV in the presence of 8-hydropsoralen (xanthotoxol).

FIG. 3. Fluence–response curves for four *E. coli* strains treated with broad-spectrum NUV in the presence of angelicin.

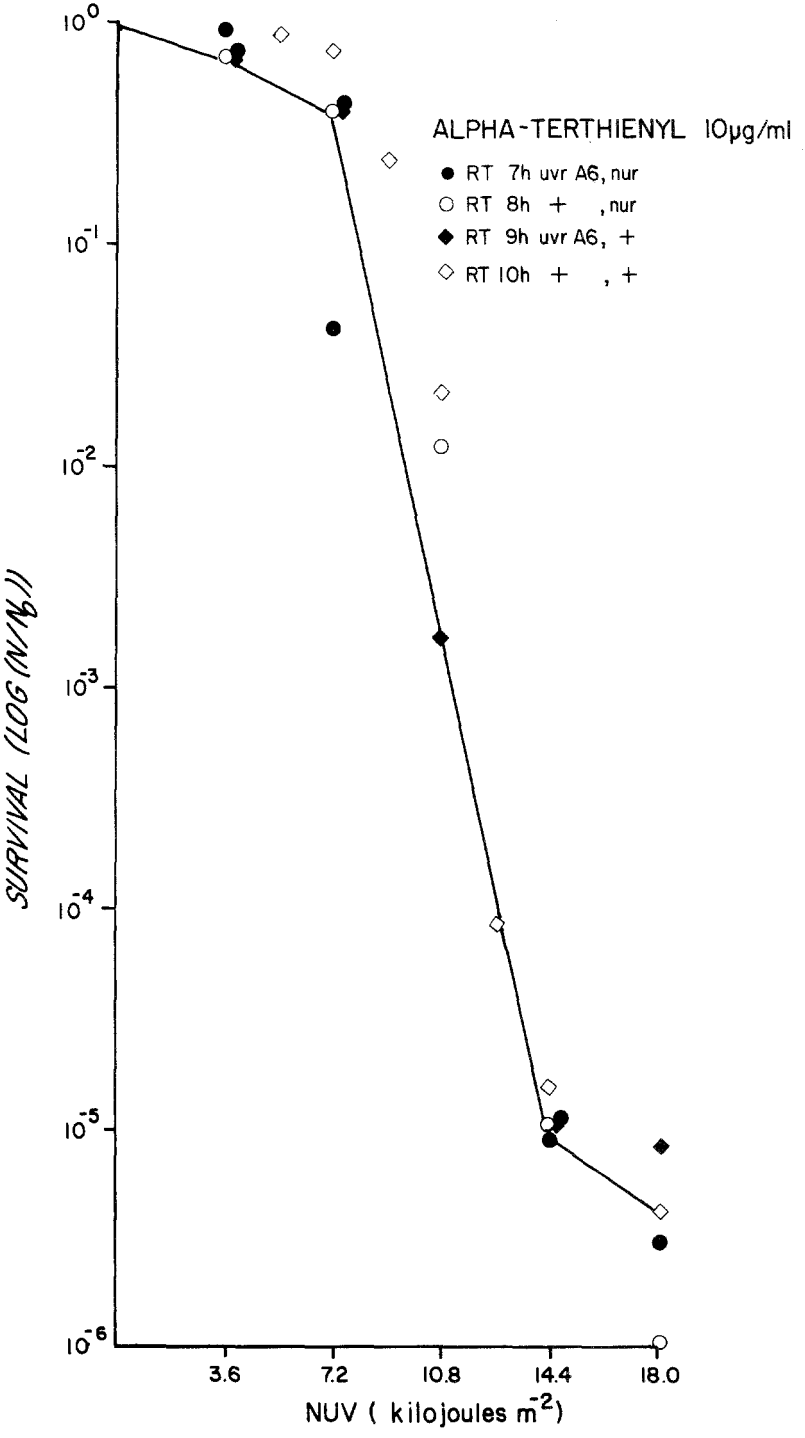

ALPHA-TERTHIENYL 10µg/ml
RT 7h uvr A6, nur
RT 8h + , nur
RT 9h uvr A6, +
RT 10h + , +
SURVIVAL (LOG (N/N0))
NUV (kilojoules m-2)
3.6
7.2
10.8
14.4
18.0

TABLE 1. PROPERTIES OF PHOTOTOXINS INVESTIGATED WITH *Escherichia coli* STRAINS RT7H, 8H, 9H, AND 10H

Phototoxin[a]	Mutagenicity	Concentration (μg/ml) used for inactivation experiments (conc./maximum conc. used)	F_{37} kilojoules for strain RT10h[a] (F_{37}/maximum F_{37})
Psoralen (Ps)	+	0.2 (0.02)	1.62 (0.03)
Xanthotoxin (8-methoxypsoralen, 8-MOP)	+	0.2 (0.02)	3.24 (0.60)
Angelicin (An)	+	10.0 (1.0)	2.70 (0.05)
Xanthotoxol (8-hydroxypsoralen, 8-HOP)	−	10.0 (1.0)	54.0 (1.0)
α-Terthienyl (α-T)	−	10.0 (1.0)	7.2 (0.13)

[a]Relative efficiency of inactivation: Ps > 8-MOP > α-T > An > 8-HOP.
[b]RT10h was used to compare F_{37} (fluence resulting in 37% survival) since it is repair proficient.

and March, 1980). This observation suggests that the *nur* gene sensitizes cells to oxidative damage involving DNA. When α-T is used as a photosensitizer, however, the *nur* gene has no sensitizing effect, as would be expected if the lethal effects of this compound plus NUV are restricted to the membrane.

When either imperatorin (Fahmy and Abu-Shady, 1947) or berberine (Philogène et al., 1984) were tested as possible phototoxins with the four *E. coli* strains RT7h, RT8h, RT9h, and RT10h, inactivation beyond that which could be accounted for by the NUV light system alone could not be demonstrated. In this assay system neither imperatorin nor berberine is phototoxic.

In all of the experiments just described (Figures 1–5), selection was carried out for histidine independence at the *his-4* locus. The comparison of the mutational results is difficult since the concentration of photosensitizer and the fluence of NUV used differs for each photosensitizer (Table 1). It seems most appropriate to compare the mutational results at equivalent survival levels assuming, therefore, the same number of lethal lesions are being produced by the phototoxins being compared. As a standard for mutagenicity, we elected to use FUV, which is highly mutagenic for the *his-4* allele carried by strains RT7h–RT10h (Kato et al., 1977). The comparison of the mutagenic effects of the various phototoxins tested and FUV are presented in Figure 6. It is apparent that psoralen, xanthotoxin (8-MOP), and angelicin are capable of inducing *his-4*$^+$ mutations but are not as efficient as FUV for the induction of such mutations. Mutations to histidine independence were not detected in the experiments in-

FIG. 5. Fluence–response curves for four *E. coli* strains treated with broad-spectrum NUV in the presence of α-terthienyl.

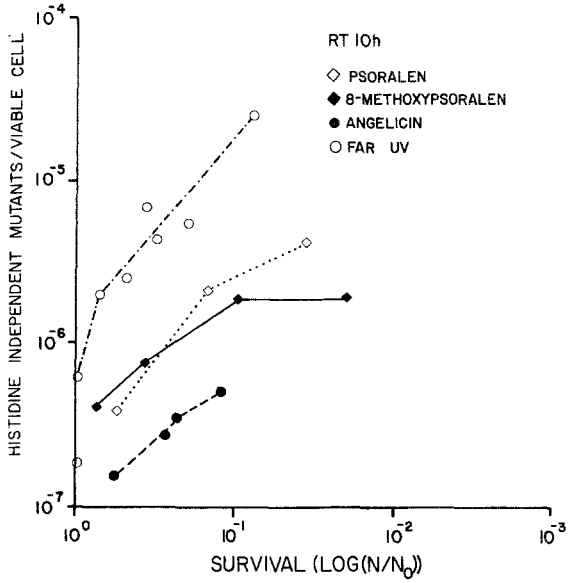


FIG. 6. Histidine-independent mutations per viable cells induced in strain RT10h with broad-spectrum NUV in the presence of phototoxins compared at equivalent survival levels.

volving xanthotoxol (8-HOP) or α-T. The mutation results with xanthotoxol were not unexpected since the inactivation observed with RT10h could be accounted for by the NUV radiation alone and NUV is not particularly mutagenic (Tyrrell, 1978; Leonardo et al., 1984). Even with RT9h, in which some fraction of the inactivation is the result of NUV and xanthotoxol, *his-4*$^+$ mutants were undetectable. The fact that *his-4*$^+$ mutants were not detectable with α-T plus NUV is what would be expected assuming the lethal target is the membrane rather than DNA.

In Table 1, the results are summarized and an evaluation of the relative efficiency of the phototoxins plus NUV for inactivation is presented. In this evaluation we have attempted to take into account the fact that the inactivation kinetics are determined by two variables: (1) the phototoxin concentration employed and (2) the fluence required to obtain 0.37 survival. As might have been expected, psoralen and xanthotoxin are the most efficient for inactivation and xanthotoxol the least efficient. In fact, the inactivation involving xanthotoxol is probably totally independent of the chemical and simply represents NUV inactivation.

The results reported here suggest that the four *E. coli* strains described (RT7h–RT10h) can be used to give a preliminary indication of the mechanism(s) by which a suspected phototoxin might inactivate cells. Furthermore, some indication of the mutagenicity of the presumptive phototoxin can be as-

sessed by selecting *his-4*$^+$ independent mutants in the same experiment in which inactivation kinetics are being assessed. Finally, identical inactivation kinetics for all four strains, as was seen with α-T (Figure 5), can be taken as preliminary evidence that the principal lethal target is the membrane.

Ashwood-Smith et al. (1983) have devised a method for assaying phototoxins involving transfer of presumptive phototoxins from TLC plates onto nutrient agar plates followed by overlaying the agar plates with soft agar (0.8%) containing bacteria (10^5–10^6 cells/ml). After the overlay solidifies, the plates are exposed to various NUV fluences. Where phototoxins were present on the TLC plate, a zone of inhibition will appear in the bacterial lawn on plates exposed to NUV before overnight incubation at 37°C. We ran an experiment to see whether similar procedures might be used with *E. coli* strains RT7h–RT10h. The results of this experiment are presented in Figure 7. It appears that the four strains described here can be used in a bioassay system comparable to that

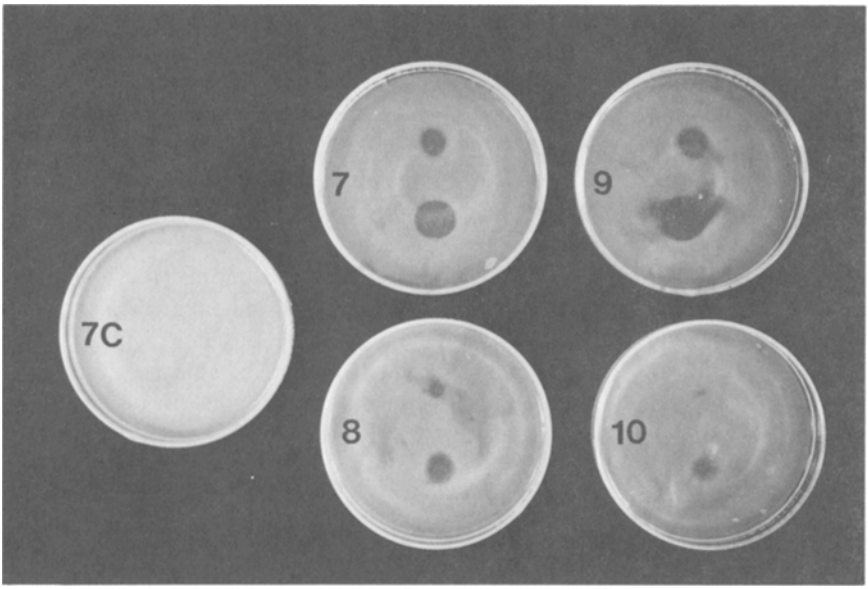


FIG. 7. Photograph of Petri dishes containing LB agar onto which TLC plates had been pressed for 2 min, following which a 4-ml overlay of LB agar seeded with indicator bacteria had been poured. 7 = RT7h, 8 = RT8h, 9 = RT9h, 10 = RT10h. Only a single unirradiated control is shown (7C = RT7h not exposed to broad-spectrum NUV). All other plates were exposed to broad-spectrum NUV. Upper clear zone in bacterial lawn = α-T; lower clear zone = psoralen.

described by Ashwood-Smith et al. (1983). The upper clear zone in the plates exposed to NUV marks the position of the α-T spot on the TLC plate and would be expected to be the same size for all four strains (Figure 5). However, R10h seems to be the least sensitive of the four strains to inactivation by α-T and NUV. The lower clear zone in the plates exposed to NUV marks the position of the psoralen spot on the TLC plate. Strains RT7h and RT9h exhibit larger zones of inactivation than do strains RT8h and RT10h, as expected, since these strains carry the *uvrA6* allele. These results imply that this qualitative bioassay can be used with the four strains described here to draw preliminary conclusions concerning the possible inactivation mechanism(s) characteristic of a particular phototoxin. However, firm conclusions concerning the inactivation mechanism(s) for such a phototoxin will require quantitative inactivation experiments.

The control of insect pests using synthetic chemicals is coming to a close. Alternative methods are needed to accomplish this objective. Clearly, there is not going to be any single method which will prove satisfactory under all circumstances. One possible method of control for insect pests would be to utilize the chemicals evolved by plants to control insect pests, such as phototoxins. These compounds, when ingested by insects feeding on plants, become toxic with exposure to sunlight. In principle, since these are "natural products," they are biodegradable and could be developed as ecologically sound "insecticides." To begin an evaluation of phototoxins, a rapid method is needed to evaluate the mechanism(s) by which the compound acts and its potential mutagenicity. Using the four *E. coli* strains we have constructed (RT7h, RT8h, RT9h, and RT10h), it should be possible within not more than two weeks to obtain some information as to the mode of action and the mutagenicity of newly isolated and purified phototoxic plant-derived compounds. This system is qualitative and reproducible, eliminating the need to do the initial phototoxicity assessment of new plant products with insect-feeding experiments. Should the bacterial experiments prove positive, then efforts to evaluate the efficacy of the new compound with insects could be undertaken. Since insect experiments are slow and exhibit high variability, the bacterial systems should accelerate the evaluation of potentially important compounds as phototoxins for insects.

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Editors' Note

The field of chemical ecology considers chemical–biological interactions in the context of the organism's surrounding environment. As techniques become increasingly sophisticated, we are able to probe more deeply into the chemical, biochemical, and genetic basis of these interactions. The International Society of Chemical Ecology, founded in 1983, is a forum for the discussion of the latest developments in this field: an area wherein the subject of chemical ecology is the focal point that brings together scientists regardless of the system in which they work (e.g., plant or animal, terrestrial or marine).

When the second annual meeting of the International Society of Chemical Ecology convened in Madison, Wisconsin on June 8–12, 1985, four symposia highlighted recent progress in various aspects of chemical ecology. These were: Marine Chemical Ecology, Chemical Aspects of Symbiosis, Behavioral Adaptations to Chemical Cues, and the Evolutionary Ecology of Chemical Defense in Insects. The *Journal of Chemical Ecology*, as the official journal of the society, is dedicating this issue to the proceedings of those symposia.

Each paper has undergone peer review in accordance with the standard review policy of the Journal. We are especially grateful to symposium moderators: D. Rittschof, D. Norris, W. Burkholder, and J. Pasteels; and to reviewers: E.A. Bernays, M.S. Blum, M.D. Bowers, W.E.S. Carr, L.S. Ciereszko, S.S. Duffey, W.B. Heed, T. Jermy, G.W. Ordal, J.G. Rodriguez, G.G.E. Scudder, and R.A. Werner.

We acknowledge the cooperation of Plenum Publishing Corporation in producing extra copies of this issue to accommodate registrants of the symposium and others interested in the proceedings under this single cover.

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CHEMICAL ECOLOGY OF MARINE ORGANISMS: AN OVERVIEW

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Abstract—An overview of marine chemical ecology is presented. Emphasis is placed on antipredation, invertebrate–toxic host relationships, antifouling, competition for space, species dominance, and the chemistry of ecological interactions.

Key Words—Chemical ecology, ecological chemistry, defense, predation, antipredation, symbiosis, biological associations, fouling, biofouling, antifouling, communities, succession, dominance, competition, marine chemistry, medicine, toxin, noxin, venom, poison, marine natural products chemistry, natural products chemistry.

INTRODUCTION

The chemical ecology of marine organisms is described by a vast assemblage of papers and books, representing a wide variety of subdisciplines within biology and chemistry. Much of the recent interest in marine chemical ecology has developed historically as offshoots from natural products chemistry (i.e., novel compounds, Manes et al., 1985) and drug and health-related studies. Among some of the most interesting applied topics, in which considerable basic research has been and is being done, are health problems such as worldwide human marine poisonings, numbering over 20,000 cases and 150 deaths annually (Russell, 1984). Paralytic shellfish poisoning or PSP, including saxitoxin, annually numbers about 2000 cases and 33 deaths (Hashimoto, 1979;

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Russell, 1984; Steidinger and Baden, 1984). Ciguatera or fish poisoning causes deaths in the Indo-Pacific region in particular (Randall, 1980). Poisonings from eating other dangerous seafoods (e.g., snails and crabs) number about 530 cases and 37 deaths annually; (Garth and Alcala, 1977; Yasumoto et al., 1983a,b; Russell, 1984).

Swimmer's itch from the blue-green *Lyngbya majuscula* (now *Microcoleus lyngbyaceus*), is caused by the inflammatory agent debromoaplysiatoxin, one of the most potent skin irritants known (Russell, 1984). Some 1.5×10^6 humans are stung every year as a result of contact with marine organisms (Kohn, 1958; Plessis, 1975; Russell, 1984). Dermatitis from haptens in bryozoans is also common (Russell, 1984). In addition to the above poisons and irritants, a variety of antibiotic (i.e., antibacterial, antifungal, antiviral) agents, antitumoral compounds such as didemnins or depsipeptides (Burkholder, 1973; Mynderse et al., 1977; Weinheimer et al., 1978; Rinehart et al., 1981a,b; Jacobs et al., 1985; Thompson et al., 1985), prostaglandins (Weinheimer and Spraggins, 1969; Ruggieri and Thoroughgood, 1985), and antiinflammatory agents such as potassium fluorosilicate are known to occur in marine species (Gregson et al., 1979).

Other topics of interest in chemical ecology include mutualism (Muscatine and Porter, 1977; Wilkinson and Vacelet, 1979; Glynn, 1983), chemical communication (Reiswig, 1970; Barbier, 1981; Liley, 1982), chemoreception (e.g., in larval settling, Hadfield, 1977; Hadfield and Ciereszko, 1978; Crisp, 1984; Morse and Morse, 1984; food recognition and detection of predators, Noakes and Ward, 1981; Croll, 1983), bioluminescence (Ruby and Morin, 1978; Morin, 1981), immunological responses such as the death of subordinate corals and graft acceptance in sponges (Hildemann et al., 1981; Jokiel et al., 1982; Neigel and Avise, 1983; Kaye and Reiswig, 1985), growth controls (Sullivan et al., 1983; Rinkevich and Loya, 1985), the use of marine pesticides (Padan or nereistoxin; Ruggieri, 1976), the weakening of calcareous structures by boring organisms (Goreau and Hartman, 1963; Anderson and Stonard, 1979; Young and Nelson, 1985), the use of marine bioactive substances in neurophysiology (McClure and Martin, 1983; Koenig et al., 1984), natural shark repellents (pardaxin, see discussion below; Bakus, 1983a; Bakus et al., 1983; Tachibana et al., 1985), natural antifouling agents (Riegle, 1982; Targett et al., 1983; Bakus and Kawaguchi, 1984), the stunning of fishes (Plessis, 1975), and chemotaxonomy (Bergquist and Wells, 1983; Gerhart, 1983; Bergquist et al., 1984; Lee and Gilchrist, 1985; Tymiak et al., 1985).

In addition, there are fascinating cases of symbiosis. For example, the clownfish, *Amphiprion clarkii*, that forms a mutualistic association with the sea anemone *Stechodactyla haddoni*, has a mucus layer of relatively inert glycoprotein that is three to four times thicker than that of many other reef fishes (Lubbock, 1980; Brooks and Mariscal, 1984). This mucus layer protects the fish from stings by nematocysts of the sea anemone. Another example is the

juvenile damselfish, *Abudefduf leucogaster*, that lives among the soft coral *Litophyton viridis* in the tropical Pacific. These fish are protected from potential predators by chemicals released by the soft coral which are noxious to most fish but to which the damselfish have developed a tolerance (Tursch, 1982).

An exhaustive review of marine chemical ecology is not possible at this time. Instead, we will provide an overview, concentrating on the following aspects of chemical ecology that represent our different principal interests, that is, antipredation, invertebrate–toxic host relationships, antifouling, competition for space, species dominance, and the chemistry of ecological interactions. The reader is referred to other recently published reviews for additional information (e.g., Herring, 1979; Fenical, 1982; Norris and Fenical, 1982; Faulkner and Ghiselin, 1983; Faulkner, 1983; 1984; Wright, 1984; Scheuer, 1985; Coll et al., 1985).

ANTIPREDATION

Predators (in the broad sense) are widespread in the marine environment. This is reflected in numerous types of offensive and defensive mechanisms among their prey. Protective and defensive mechanisms evolved by the biota against vertebrates and invertebrates include rapid growth and growth in wave-washed and cryptic areas by benthic algae, unpredictable occurrences in time and space, burrowing, boring, large size, structural defense, evasive movement, cryptic coloration and form, nocturnal activity, symbiotic associations, parental protection, and chemical defenses (Bakus, 1964, 1969, 1983b; Menge and Lubchenco, 1981, Norris and Fenical, 1982). Many interactions of attack, defense, and behavioral response involve not physical force but chemical agents. A great many of these chemical agents can be assigned two or three adaptive roles (Whittaker and Feeny, 1971; Janzen, 1979). For example, secretion of mucus may serve to clear a hard coral of sediment but may also provide a mechanism for concentration of allelochemicals, preventing excessive dilution by currents (Jackson and Buss, 1975; Sullivan et al., 1983). This may also be the case in Maldivian soft corals (e.g., *Dendronephthya* and *Sarcophyton*, Bakus, personal observation). The retention of pigments is a passive chemical defense mechanism, effective in cryptic coloration, warning coloration, and countershading. For example, juveniles of the burrfish *Chilomycterus antennatus* reportedly are Batesian mimics in shape and coloration of the sea slug *Aplysia dactylomela* (Norris and Fenical, 1982).

Consumer pressure is severe in tropical rocky intertidal communities, where most organisms are located in holes and crevices for protection from predators (Menge and Lubchenco, 1981). The ultimate in consumer pressure occurs on coral atolls (Bakus, 1967, 1969). However, chemical defenses are not limited to species occurring in low latitudes. The kelp *Alaria marginata* produces an-

tiherbivore phenolic compounds (Steinberg, 1984) as does the Caribbean brown alga *Stypopodium zonale*. The holothurian *Psolus chitinoides* in the San Juan Islands, Washington, is highly unpalatable to fish and has the highest saponin content of the four species of sea cucumbers examined (Bingham and Braithwaite, 1985). Many species of the green algae *Caulerpa* and *Halimeda* are ichthyotoxic (Gerwick and Fenical, 1981; Norris and Fenical, 1982; Russell, 1984). Genera within the Udoteaceae and Caulerpaceae are highly resistant to fish grazing (Lewis, 1985). In particular, certain *Halimeda* species contain halimedatriol, a diterpenoid trialdehyde, which shows antibiotic activity, inhibits cell division and sperm motility, and is ichthyotoxic (Paul and Fenical, 1984). Some *Halimeda* and *Caulerpa* species also produce diacetoxybutadiene-containing terpenes which deter fish feeding even when coated onto their preferred food, the seagrass *Thalassia testudinum* (Targett et al., 1986). However, the reef flat blue-green *Schizothrix calcicola* contains a major toxic metabolite (debromoaplysiatoxin) yet is consumed in large quantities by surgeonfishes at Enewetak Atoll (Bakus, 1967; Mynderse et al., 1977). This tends to support Lewis' (1985) conclusion that the susceptibility of tropical algal species to fish grazing is not clearly correlated with morphological (e.g., calcification) or chemical (e.g., allomones) characteristics that have been previously suggested as plant defenses against herbivory.

Similarly, toxic sponges and probably soft corals are consumed by certain fishes (Randall and Hartman, 1968; Anderson et al., 1981; Tursch and Tursch, 1982). The sponges *Neofibularia* spp. contain some of the most painful undescribed marine chemicals known to man (Hartman, 1967). Several flatworms including *Thysanozoon* spp. and even certain brittlestars secrete an acid, as do some opisthobranchs (Marbach and Tsurnamal, 1973; Cimino et al., 1983; Faulkner and Ghiselin, 1983; Russell, 1984). Opisthobranchs also release terpenes and a variety of other compounds which are thought to function in defense (Lewin, 1970; Faulkner, 1983; Mebs, 1985). Faulkner and Ghiselin (1983) believe that the loss of the shell in dorid nudibranchs and other opisthobranchs is correlated with defense chemicals, the chemical defenses being elaborated gradually as the shell was being lost.

Coelenterates employ nematocysts offensively and defensively. The most toxic are cubomedusae such as the sea wasp, *Chironex fleckeri*, which has been known to cause human death in less than five minutes (Russell, 1984; Halstead, 1985). Nematocysts in hydroids (*Lytocarpus* spp. and *Millepora* spp.) and some hard corals (*Acropora palmata*) are known to sting divers (Halstead, 1985). Zoanthids containing palytoxins are reportedly among the most toxic marine organisms known (Moore and Scheuer, 1971; Moore and Bartolini, 1981; Russell, 1984; Uemura et al., 1985). The sea anemone (*Stoichactis* spp.) contains polypeptides that are proteinase inhibitory, toxic, and hemolytic (Mebs and Gebauer, 1980). Many sea anemones have cytolytic toxins, principally proteins and peptides (Russell, 1984). However, not all cnidarian defenses are contained

within the nematocysts. For example, gorgonians (*Lophogorgia* spp., *Plexaura* spp.) have terpene and prostaglandin defense toxins (Fenical et al., 1981; Gerhart, 1984).

The polychaete *Eurythoe* has hollow, sometimes fluid-filled stinging chaetae; the sea hare *Aplysia brasiliana* produces antishark chemicals (Kinnel et al., 1979); and the blue-ringed octopus in Australia (*Hapalochaena maculosa*, which contains the venom maculotoxin, identical to the chemical poison tetrodotoxin) has caused human fatalities (Russell, 1984). Even the eggs and/or larvae of some seastars (*Asterias rubens*, *Acanthaster planci*) are protected by saponins or glycosides (Lucas et al., 1979). In fact, it is likely that the larvae of many asteroids may contain saponins that act against predators such as polychaete worms and fishes. Palytoxin also occurs in the eggs of the zoanthid *Palythoa tuberculosa* (Endean and Cameron, 1983).

Russell (1984) has questioned whether offensive sponge odors and taste play a defensive role. Green (1977) found that the sponge *Iotrochota birotulata* has a colored, strong-smelling exudate that, when released in water, is avoided by fishes. Green and Bakus (personal observation), in 1973 found that several species of foul-smelling gorgonians from the region of Veracruz, Mexico, were ichthyotoxic.

Aposematism is poorly studied in marine invertebrates. Some zooanthids are aposematically colored (Lewis, 1982), but sponges seem to display a large variety of colors whether toxic or not (Bakus and Thun, 1979). Some fishes have what appears to be warning coloration and erectile caudal spines as defensive mechanisms (surgeonfishes or Acanthuridae). Others secrete a mucus that prevents predaceous fishes from eating them (Kerstitch, 1984). Perhaps the most incredible case of antipredation occurs in the Moses sole found in the Red Sea. This diminutive flatfish secretes a milky toxin that in some instances paralyzes the jaws of a biting shark (Clark, 1983; Dr. Eugenie Clark, personal communication).

Many marine toxins appear to be feeding deterrents also. Echinoderm noxins and toxins appear to be the same chemically, that is, triterpenoid and steroidal saponins (Burnell and ApSimon, 1983; Dr. G. G. Habermehl, personal communication). Terpenoids in sponges may be unpalatable to predators (Manes et al., 1984, 1985; Russell, 1984; Bescansa et al., 1985). More than half of nontoxic soft corals are noxious to fishes, their chemicals causing 48–88% feeding deterrency in fish (La Barre et al., 1986b). Feeding deterrency is as common among nontoxic fish corals as among toxic soft corals. The herbivorous fish *Sparisoma radians* avoided the milky exudate released from injured tips of the green alga *Halimeda incrassata* (Targett et al., 1986).

A variety of marine organisms are venomous, including octopuses (via their salivary glands), snails (especially *Conus geographus* and *C. textile*), sea urchins (*Toxopneustes*), sea stars (*Acanthaster planci*), fishes (scorpaenids or rockfishes such as the turkeyfish, stingrays; Roche and Halstead, 1972), and of

course, all of the 51 species of sea snakes (Family Hydrophiidae, Voris, 1972). There are about 700 species of toxic (i.e., venomous and poisonous) fishes known. Rather few of the fishes (3%) on the Great Barrier Reef are venomous (Cameron, 1976). Many species are poisonous, either with toxic skin (about 50 species, e.g., crinotoxic soapfish, boxfish, puffers, and stonefish) or nocturnal mucus cocoons (wrasses and parrotfishes). There are even cases of fishes with toxic models (*Canthigaster*) and edible mimics (*Paraluteres*, Cameron, 1976). Recent summaries of information on venomous and poisonous marine organisms are found in Habermehl (1981), Sutherland (1983), Russell (1984), Russell et al. (1984), and Halstead (1985).

Defensive toxicity is prevalent in warm waters (Table 1). This high incidence in the tropics probably is an evolutionary response to a sessile or slow-moving habit, exposure coupled with high-diversity and high-intensity predation (especially by fishes), and rapid turnover rates (Bakus, 1969, 1983b; Cameron, 1976; Miller, 1982; Hay, 1984a,b). Recent findings indicate that the toxicity of sponges to fishes is relatively high in northern France and even the Antarctic (Hugsecom and van de Vyer, 1985; James B. McClintock, personal communication). This toxicity may represent adaptive responses to invertebrate predators, the broad spectrum toxins also affecting fishes.

What we do not know is often more interesting than what is known. Although exposed coral reef holothurians are toxic to fishes, and cryptic sea cucumbers in the same area are not, some cryptic sponges are toxic to fishes (Green, 1977; Bakus, 1981). Is this an accidental metabolic byproduct resulting from transposons, a feature selected in the past that has not yet been eliminated from the gene pool, or does this represent a species that occurs cryptically in some regions and exposed in others? Green (1977) found that most nontoxic sponges were unexposed in the tropics but exposed in cold temperate waters. As a general rule, opisthobranchs are both distasteful and cryptic (Faulkner and Ghiselin, 1983), the latter especially common in the tropics (Bakus, personal observation). Only seldom has it been demonstrated that even suspected toxins vary seasonally (e.g., saponins in the seastar *Asterias amurensis*, Yasumoto et al., 1966). How widespread is this phenomenon? Is it simply a Q_{10} response or could it be an adaptation to a seasonal increase in predation pressure, or both?

Bakus (1969) illustrated how the holothurian *Holothuria atra* secretes a toxin when disturbed. Parrish (1972) reported that the holothurian *Actinopyga agassizi* releases a noxious substance from the body surface when attacked by the snail *Charonia variegata*. Advanced hunger and periods of exposure in close proximity to the holothurian appear to give *C. variegata* sufficient resistance to the noxin to permit successful predation. Green (1977) suggested that sponge toxins may be released continuously into the surrounding water and serve as a warning deterrent to predators. Lucas et al. (1979) stated that saponins diffuse from starfish and are detected by their prey. They also serve as defense chemicals in asteroids and holothurians. The first chemically demonstrated release

of an allelochemical from a marine organism was from an Australian soft coral (Coll et al., 1982a). Toxins are secreted more or less continuously in several species of soft corals (Sammarco et al., 1982; Coll and Sammarco, 1983) and in at least two species of sponges from California (*Spongia idia* and *Aplysina fistularis*, Walker et al., 1980; Thompson, 1985). We know nothing about this phenomenon in hundreds of other species of toxic sponges, gorgonians, and holothurians. The intensity of fish grazing on the benthos certainly appears to be greater in the tropics in general than that of higher latitudes (e.g., note the high-standing crops of algae in the latter, Bakus, 1969; Choat, 1982). However, is the encounter rate between predators and prey also greater in the tropics? This has never been investigated. How does a fish learn that a species is toxic? Presumably by trial and error feeding and/or by chemoreception (Bakus, 1981). This interaction needs considerably more study in the field.

Some marine toxicity studies have examined whole organisms from diverse geographical areas and pooled results. Questions regarding variations in toxicity with geography, age, sex, body part, depth, and so forth, need to be addressed in more detail. Based on analogies with terrestrial systems, one would expect to find variations in quality and quantity of allelochemics over the range of some species in response to changes in the environment or shifting selection pressures (Whittaker and Feeney, 1971). Dr. Valerie Paul (personal communication) is currently studying these subjects in marine algae, but we know virtually nothing about this in marine benthic invertebrates. There do not appear to be significant changes in the toxicity of soft corals with latitude along the Great Barrier Reef of Australia (Coll and Sammarco, 1983). This suggests that there is no significant change in the intensity of predation as long as coral reef communities persist. Toxicity in holothurians is weaker in cold waters than in the tropics (Bakus, 1974). Sponges that are toxic in colder waters were moderately to highly toxic whereas those in the tropics ranged from slightly to highly toxic (Green, 1977). Certain species of sponges and holothurians apparently are toxic in some localities and nontoxic in others (Green, 1977; Bakus, 1981). A sample of two species of blue-greens collected from the seaward side of Enewetak Island was much more toxic than a specimen collected on the lagoon side of the island (Mynderse et al., 1977), perhaps an adaptive response to high intensity grazing on the outer reef flat (Bakus, 1967). Two antimicrobial metabolites occur in the intertidal sponge *Aplysina fistularis* but not in the same species at depths of 5–15 m (Thompson et al., 1983; Thompson, 1985).

Several different toxic saponins can be found within an individual holothurian (Dr. G. G. Habermehl, personal communication), yet we know almost nothing about the adaptive advantage of having several compounds. One suggestion is that saponins are important in reproduction, antipredation, and antifouling. Prostaglandin A_2 in the gorgonian *Plexaura homomalla* (representing about 10% of the dry weight) is one million times more concentrated than in most other marine invertebrates. It has emetic properties in a very wide range

TABLE 1. TOXICITY OF SHALLOW-WATER MARINE ORGANISMS AT VARIOUS LATITUDES[a]

Locality	Latitude	Taxonomic group	No. of Species		Species (%)	Source
			Tested	Toxic		
Canada	48°N to 78°N	freshwater and marine fish	?(770)[b]	12	1.6	McAllister, 1968
San Juan Islands, Washington	48°N	sponges	34	3	9	Bakus and Green, 1974; Green, 1977
		holothurians	12	3	25	Bakus, 1974; Bakus and Green, 1974
Nontropics	less than 20°N & S	marine organisms	937	262[b]	28	Various authors, in Bakus, 1969
Onagawa, Japan	38°N	holothurians	5	4	80	Yamanouchi, 1955
Seto, Japan	35°N	holothurians	9	7	78	Yamanouchi, 1955
Santa Catalina Island, California	33°N	sponges	44	9	21	Bakus and Green, 1974; Green, 1977
		holothurians	2	1	50	Bakus, 1974; Bakus and Green, 1974
Guaymas, Mexico	28°N	holothurians	6	5	83	Bakus, 1974; Bakus and Green, 1974

Zihuatenejo Bay, Guerrero, Mexico	17°N	sponges	11	7	64	Bakus and Green 1974; Green, 1977
La Blanquilla Reef, Veracruz, Mexico	19°N	sponges	36	37	75	Bakus and Green 1974; Green, 1977
Tropics	Approx. 20°N to 20°S	marine organisms	937	675[b]	72	Various authors, in Bakus, 1969
Cozumel, Mexico, to Belize, Belize	20°N to 17°N	sponges	54	31	57	Bakus and Thun, 1979
Eniwetok, Marshall Islands	12°N	holothurians	4	4	100	Bakus, 1968
Palau Islands, Pacific Ocean	7°N	holothurians	11	11	100	Yamanouchi, 1955
Cocos Island, eastern Pacific	6°N	holothurians	7	6	86	Bakus, 1974
Lizard Island, north Great Barrier Reef, Australia	14°S	sponges, soft corals, gorgonians, asteroids, criniods, holothurians, ascidians	42	25	60[c]	Bakus, 1981

[a] Organisms toxic to fish unless otherwise indicated.
[b] Organism toxic to humans.
[c] 73% of exposed benthic species.

of organisms, causes learned feeding aversion in fish, and is ichthyotoxic (Gerhart, 1984). Many other gorgonians are ichthyotoxic (see above and Bakus, 1981). Toxins in soft corals, principally terpenes, constitute about 1% of their dry weight (Dr. John Coll, personal communication). About half of soft coral species are ichthyotoxic (Coll et al., 1982b; La Barre et al., 1986b). Siphonodictidine, a sponge chemical that kills coral polyps around the sponge *Siphonodictyon* spp., represents about 1% of the dry weight of the sponge. A congeneric sponge contains the chemical siphonodictyal, a very different metabolite that also kills nearby coral polyps (Sullivan et al., 1983). To what extent do inquilines occur in toxic animals such as sponges? The mildly toxic (to fish) Caribbean sponge *Spheciospongia vesperium* is teeming with inquilines (Pearse, 1932, 1950; Bakus and Thun, 1979), but the more toxic sponge *Ircinia campana* seems to have far fewer (Bakus, personal observation).

There is a dearth of information on whether the compounds toxic to fishes also serve other adaptive roles, such as antifouling agents or species spacing allomones. Sammarco et al. (1982) suggest that toxic compounds from soft corals serve both an antipredator function and a role in competition for space. However, it is not known whether chemically identical terpenes are involved in multiple roles. Coral reef sponges, soft corals, and ascidians that lack allelochemicals have a hardened body or secrete copious amounts of mucus (Bakus, 1969, 1981; Green, 1977; Bakus and Thun, 1979; Dr. John Coll, personal communication). The behavior of fishes exposed to benthic invertebrate toxins suggests that the chemicals are interfering with oxygen transport across gill membranes and/or that a strong chemoreceptive response is occurring. Mackie et al. (1975) reported that the toxic effects of saponins are similar to those of synthetic surfactants. They damage the gill epithelium, reducing gas exchange across the gill surface. Pardaxin, a polypeptide of the size of about 3800 daltons in the Moses sole, is hemolytic and ichthyotoxic (Primor and Zlotkin, 1976; Dr. Samuel Gruber, personal communication). It is a potent gill ATPase inhibitor and clearly causes structural damage to gill tissues (Primor et al., 1980). It also acts as a surfactant (Primor et al., 1983; Gruber and Tachibana, 1986). These appear to be the only instances where the physiological effects of antipredatory toxins are known.

INVERTEBRATE-TOXIC HOST RELATIONSHIPS

Benthic marine organisms containing bioactive compounds, such as some sponges, cnidarians, mollusks, holothurians, and asteroids, are probably never completely devoid of either predators or symbionts. Because of this, it is necessary to examine how predators and symbionts (i.e., associated species) handle the toxic or noxious compounds that their hosts produce. A toxic compound is thought to provide general protection to the host; therefore, any associate would be expected to be specialized in their adaptations to the host. As an example of

this, Coll et al. (1983) briefly describe the relationship of the prosobranch mollusk *Ovula ovum*, an egg cowry, to the highly allelotoxic soft coral *Sarcophyton* spp. The prosobranch grazes on the corals' tissue without apparent harm. By way of a reduction–elimination reaction believed to be enzyme-mediated, the egg cowry transforms sarcophytoxide, the most abundant terpene in the soft coral, into a much less toxic, 7,8 deoxysarcophytoxide. This process probably occurs in the digestive diverticula/stomach region. Similarly, in a review article, Faulkner and Ghiselin (1983) discuss numerous cases in which nudibranchs selectively incorporate sponge toxins that are capable of acting as feeding inhibitors, the only exception being the *de novo* synthesis of polygodial in *Dendrodoris limbata* (Cimino et al., 1983). The secondary metabolites of the nudibranch *Cadlina luteomarginata* are found only in the dorsum and act as antifeedant chemicals to fish (Thompson et al., 1982). Additionally, herbivorous marine mollusks (e.g., *Aplysia*, saccoglossans) contain protective compounds dietarily derived from their algal food sources (Norris and Fenical, 1982; Faulkner, 1984).

In many other cases, however, the means by which an associate handles the toxins of its host are not well understood. Sponge-zoanthid associations appear to be of two distinct types in the central Caribbean. One group consists of dull-colored zoanthids, lacking toxicity, thus offering no protection to their sponge host. Brightly pigmented zoanthids, toxic in nature, comprise the second group, possibly representing a mutualistic association in which the toxic zoanthid reduces predation on host sponges (Lewis, 1982). It seems likely that sponge hosts are never exposed to zoanthid toxins. This may also be the case in zooxanthellae-coelenterate relationships. Many toxic alcyonaceans and gorgonians contain algal symbionts which contribute to the nutrition of the host (Muscatine, 1980). Kokke et al. (1984) present strong evidence that the zooxanthellae do not synthesize these bioactive compounds nor do they provide an immediate precursor to toxin (terpene) synthesis (e.g., a mevalonic precursor). Since these terpenes are probably stored in membranous vacuole-type structures similar to those proposed in sponges (Simpson, 1984), any toxin-resistance hypothesized from the mere existence of the algal–toxic host relationship might simply be explained by stating that the zooxanthellae are never exposed to these bioactive compounds (Dr. William Fenical, personal communication).

Other toxic host–associate interactions simply have not been examined in detail. A variety of animals (worms, sea urchins, crustaceans, and fishes) feed on the seastar *Acanthaster* in spite of its toxic saponins (Glynn, 1984). The prosobranch gastropods *Cyphoma* and *Neosimnia* feed on gorgonians containing bioactive compounds without deleterious effects (Dr. Eric Jordan, personal communication). Sundial shells (*Heliacus* spp.) eat some of the polyps of the zoanthid *Palythoa* (Endean and Cameron, 1983). The seastar *Evasterias troschelii* has a commensal polychaete, *Arctonoe fragilis*, that is immune to the hosts' saponin, whereas the noncommensal polychaete *Arctonoe pulchra* is not

(Patterson et al., 1978). Some tropical fishes feed on toxic sponges with no visible negative reactions (Randall and Hartman, 1968). These are only a few examples of the wide variety of interactions between an associate and its toxic host yet to be explored and understood.

In summary, a species that closely associates with (i.e., lives within in some manner or feeds on) a toxic host species may handle bioactive compounds by one of the following means: (1) excrete or secrete the toxins unchanged (e.g., some opisthobranchs), (2) incorporate the toxins or a derivative thereof, possibly for their own defensive benefit (e.g., many sponge-feeding nudibranchs; *Ovula ovum*), (3) be immune to any toxic effects yet forced to handle bioactive compounds in some manner, and (4) avoid contact with its host's toxins (e.g., possibly zooxanthellae).

ANTIFOULING AND SUCCESSION

The initial stages of marine community development involve several steps: (1) adsorption of biopolymers in water to a surface (Mitchell, 1977; Baier, 1984; Lewin, 1984), (2) chemical attraction of bacteria, (3) reversible adsorption of bacterial populations, the bacteria adhering within minutes after settling onto a surface (Colwell, 1984), (4) irreversible adsorption of bacteria involving macromolecular fibrils, the attachment occurring by flagella and other cellular structures (a typical adhesion polymer on a bacterial surface might be making at least 10,000 contacts with a surface; Robb, in Lewin, 1984), (5) agglomeration and colony formation, and (6) growth of a secondary bacterial population, pennate diatoms, protozoans, and adhesion of particulate matter (Cuba and Blake, 1983; Mitchell and Kirchman, 1984). Whether microfouling is a general prerequisite to subsequent settling has not been resolved (Little, 1984).

Larval settling behavior is dependent on substratum type, substratum rugosity, light intensity, existing surface films, host inductance, larval chemoreception and other factors (Meadows and Campbell, 1972; Birkeland, 1977; Chia and Rice, 1978; Costlow and Tipper, 1984; Crisp, 1984; Morse and Morse, 1984). Three ecological models have been developed to explain the causes of succession or the replacement of species over time. They include: (1) "facilitation" or alteration of conditions by early species that allow later species to replace them, (2) "inhibition," that is, early colonists inhibit the invasion of later species, and (3) "tolerance," that is, early colonists do not affect the recruitment and growth of later species. There is ample evidence to support both facilitation (Gallagher et al., 1983; Turner, 1983; Harris et al., 1984) and inhibition (Sutherland and Karlson, 1977; Sousa, 1979; Standing et al., 1982; Rittschof et al., 1985). Breitburg (1985) recently concluded that examples of all three models appear to occur in marine community development. She and Dean and Hurd (1980) showed that more than one mechanism may be present

in the same community. Inhibition seems to be more prevalent than facilitation, and tolerance appears to be relatively rare (Bergen, 1985).

Replacement of species during succession has been reported as orderly (Anger, 1978; Murray and Littler, 1978; Sousa, 1980; Sutherland, 1981) and disorderly (Fager, 1971; Osman, 1977; Sutherland and Karlson, 1977). Species composition in succession is affected by prior residents, grazing, temporal variability in recruitment and growth, small-scale differences in settlement, growth and/or survival of colonists, and the physical regime (i.e., disturbance ranging from mild to severe) under which they occur (Bergen, 1985; Breitburg, 1985). However, if successional studies are examined in detail, the actual mechanisms by which species growth is enhanced or inhibited are poorly known. To predict changes in species composition, interactions between the various pairs of early and late colonizers must be taken into account (Breitburg, 1985).

Woodin and Jackson (1979) suggested that different species of the same functional group (i.e., guild) may help each other in competition with species in different functional groups. This may be related in part to the fact that there is more evidence for competition between distantly related taxa than between closely related forms. Thompson (1984) proposed that communities develop by certain species secreting allomones in concert, preventing other species from settling. Thompson (1985) demonstrated that exudates from the sponge *Aplysina fistularis* inhibited metamorphosis of gastropod veliger larvae, reduced settlement of larvae, and caused behavioral modifications in five species of adult invertebrates, but were not toxic to dorid nudibranchs. They also reduced tissue damage from browsing animals by repelling them.

Walker et al. (1985) reported that the sponge *Aplysina fistularis* responded to a simulated injury by exuding 10–100 times more antimicrobial metabolites within about the first 5 min than normally. They found that marine sponges with clean surfaces exhibited greater antimicrobial activity than those with biofouled surfaces. This phenomenon was also reported from Australia (McCaffrey and Endean, 1985). Nakatsu et al. (1983) suggest that sterol sulfates may be responsible in part for the lack of fouling organisms on the sponge *Toxadocia zumi*. Crinoids contain polyketide sulfates which may serve not only as a defensive mechanism against fish (Rideout et al., 1979), but as antifouling agents. Even asteroids may discharge saponins into water in part to prevent fouling (Russell, 1984). Certainly one of the most unusual cases of antifouling occurs in the stonefish (*Synanceja horribilis*) which moves so seldom that it must shed its biofouled skin periodically (Cameron, 1976).

In addition to the above, evidence is beginning to accumulate which suggests that chemistry may play an important role in succession. Kirchman et al. (1983) proposed a lectin model system (i.e., proteins or glycoproteins with carbohydrate-binding specificity) to explain biochemical processes in the settling of fouling organisms (see also Lewin, 1984; Mitchell and Kirchman, 1984). Stoecker (1980a, b) showed that acidity and high vanadium contents are

defenses against fouling in some ascidian species. Targett et al. (1983) found that the growth of the diatom *Navicula salinicola* is inhibited by the substance homarine from gorgonians, a simple 2-carboxy *n*-methyl pyridine. A furanogermacrene isolated from the gorgonian *Pseudopterogorgia americana* inhibits both *N. salinicola* and *Nitzchia* spp. at naturally occurring concentrations in *in situ* experiments (Targett, 1985). Experiments conducted by Bakus et al. (1983) indicate that extracts from tropical gorgonians and sponges can inhibit, enhance, or not affect the settling of dominant marine fouling organisms. Preliminary evidence supports the suggestion that tropical marine organisms may show greater potential as antifoulers than do temperate latitude species (Bakus and Kawaguchi, 1984). Moreover, antibiosis in sponges near Cancun, Mexico, appears to be seasonal (Green et al., 1986; Batus et al., 1986).

The occurrence of clean-surfaced animals that lack appendages, such as sponges, ascidians, soft corals, gorgonians, and holothurians, suggests that antifouling allomones may be present. This is especially true of species on coral reefs, animals potentially exposed to a great variety of algal spores and especially invertebrate larvae. We are beginning to test this hypothesis, working on the assumption that tidy tropical species remain clean by preventing the initial irreversible adsorption of bacteria on their surfaces. Thus, experiments with antibiotics in the laboratory coupled with antifouling experiments in the field may answer some important questions in this regard.

We know very little about the interaction between species pairs during marine succession, especially on the chemical level. Laboratory experiments are needed to demonstrate chemical interactions, corroborated by field experiments. We need to determine how certain species maintain clean body surfaces. If chemicals are secreted, where are they produced and where are they stored; what controls their production and release?

COMPETITION FOR SPACE

Marine organisms, in competing for space, may crowd, undercut, crush, overshadow, overgrow, digest, or poison their neighbors (Quinn, 1982; Branch, 1984). Soft corals may leave trails by moving over hard corals and killing them, offering space susceptible to secondary colonization (Benayahu and Loya, 1981; La Barre and Coll, 1982). Kittredge et al. (1974) brought to attention the role of allelochemics in the sea. Recently, Bak and Borsboom (1984) demonstrated an allelopathic interaction between the giant sea anemone *Condylactis gigantea* and benthic algae. They emphasized that the widely differing results between their laboratory and field experiments were due to the considerable dilution of toxin released by the anemone into the sea. Sheppard (1979) suggested that a toxic secretion may be responsible for the gap between certain hard corals competing for space. Benayahu and Loya (1981) reported that although massive

soft corals (e.g., *Lobophytum, Sacrophyton, Sinularia*) inhibit hard coral growth, some hard corals can inhibit the growth of smaller soft corals. There is rapidly growing interest in the biological role of terpenes in hard corals.

The role that toxins might play in competition for space among cryptic coral reef species was suggested by Jackson and Buss (1975). They proposed competitive networks or a feedback loop in an otherwise hierarchical sequence of interference competitive abilities. For example, species 1 is dominant over species 2, species 2 over species 3, species 3 over species 4, yet species 3 dominates over species 1 (feedback loop). Later they suggested that where competitive networks exist, competition for space may increase diversity (Buss and Jackson, 1979), in comparison with the generally accepted theory of competitive hierarchies disturbance. Quinn (1982) found that the competitive organization of low intertidal rock faces in Washington was essentially hierarchical. Reversals in the outcome between pairs of competing species may occur seasonally or in response to changing physical conditions or even composition of the plankton. Russ (1982), in a long-term study, demonstrated that major groups or taxa are hierarchical (e.g., ascidians and sponges overgrow everything else), that species networks did not exist, that there was no single competitively dominant species, and that larger colonies often would win in competition for space between pairs of species. He also indicated that except in one case, Jackson and Buss had not demonstrated competitive networks. Other criticisms of Jackson and Buss were reported by Quinn (1982); Russ (1982); and Walker et al. (1985). Huston (1985) suggested that an alteration of competitive hierarchies by changing environmental conditions is an alternative mechanism that could produce a nonhierarchical competitive networks on the underside of coral shelves.

Porter and Targett (1985) have quantitative evidence that the proximity of the sponge *Plakortis zygomorpha* to the scleractinian coral *Agaricia lamarki* stressed the coral to the point where in situ P/R (productivity/respiration) ratios were less than 1. Sammarco et al. (1982) demonstrated that soft corals could retard the growth of hard corals, effectively competing with them for space. They considered the secreted compounds important in both antipredation and competition for space. These discoveries have clarified some perplexing problems concerning competition for space between these two major coral taxa (Sheppard, 1979; Benayahu and Loya, 1981) and showed that contact was not essential for inhibition. Birkeland et al. (1981) found that the coral *Mycetophylla aliciae,* strongly competitive for space among Caribbean hard corals, is apparently defenseless against overgrowth by ascidians. The movement of larger didemnids is a frequent source of small-scale (about 100 cm^2) disturbance of occupation of space by hard corals, yet sponges were overgrown but not damaged. Bakus (personal observation) found that ascidians were the only organisms growing over the living basal tissues of the Maldivian soft coral *Dendronephthya* spp. Space for recruitment may not be a limiting factor for the giant

sea anemone *Condylactis gigantea* in a supposedly space-limited environment, due to the toxins it secretes (Bak and Borsboom, 1984).

Lang (1973) concluded that a strict hierarchy of aggression (i.e., one species destroying another species by exocoelenteric digestion with extended mesenteric filaments) occurs in Caribbean corals. Sheppard (1979, 1981), however, found that such a rigid hierarchy was not characteristic of scleractinian corals in the Indian Ocean. Coral aggression was not related to general morphology, taxonomic position, or corallum shape. Wellington (1980) demonstrated that the previously reported hierarchy for some Pacific corals, based on extension of mesenterial filaments, was a short-term response. Aggression by corals also occurs by catch or sweeper tentacles (Sebens, 1984; Hidaka, 1985). However, Bradbury and Young (1981) and Cope (1981) maintain that physical factors (i.e., temperature, wave action, light) rather than competition or predation, explain the distributions of scleratinian corals. Many ecologists would agree that physical–chemical factors are most important because they determine which species have the physiological ability to survive in a new habitat.

Scleractinian corals are not alone regarding agonistic behaviors. Intraspecific agonistic behavior has been reported in the large temperate sea anemone *Anthopleura xanthogrammica* (Sebens, 1984). Nonneighbor anemones exhibit an acrorhagial response, producing vesicular extensions at the column margin over a period of up to about 10 min, damaging other anemones. The chemistry of this interaction is apparently unknown; it presumably involves the secretion of digestive enzymes. All anemones with acrorhagi reportedly use them agonistically, intra- or interspecifically, except for genetically identical clonemates (Sebens, 1984). Webb and Coll (1983) showed that hard corals die within 24 hr of exposure to soft coral terpenes at a concentration of $\geqq 10$ ppm. Finally, Theodor (1966, 1971, 1975) showed that tissue necrosis resulted from contact between gorgonians, and La Barre et al. (1986a) demonstrated that soft corals move apart to avoid tissue degradation resulting from tissue contact.

It is now known that community structure can be controlled by chemical interactions from direct contact, from the release of chemicals into surrounding waters, by overgrowth (nearly a kilometer of reef at Gaum was covered by the sponge *Terpios* sp., Bryan, 1973; Plucer-Rosario, 1983), by agonistic behavior, and by "sessile" species moving apart from one another (La Barre et al., 1986a, b). We do not know what the total pattern of these interactions is for any single community nor how these patterns vary with latitude.

COMMUNITY DOMINANCE AND BIOACTIVE SUBSTANCES

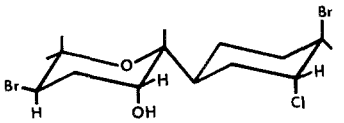
It has been accepted dogma that dominance is a distinguishing trait of species-poor ecosystems. However, Birch (1981) showed that dominance increases as marine benthic communities become more species-rich. Highly aggressive Caribbean corals reportedly are relatively minor components in all reef habitats

(why should this be?), whereas many of the most aggressive corals in the Indian Ocean are extremely dominant in certain zones (Stoddart and Yonge, 1971; Lang, 1973; Sheppard, 1979). Many ecologists have proposed that allelochemics may play an important role in contributing to the maintenance of high species diversity (e.g., Sammarco et al., 1982), but some of these chemicals may also be responsible for creating dominance in toxic tropical species (Birch, 1981). Endean and Cameron (1983) claimed that many coral reef animals that are well protected by toxins are normally rare but live long. However, Bakus maintains that many tropical species with defenses are dominant by biomass and/or number (e.g., the corals *Acropora* spp. and *Porites* spp., the surgeonfishes *Acanthurus* spp., the two-species scleractinian coral reefs of Cocos Island, off Pacific Central America, Bakus, 1967, 1975; Sheppard, 1979; Potts et al., 1985). Other species that are dominant and toxic include the algae *Halimeda* spp., *Caulerpa* spp., and *Laurencia* spp.; the sponges *Ircinia* spp. and *Terpios* spp.; the soft corals *Sarcophyton* spp. and *Dendronephthya* spp.; the stinging corals *Millepora* spp.; the sea cucumbers *Holothuria* spp.; and the diadematid sea urchins; to name but a few (Bakus, 1968; 1973; Bryan, 1973). Although these species may not reflect overall dominance, they dominate in patches. Patchy dominance, a phenomenon of scale, may be an ecologically significant factor in the tropics.

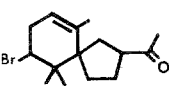
CHEMISTRY

Marine organisms often possess characteristic and/or unique chemical features. These components have served as the starting point for many chemical-ecological studies, although it has been shown that marine chemical–biological interactions are by no means restricted to uniquely marine compounds. The general classes of interest to marine chemical ecologists are outlined below.

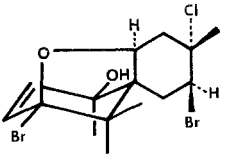
Terpenes. Terpenoid compounds have been isolated from algae, sponges, coelenterates, mollusks, and echinoderms. From algae alone more than 60 sesquiterpenes have been identified. These can be divided into 18 skeletal types, at least two thirds of which are new to science (Martin and Darias, 1978). More than 90 diterpenes have also been isolated from marine organisms. Of these, about 25% represent new skeletal classes (Fenical, 1978). Carbon–halogen bonds are characteristic of many marine terpenoids, particularly those isolated from algae. Bromine is the halogen most frequently incorporated into these marine secondary metabolites. Most typically, halogen-containing terpenes are found in algae in the genus *Laurencia* where they are thought to function in part as herbivore feeding deterrents (Figure 1) (e.g., Fenical, 1975; Erikson, 1983). The sea hare *Aplysia* spp. is one of the few herbivores adapted to grazing on *Laurencia* spp. Halogenated metabolites from the plant are concentrated in the sea hare and are thought to provide it with a means of defense against its own predators (Fenical, 1975). Other marine animals are capable of elaborating



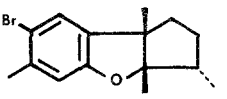
Isocaespitol



Spirolaurenone



Pacifenol

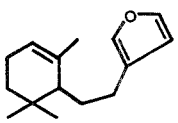


Aplysin

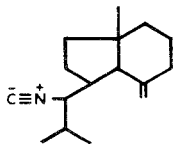
FIG. 1. Some terpenoid skeletal types typical of marine organisms include: the bisabolane, isocaespitol, from the red alga *Laurencia caespitosa* (Gonzalez et al., 1973; 1975); the chamigrane, spirolaurenone, from the red alga *Laurencia glandulifera* (Suzuki et al., 1970); pacifenol, a trihalogenated chamigrane from the red alga *Laurencia pacifica* (Sims et al., 1971); and the bromocuparane, aplysin, known from the opistobranch mollusk *Aplysia* and its algal diet, *Laurencia* (Yamamura and Hirata, 1963; Irie et al., 1969).

terpenoid metabolites de novo for use in chemical defense (Cimino et al., 1983). In sponges, terpenes represent the most abundant nonsteroidal metabolites; over 100 have been identified (Figure 2) (Minale, 1978). Sponge terpenes typically contain a multiplicity of prenyl units and furan rings (e.g., Cimino et al., 1971, 1975a,c). The naturally rare isonitrile function is also known from several sponge terpenoid compounds (e.g., Fattorusso et al., 1974, 1975). Sesterterpenes are also relatively abundant in sponges (Cimino et al., 1972; Fattorusso et al., 1972). In coelenterates, terpenes are known only from the Order Alcyonaria. These include sesquiterpenes and diterpenes. Most characteristic is the cembranolide skeleton (Figure 3) (Tursch et al., 1978). In general, terpenoid compounds are thought to function in antipredation, competition for space, and possible antifouling.

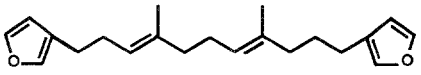
Steroids. The biosynthesis of steroids is intimately related to that of the higher terpenes, particularly the triterpenes. Marine organisms contain a far more diverse array of sterols than do terrestrial organisms. Marine organisms produce sterols with a remarkable variety of side chains, unconventional nuclear structures, and assorted hydroxylation patterns (Figure 4) (Schmitz, 1978). Sponges yield the most varied and biogenetically unprecedented array of sterols found among the invertebrate phyla (Goad, 1978). Dinoflagellate sterols are characterized by more heavily alkylated side chains and the presence of a 4-



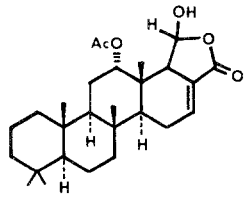
Pallescensin-1



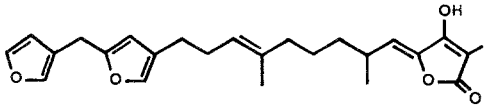
Axisonitrile-1



Anhydrofurospongin-1



Scalarin

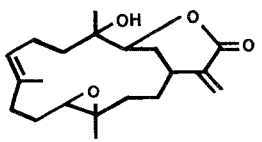


Ircinin-1

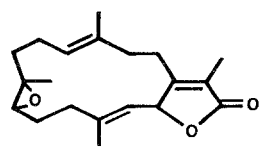
FIG. 2. Terpenoids typical of marine sponges include sesquiterpenes with furan rings (pallescensin-1, Cimino et al., 1975a–c) which are often joined with linear prenyl groups (anhydrofurospongin-1, Cimino et al., 1971), sesquiterpenes with the rare isonitrile functional group (axisonitrile-1, Cafieri et al., 1973), and linear and cyclic sesterterpenes (ircinin-1, Cimino et al. 1972; scalarin, Fattorusso et al., 1972).

alpha methyl group (Withers, 1983). The gorganians are characterized by their content of gorgosterol and related sterols (Goad, 1978; Withers, 1983), which contain a cyclopropane group in the side chain. Sterols are of interest principally because of their role in the production of steroidal hormones such as ecdysones with which regulate molting in Crustacea (Goad, 1976). They also are known to play a role in the defense of certain species (Burnell and ApSimon, 1983).

Carotenoids. Carotenoids are encountered in bacteria, algae, fungi, inver-

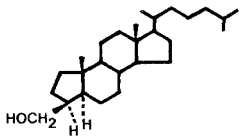


Flexibilide

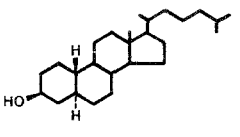


Sarcophine

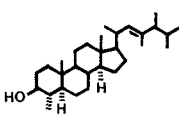
FIG. 3. Flexibilide and sarcophine, two biologically active cembranolide diterpenes from the soft corals *Sinularia flexibilis* and *Sarcophyton crassocaule* (Webb and Coll, 1983).



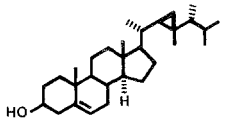
A-norstanol skeleton



19-norstanol skeleton



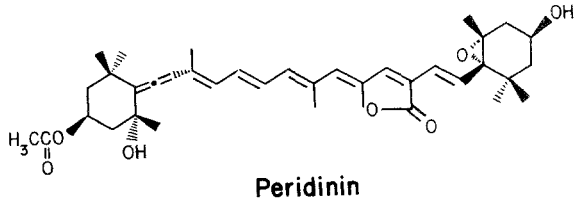
Dinosterol



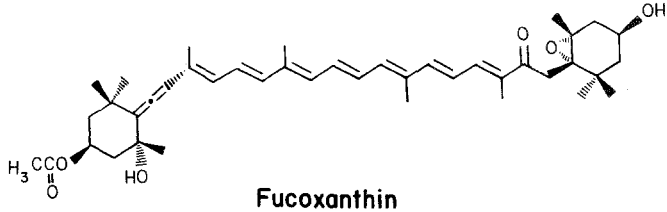
Gorgosterol

FIG. 4. Steroid types typical of marine organisms include: the A-norstanol and 19-norstanol skeletal modifications (Minale and Sodano, 1974a,b); dinosterol with the 4α-methyl group and additional side chain aklylation that is characteristic of many dinoflagellate sterols (Shimizu et al., 1976); and gorgosterol which shows the side chain with the cyclopropyl bridge typical of gorgonians and their dinoflagellate symbionts (Ciereszko et al., 1968).

tebrates, and vertebrates. More than 100 carotenoids have been isolated from marine sources and of these, about 40% are exlusively marine (Liaaen-Jensen, 1978). The most abundant are two carotenoids found in phlytoplankton, peridinin and fucoxanthin (Figure 5) (Strain et al., 1976). In general, marine carotenoids have more complex structures and more structural variation than do



Peridinin



Fucoxanthin

FIG. 5. Two exclusively marine carotenoids. Peridinin has a unique C_{37} skeletal structure bearing lactone, allenic, epoxy, acetoxy, and alcohol functions (Rapoport, 1971; Strain, 1976; Kjoesan et al., 1976).

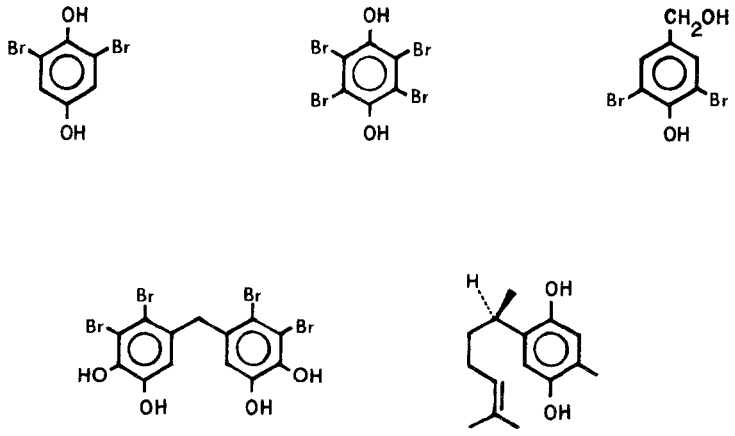


FIG. 6. Phenols arising from a variety of biosynthetic pathways are characteristic of many marine plants and invertebrates (Craigie and Gruenig, 1967; Kurata and Amiya, 1977; McEnroe and Fenical, 1978; Higa et al., 1980).

their terrestrial counterparts (Liaaen-Jensen, 1978). Marine carotenoids have a photoprotective role in photosynthetic bacteria and algae and play an important role in cryptic coloration and form (Burnett, 1976). The presence of oxygenated carotenoids is correlated with the tolerance of certain marine mollusks to environmental pollution, suggesting that they provide an intracellular reserve of oxygen (Karnaukhov et al., 1977). Carotenoids and carotenoproteins may also have a functional role in the reproduction of brown algae, crustaceans, and sea urchins (Lee, 1977; Hallenstvet et al., 1978). They are also useful in chemotaxonomy (Lee and Gilchrist, 1985).

Phenolics. Phenolic compounds occur in bacteria, algae, seagrasses, sponges, coelenterates, annelids, echinoderms, hemichordates, and protochordates. They are best known in brown and red algae, sponges, and echinoderms (Higa, 1981). More than 220 phenolics have been described from marine organisms (Figure 6). They arise via a variety of biosynthetic pathways. Their most obvious characteristic is the presence and abundance of halogenated substituents. Bromine is the halogen most frequently incorporated. Halogenated and nonhalogenated phenolics have been shown to have a multiplicity of potential ecological roles. For example, they are known to function as feeding deterrents in certain marine macrophytes (Valiela et al., 1979; Geiselman, 1980; Phillips and Towers, 1982a,b; Steinberg, 1984, 1985) thought to be responsible for defense or recognition in several species of enteropneusts (Higa, 1981).

Nitrogeneous Compounds. A wide variety of nitrogeneous compounds have been isolated from marine organisms, ranging from simple compounds such as tetramine to complex ones such as tetrodotoxin and saxitoxin (Figure 7). The latter are powerful neutrotoxins which inhibit sodium passage through axonal

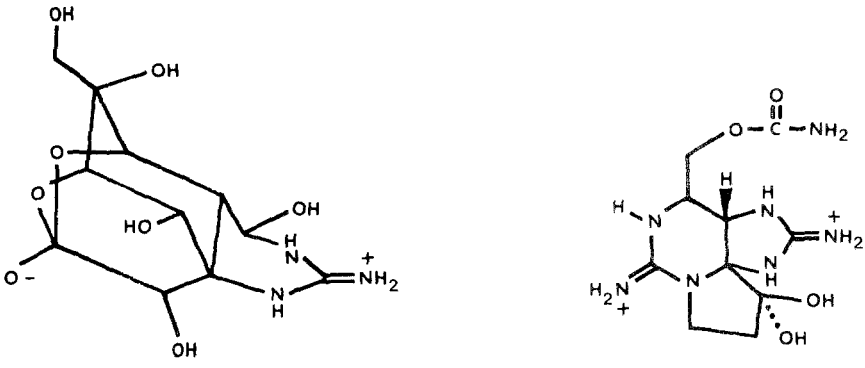


FIG. 7. Tetrodotoxin and saxitoxin are the two most widely known marine guanidino compounds (Woodward, 1964; Shimizu, 1978). Both have the high ratio of heteroatoms to carbon that is typical of many marine toxins.

membranes (Chevolot, 1981). Other simpler nitrogenous compounds play important roles in marine biological interactions. For example, gamma-aminobutyric acid (GABA), produced by encrusting algae (*Lithothamnium* and *Lithophyllum*), induces settlement and metamorphosis in abalone larvae (Morse et al., 1979; Morse and Morse, 1984). Alkaloids unique to the marine environment are being isolated and identified in growing numbers. They are often characterized by a bromine substituent. Haloindoles isolated from marine acorn worms (*Ptychodera flava* and *Glossobalanus* spp.) are responsible for the characteristic odor of these species and are thought to function as a chemical defense against micro- and macroorganisms (Figure 8) (Higa et al., 1980; Christopherson, 1983).

Compounds of Mixed Biogenesis and Miscellaneous Compounds. Many compounds are derived from the condensation of products arising from different biogenetic pathways (Figure 9). For example, asterosaponin A is an oligosaccharide with a steroidal aglycone. It is responsible for the pronounced avoidance behavior of numerous marine species when they are placed in the vicinity of a

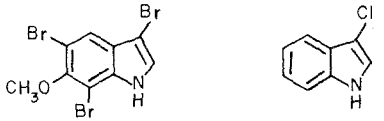
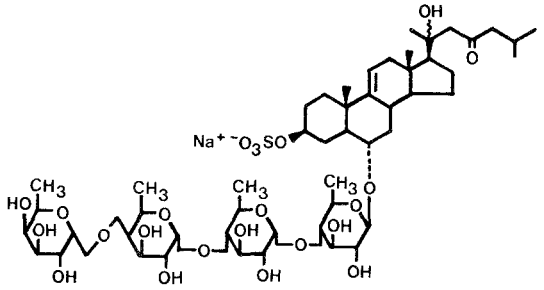


FIG. 8. Examples of halogenated indoles, these are found in marine acorn worms in the genera *Ptychodera* and *Glossobalanus* (Higa and Scheuer, 1977; Higa et al., 1980).

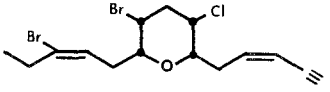


Asterosaponin A

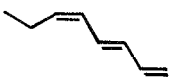
FIG. 9. A biologically active compound of mixed biogenesis, asterosaponin A is an oligosaccharide β-linked to a steroidal aglycone (Ikegami et al., 1972a–c).

$CHBr_3$, $CHBr_2I$, $CHBr_2Cl$
Haloforms

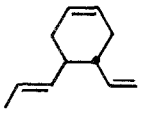
Cyclic polysulfides



Dactylyne



Fucoserratene



Aucantene

FIG. 10. Examples of miscellaneous compounds from marine organisms: Haloforms from the red alga *Asparagopsis* (Burreson et al., 1975, 1976); cyclic polysulfides from the red alga *Chondria californica* (Wratten and Faulkner, 1976); dactylyne, an enyne containing compound from the digestive gland of the sea hare *Aplysia dactylomela* (McDonald et al., 1975); and fucoserratene and aucantene, hydrocarbon pheromones from the brown algae *Fucus serratus* and *Cutlaria multifida*, respectively (Jaenicke et al., 1974).

starfish (Burnell and ApSimon, 1983). Miscellaneous compounds include compounds such as haloforms, acetylenes, sulfur compounds prostaglandins, and unusual lipids (Figure 10). To these compounds have been attributed a variety of roles, including chemical defense and species recognition (Jaenicke et al., 1974; Mueller, 1979; Gerhart, 1984).

Despite the considerable work on natural products chemicals since the early 1960s, many questions concerning the chemical nature of biological interactions remain unanswered. We need information on the origin of the compounds (de novo synthesis, diet, or symbiosis, e.g., Barrow, 1983; Catalan et al., 1985), whether the compounds are continuously produced or induced in response to an external stimulus, whether they are sequestered or free within their hosts, whether they are directly released into the sea, the nature of the released form compared with the stored form, and what the modes of action are. In an applied sense, we need to know more about the potential for these compounds as prototypes for antifoulants, pharmaceuticals, and agrichemicals (Colwell, 1983; Colwell et al., 1984).

CONCLUSIONS

For chemical ecologists, natural products chemistry has little meaning without a framework based on ecological and evolutionary theory. Similarly, understanding the fundamental mechanisms of many ecological interactions can be achieved only with an investigation of the chemistry involved. We are beginning to realize that current ecological theory may be too general or insensitive (e.g., in marine succession), that generalizations about populations will be made at a lower level of generality with much more detail and more restrictive conditions than previously anticipated (Krebs, 1985). It is also apparent that interdisciplinary studies between ecologists and chemists are rewarding because they seek the fundamental mechanisms behind ecological phenomena. Marine chemical ecology, an emerging discipline, seeks to create an interdisciplinary understanding of chemical–biological interactions occurring in the sea.

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CHEMICALLY STIMULATED FEEDING BEHAVIOR IN MARINE ANIMALS
Importance of Chemical Mixtures and Involvement of Mixture Interactions

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Abstract—A review is provided of the chemical components in tissue extracts that elicit feeding behavior in marine fish and crustaceans. For most species, the major stimulants of feeding behavior in excitatory extracts are an assemblage of common metabolites of low molecular weight including amino acids, quaternary ammonium compounds, nucleosides and nucleotides, and organic acids. It is often mixtures of substances rather than individual components that account for the stimulatory capacity of a natural extract. Recent studies using a shrimp, *Palaemonetes pugio*, are described in which behavioral bioassays were conducted with complex synthetic mixtures formulated on the basis of the composition of four tissue extracts. These results indicate that synergistic interactions occur among the mixture components. The neural mechanisms whereby marine crustaceans receive and code information about chemical mixtures are also reviewed. Narrowly tuned receptor cells, excited only by particular components of food extracts such as specific amino acids, nucleotides, quaternary ammonium compounds, and ammonium ions, are common in lobsters and could transmit information about mixtures as a labeled-line code. However, since physiological recordings indicate that most higher-level neurons in the brain each transmit information about many components of mixtures, rather than about a single component, it is suggested that information about a complex food odor is transmitted as an across-fiber pattern, instead of a labeled-line code. Electrophysiological recordings of responses of peripheral and central neurons of lobsters to odor mixtures and their components reveal that suppressive interactions occur, rather than the synergistic interactions noted earlier in the behavioral studies.

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Possible reasons for these differences are discussed. Evidence from the behavioral study indicates that the "direction" of a mixture interaction can be concentration-dependent and the synergism may occur at low mixture concentrations, while suppression may occur at high concentrations.

Key Words—Feeding behavior, chemoreception, mixture interaction, synergism, suppression, neural coding, chemical senses, crustaceans, fish, marine animals.

INTRODUCTION

Many marine animals have extremely well-developed chemical senses. The detection of external chemicals by the chemical senses affects many facets of the behavior of marine animals including the following: (1) elicitation of feeding behavior, including food recognition and ingestion, by scavengers (Carr, 1982), selective predators (Rittschof et al., 1984), and herbivores (Harada et al., 1984); (2) deterrence of feeding behavior (Sun and Fenical, 1979; Stoecker, 1980); (3) mate recognition and copulatory behavior (Dunham, 1978; Gleeson, 1980); (4) predator avoidance and alarm responses (Mackie and Grant, 1974; Sleeper et al., 1980); (5) selection of surfaces for settlement by larvae (Morse et al., 1979); (6) homing by migratory species (Stabell, 1984); (7) species recognition and social behavior (Liley, 1982); and (8) maintenance of symbiotic associations (Ache, 1974; Davenport, 1966).

In this presentation, we will focus upon studies of stimulants of aspects of feeding behavior in fishes and crustaceans. We will not attempt to review all of the earlier studies on feeding behavior in marine animals. For these, the reader should refer to the following: coelenterates (Lenhoff and Lindstedt, 1974); gastropods (Croll, 1983); crustaceans (Ache, 1982); fish (Hara, 1975; Atema, 1980, Carr 1982); general review (Mackie and Grant, 1974). More particularly, the focus will be upon studies of fishes and crustaceans in which quantitative bioassay procedures were employed to identify and to measure stimulatory chemicals that occur in the tissues of various prey organisms. We will show that, in most cases, the perceived "odors or tastes" of prey organisms are due to complex mixtures of chemicals. Recent behavioral data will then be presented to show that synergistic interactions occur among the components of several complex mixtures. Finally, we will discuss neural mechanisms identified in crustaceans for the reception and integration of information about complex odorants.

METHODS USED TO STUDY FEEDING BEHAVIOR

Several types of behavioral responses have been used with fishes to measure the stimulatory capacity of the active components of food odors or tastes.

These responses include the biting of an inert object emitting a chemical stimulus (Carr et al., 1976), ingestion of food pellets containing chemical stimulants (Mackie, 1982; Ohsugi et al., 1978; Ina and Higashi, 1978), attacking of food balls (Goh and Tamura, 1980; Hidaka, 1982), attraction and entrance into compartments containing stimulants (Hashimoto et al., 1968; Tsushima and Ina, 1978), and other aspects of behavior associated with food searches (Atema et al., 1980; Holland, 1978; Pawson, 1977). In crustaceans, the types of behavioral responses used to study food-related chemicals include the attraction to and grasping of a delivery device releasing a stimulant (Carr et al., 1984), movement toward the site of release of a stimulant (Ache et al., 1978); Mackie and Shelton, 1972; McLeese, 1970), and the onset of a series of components of behavior associated with the detection of food including antennular flicking, leg probing, movements of mouthparts, and locomotion (Derby and Atema, 1981; Pearson and Olla, 1977; Pearson et al., 1979; Zimmer-Faust and Case, 1983).

SUBSTANCES IN NATURAL EXTRACTS EVOKING ASPECTS OF FEEDING BEHAVIOR

Aqueous extracts prepared from the tissues of diverse organisms often prove to be highly excitatory stimulants of feeding behavior in marine animals. Several investigators have addressed the problem of fractionating and analyzing an excitatory extract in an effort to reproduce its activity with synthetic solutions. Table 1 summarizes the results of studies in fishes and crustaceans in which a substantial portion of the stimulatory capacity of an extract has been mimicked with synthetic solutions. The following three important generalities emerge from an inspection of the material presented in Table 1:

1. In the case of each extract described here, the principal stimulants of feeding behavior are common metabolites of low molecular weight that include amino acids, quaternary ammonium compounds, nucleosides and nucleotides, and organic acids. In some species of crustaceans, there are examples in which particular extracts or biological fluids contain attractants or feeding stimulants that are high-molecular-weight substances, such as proteins (Carr and Gurin, 1975; Zimmer-Faust et al., 1984). However, in most cases it is clear that the principal stimulants are low-molecular-weight substances of the sort cited in Table 1.

2. When a single excitatory extract is analyzed and tested behaviorally in more than one species, the different test species may respond to different substances in the same extract. Note in Table 1 that a similar squid extract was used in studies with the turbot (No. 3), rainbow trout (No. 4), and plaice (No. 11). With each fish species, the major stimulants in the squid extract were different. For the turbot, inosine was the major stimulant, whereas the rainbow

TABLE 1. STUDIES OF CHEMICALLY STIMULATED FEEDING BEHAVIOR IN FISH AND CRUSTACEANS IN WHICH A SUBSTANTIAL PART OF THE ACTIVITY OF AN EXTRACT WAS ACCOUNTED FOR WITH A SINGLE SUBSTANCE OR A MIXTURE OF SUBSTANCES

Animal studied[a]	Substances required to mimic activity of extract	Source of extract	Behavioral response
A. Fish	Single substances		
1. Dover sole	Betaine or betaine + glycine	Mussel	Ingest pellet
2. Pigfish	Betaine	Oyster	Biting response
3. Turbot	Inosine	Squid	Ingest pellet
	Mixture of substances		
4. Rainbow trout	Amino acids	Squid	Ingest pellet
5. Japanese eel	Amino acids	Clam	Attraction
6. European eel	Amino acids	Squid	Ingest ration
7. Red sea bream	Amino acids	Worm	Ingest pellet
8. Pigfish	Amino acids and betaine	Crab, shrimp	Biting response
9. Pinfish	Amino acids and betaine	Shrimp[b]	Biting response
10. Puffer	Amino acids and betaine	Clam	Ingest pellet
11. Plaice	Amino acids, quaternary ammonium compounds, nucleotides, nucleosides and lactate	Squid	Ingest pellet
B. Crustaceans			
12. Lobster	Amino acids, quaternary ammonium compounds, nucleotides, nucleosides and lactate	Squid	Attraction
13. Shrimp	Amino acids, quaternary ammonium compounds, nucleotides, nucleosides and lactate	Crab, shrimp	Grasp delivery device

[a] References for animal number cited: 1—Mackie et al., 1980; Mackie and Mitchell, 1982. 2—Carr et al., 1977. 3—Mackie and Adron, 1978. 4—Mackie, 1982. 5—Hashimoto et al., 1968; 6—Mackie and Mitchell, 1983. 7—Fuke et al., 1981. 8—Carr, 1976; Carr et al., 1977. 9—Carr and Chaney, 1976. 10—Ohsugi et al., 1978; Hidaka, 1982. 11—Mackie, 1982. 12—Mackie, 1973. 13—Carr et al., 1984; Carr and Derby, 1986.

[b] For the pinfish, a synthetic mixture or amino acids and betaine accounted for about 20% of extract's activity.

trout required a mixture of amino acids, and the plaice required an even more complex mixture of substances in the same extract.

3. The stimulatory capacity of most extracts is due to a mixture of substances acting in concert rather than to a single dominant substance. In 10 of the 13 examples cited in Table 1, mixtures were necessary to yield synthetic stimulants with stimulatory capacities similar to those of the natural extracts.

EVIDENCE OF MIXTURE INTERACTIONS IN RESPONSES OF SHRIMP TO MIXTURES OF FOOD COMPONENTS

Mixtures—Additive vs. Interactive Effects

Rifkin and Bartoshuk (1980) described the following two methods whereby a mixture of components could be treated in an additive manner by a chemosensory system: (1) addition of perceived intensities (referred to below as response summation), and (2) stimulus summation (= stimulus substitution). Response summation would be more likely when two or more stimulants activate different receptor sites. Stimulus summation would be expected to occur when two or more stimuli activate the same receptor sites.

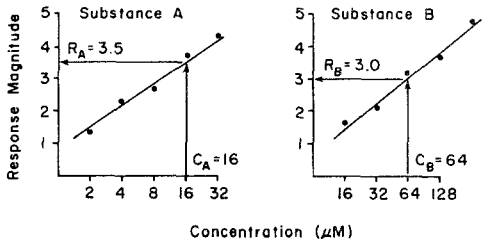
Figure 1 depicts the application of these additive methods toward predicting the effects of a mixture of two substances whose individual dose–response (D-R) functions have been measured. The response summation method (IIA in Figure 1) predicts that when the two substances are mixed at known concentrations, the response elicited will be equal to the simple sum of the responses elicited by the unmixed components. The stimulus summation method (IIB in Figure 1) treats the mixture as a higher concentration of one component and uses the D-R function of that component to predict the response to the "higher concentration" of that component.

A mixture interaction, which may express itself as either synergism or suppression, is considered to have occurred when a mixture of substances is observed to evoke a response that is different in magnitude than the response predicted by applying the additive methods described above (Rifkin and Bartoshuk, 1980; Derby and Ache, 1984b). Whereas the results of some of the behavioral studies cited in Table 1 suggest that mixture interactions may indeed be occurring, only the results of the following study with a marine shrimp have been subjected to the quantitative analyses required to document such an interaction.

Analysis of Effectiveness of Chemical Mixtures and Components

The synthetic chemical mixtures shown in Table 2 were used to study behavioral chemoattractants of the shrimp, *Palaemonetes pugio*. This shrimp is an opportunistic scavenger that feeds upon detritus (Welsh, 1975) and small

I. DOSE RESPONSE CURVES FOR SUBSTANCES A AND B.



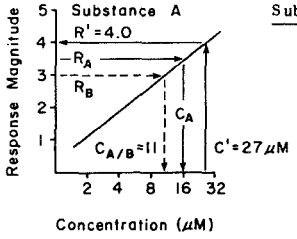
II. PREDICTING THE RESPONSE TO A STIMULATORY MIXTURE CONTAINING: 16μM A PLUS 64 μM B

A. Response Summation: Sum responses elicited by individual substances.

1. Concentration of A(=C_A) = 16 μM; Response to C_A = 3.5 (=R_A)
2. Concentration of B(=C_B) = 64 μM; Response to C_B = 3.0 (=R_B)

Sum of R_A + R_B = 6.5 =PREDICTED RESPONSE

B. Stimulus Summation: Use the response elicited by B (=R_B) to express the mixture as a higher concentration of A.



Substance	Response to Substance	Concentration of A Eliciting Same Response
A	3.5(=R_A)	16 μM (=C_A)
B	3.0(=R_B)	11 μM (=$C_{A/B}$)

Sum of C_A + $C_{A/B}$ = 27 μM (=C')

Response to C' = 4.0 (=R') = PREDICTED RESPONSE

FIG. 1. Using the methods of response summation and stimulus summation to predict the response to a mixture of substances whose effects are additive. See text for further explanation.

invertebrates (Bell and Coull, 1978) in the field and is attracted to and feeds upon the soft tissues of a broad array of organisms in the laboratory. For behavioral bioassays, groups of shrimp were tested in compartmented boxes into which solutions of defined concentrations were delivered at a controlled rate. Each solution was tested over a range of concentrations, and the number of animals giving a positive response was recorded. A positive response consisted of a shrimp moving to, and grasping, the delivery device within a 2-min period. The percentage of animals giving a positive response was then transformed to the percentage of the maximum response obtained during the study. Further procedural details are given in Carr and Derby (1986).

Table 2 shows the results of analyses of the amino acids, nucleotides and related substances, quaternary ammonium compounds, and organic acids in

aqueous extracts of crab, shrimp, oyster and mullet. These analyses were used to prepare synthetic mixtures containing substances at the same relative concentrations as present in the natural extracts. The "extracts" that were analyzed in the current study were prepared by the gentle shaking in cold water of diced tissue obtained from live organisms. Unlike extracts prepared by blending or homogenizing tissues, the extracts obtained by the current procedure include only those molecules that readily diffuse from recently injured tissues, thereby providing a stimulus that is potentially excitatory to an array of opportunistic scavengers such as *P. pugio*.

Figure 2 shows the dose–response (D-R) regressions obtained for the artificial mixtures based on the composition of oyster (AOM), shrimp (ASM), crab (ACM), and mullet (AMM), and for eight compounds that both occur in the mixtures and elicit behavioral responses when tested individually at concentrations of 1 mM or less. These stimulatory compounds were the nucleotides AMP, XMP, GMP, ADP, and IMP, the amino acids glycine and taurine, and a quaternary ammonium compound, betaine. Other than these eight compounds, only three compounds in Table 1 (the amino acids alanine, hydroxyproline, and phenylalanine) had measurable activities when tested individually. These latter substances were omitted from Figure 1 and from the analyses of interactions given below because they occur in the mixtures at subthreshold concentrations.

Evidence for Synergistic Interactions among Components of Synthetic Mixtures

Figure 3 depicts how the methods of response summation and stimulus summation described earlier are applied to provide predicted responses to a single dose of one of the complex mixtures cited in Figure 2. In the example (Figure 3), the stimulatory mixture is a 96 μM dose of ASM, a dose that evokes 90% of the maximum response. The figure shows the D-R functions for the only four components of ASM (AMP, Gly, Tau, and Bet) that are present at suprathreshold concentrations within the range of concentrations at which ASM was tested. The concentrations of AMP, Gly, Tau, and Bet in 96 μM ASM are 1.39, 26.72, 9.41, and 14.37 μM, respectively, and are represented in this figure as C_{AMP}, C_{Gly}, C_{Tau}, and C_{Bet}. The responses elicited by C_{AMP}, C_{Gly}, C_{Tau}, and C_{Bet}, as determined by their individual regression lines, are 43.0, 19.7, 3.8, and 0%, respectively, and are represented on the y axis as R_{AMP}, R_{Gly}, and R_{Tau}. (C_{Bet} is a subthreshold concentration and thus is not stimulatory by itself at the concentration found in 96 μM ASM.)

Response Predicted by Response Summation. The sum of the individual responses is 66.5% (ΣR in Figure 3), which is less than the measured response value of 90%.

Response Predicted by Stimulus Summation. The active components are expressed in terms of a higher concentration of AMP, the most contributory

TABLE 2. COMPOSITION OF EXTRACTS[a]

Substance (abbreviation)[b]	Concentration (mM) in extract[c]			
	Crab	Shrimp	Oyster	Mullet
I. Amino acids[d]				
Alanine	2.14	3.54	1.22	0.440
β-Alanine			0.287	0.004
α-Aminobutyric acid				0.005
Arginine	2.29	1.47	0.103	0.046
Asparagine	0.195	0.167	0.038	0.057
Aspartic acid	0.036	0.138	0.164	0.056
Cysteine	0.120		0.021	
Glutamic acid	0.284	0.275	0.169	0.070
Glutamine	2.95	1.05	0.129	0.085
Glycine (Gly)	11.34	15.62	0.798	0.509
Histidine	0.138	0.054	0.016	1.12
Hydroxyproline			0.041	0.010
Isoleucine	0.107	0.176	0.006	0.024
Leucine	0.245	0.317	0.013	0.043
Lysine	0.227	0.081	0.055	0.196
Methionine	0.392	0.173	0.004	0.012
3-Methylhistidine				0.020
Ornithine			0.031	0.045
Phenylalanine	0.093	0.090	0.001	0.014
Phosphoserine				0.015
Proline	5.57	1.90	0.409	0.079
Serine	0.265	0.224	0.112	0.082
Taurine (Tau)	1.88	5.50	5.52	1.90

Threonine	0.404	0.106	0.019	0.080
Tryptophan	0.131			
Tyrosine	0.101	0.153	0.009	0.006
Valine	0.318	0.384	0.014	0.046
Total	29.226	31.418	9.179	4.964
II. Nucleotides, -sides and related				
Adenosine 5′-monophosphate (AMP)	0.025	0.813	0.099	0.003
Adenosine 5′-diphosphate (ADP)	0.182	0.238	0.008	0.002
Adenosine 5′-triphosphate (ATP)	0.878	0.066		
Guanosine 5′-monophosphate (GMP)			0.013	0.010
Inosine 5′-monophosphate (IMP)	0.158	0.333	0.019	0.688
Xanthosine 5′-monophosphate (XMP)				0.029
Hypoxanthine	0.033	0.038		0.012
Inosine	0.069	0.010	0.012	0.166
Total	1.345	1.463	0.136	0.900
III. Quaternary ammonium compounds				
Betaine (Bet)	3.70	8.40	3.38	1.04
Homarine	0.469	1.15	0.332	
Trimethylamine oxide (TMO)	3.38	9.0		0.990
Total	7.549	18.55	3.712	2.03
IV. Organic acids				
L-Lactic acid	4.35	4.58	0.091	5.72
D-Lactic acid			0.015	
Succinic acid			0.357	
Grand total	42.47	56.06	13.49	13.614

[a] From Carr and Derby, 1986.
[b] Abbreviation of substance that is used in text.
[c] A blank space indicates the substance was not detected in the extract.
[d] L-Amino acids were used in artificial mixtures.

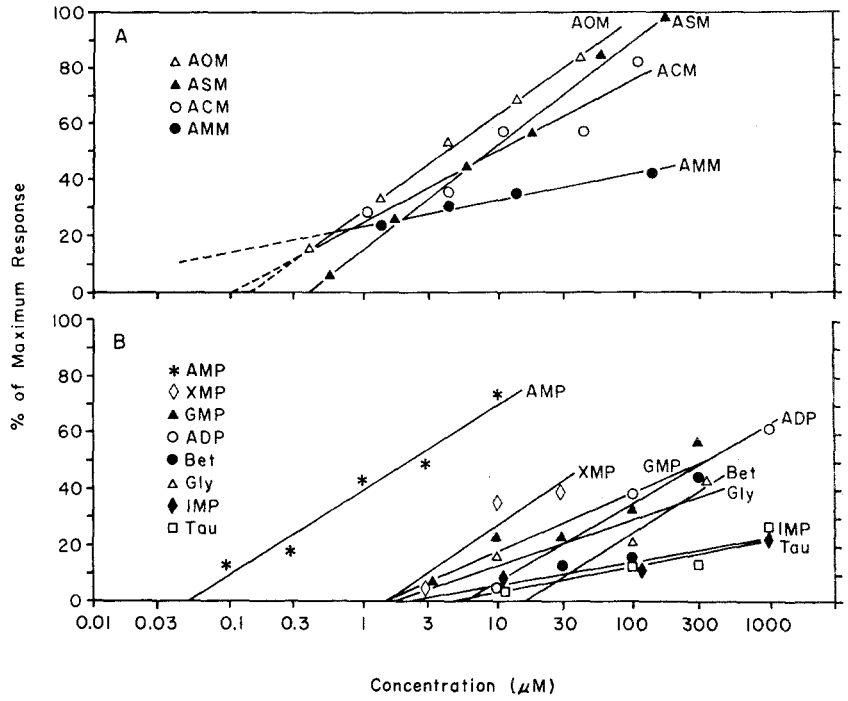


FIG. 2. Dose–response functions for mixtures and individual components tested with *P. pugio*. Responses are expressed as a percentage of the maximum response evoked by any stimulus. (A) Mixtures: artificial oyster mixture (AOM), artificial shrimp mixture (ASM), artificial crab mixture (ACM), artificial mullet mixture (AMM). (B) Components: AMP, XMP, GMP, ADP, IMP, betaine, glycine, taurine. In both A and B, regression lines of best fit for the data are shown for each mixture and component. Abbreviations for components are as shown in Table 2. Modified from Carr and Derby (1986).

component in the mixture. The AMP-equivalent concentrations for R_{AMP}, R_{Gly}, and R_{Tau} are 1.39, 0.24, and 0.07 μM, respectively (represented in Figure 3 by C_{AMP}, $C_{AMP/Gly}$, and $C_{AMP/Tau}$). The sum of these concentrations is depicted as C′ (= 1.70 μM). This represents a "mixture" of AMP at a total concentration equivalent in effect to the amounts of AMP, Gly, and Tau in a 96 μM dose of ASM. The response elicited by concentration C′ is determined from the AMP regression line to be 45.6% (ΣS) which is much less than the measured response of 90% that is actually evoked by a 96 μM dose of ASM.

Occurrence of Synergistic Interactions over a Range of Concentrations. In Figure 4, the D-R functions observed during the bioassays of the four artificial mixtures are compared with the predicted functions obtained by applying the additive methods of response summation (ΣR) and stimulus summation (ΣS). Synergistic interactions occurred over the entire response range at which the

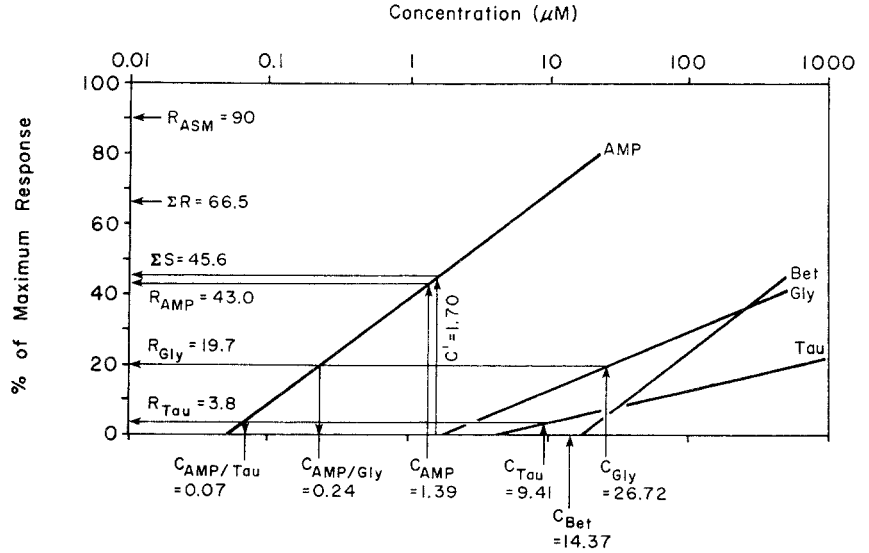


FIG. 3. Application of additive methods to calculate predicted responses to a mixture of components. The stimulatory mixture is 96 μM ASM (artificial shrimp mixture), a dose that evokes a 90% maximal response (see Figure 1). Magnitude of response to 96 μM ASM is shown as R_{ASM}. Calculations are based on D-R lines of best fit for each component as shown in Figure 2. See text for description of how methods are used to obtain a predicted response. Modified from Carr and Derby (1986).

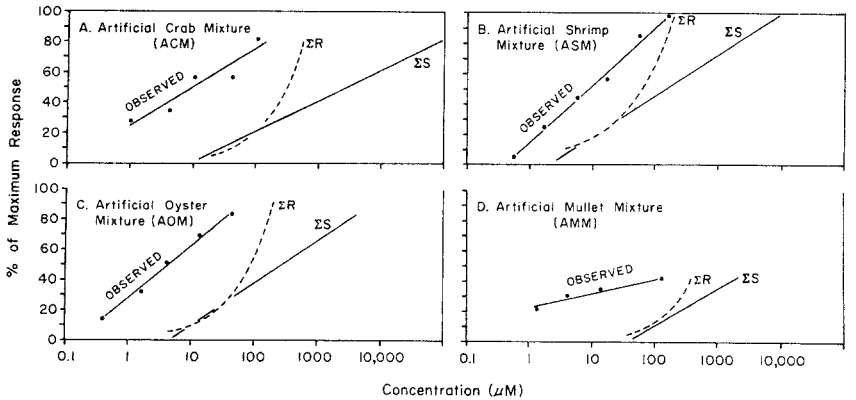


FIG. 4. Comparison of the observed and predicted D-R functions for four artificial mixtures tested with the shrimp, *P. pugio*. (A) Crab; (B) shrimp; (C) oyster; (D) mullet. For each mixture, the observed D-R curve is compared to the curves predicted by response summation (ΣR) and stimulus summation (ΣS). Modified from Carr and Derby (1986).

mixtures were tested since each mixture was far more effective than predicted by either of the additive models. In each of the four mixtures, the D-R function for the observed result is situated to the left of the functions for the predicted results. Hence, in each case the mixture concentration predicted to elicit a response of a particular magnitude is a greater concentration than that actually required to elicit such a response. A major consequence of synergistic actions such as those shown in Figure 4 is that they provide an organism with the capability for detecting and responding to mixtures at concentrations below the response thresholds for even the most stimulatory individual components in the mixture. Note that responses as high as 20–40% of the maximum response are elicited by mixture concentrations that are predicted by the additive D-R functions to be subthreshold.

NEURAL MECHANISMS IN CRUSTACEANS FOR EXTRACTING INFORMATION FROM CHEMICAL MIXTURES

Chemosensory Systems of Crustaceans

Chemosensory sensilla, sensory hairs containing chemoreceptor cells, are located on much of the body surface of aquatic crustaceans, including both pairs of antennae (antennules and antennae), the six pairs of mouthparts, and the claws and walking legs (Ache, 1982). The antennules, containing aesthetasc sensilla, are usually referred to as olfactory (smell) organs, whereas antennae, walking legs, and mouthparts are usually referred to as gustatory (taste) organs. The distinction between organs of olfaction and gustation in aquatic animals cannot be made on the basis of the carrier medium, as with terrestrial animals. Nonetheless, a distinction may be possible on the basis of other criteria, including differences in central projections of axons of receptor cells from different organs and differences in behavioral functions governed by different organs (Atema, 1977, 1980).

Neural Coding of Single Compounds

Because of their large size, lobsters have frequently been selected for electrophysiological studies of crustacean chemosensory systems. Electrophysiological responses given by chemosensory neurons have been recorded during the application of the individual components of excitatory mixtures onto sensilla on the antennules and walking legs. This provides a first approach to understanding how these systems encode and decode the information contained in chemical mixtures. Recordings from the antennules or the walking legs of the spiny lobster (*Panulirus argus*) and the American lobster (*Homarus americanus*) indicate that many of the chemoreceptor cells possess narrow excitatory response spectra (see Figure 5). Receptor cells with narrow excitatory response

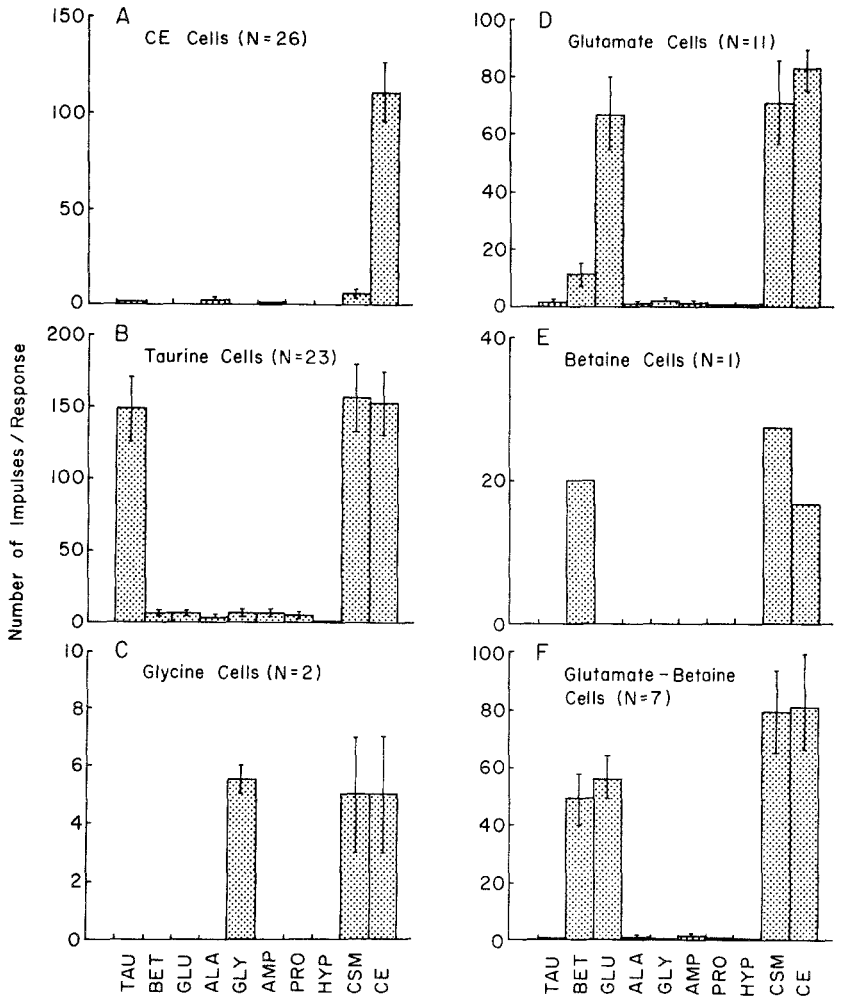


FIG. 5. Excitatory response spectra for six types of chemoreceptor cells in the antennules of the spiny lobster, *P. argus*. Responses are to taurine, betaine, glutamic acid, alanine, glycine, AMP, proline, hydroxyproline, artificial crab mixture (CMS, this mixture is similar but simpler than that in Table 1 and has a total concentration of 0.33 mM), and crab extract (CE). Each individual compound was tested at the concentration that it occurs in the mixture and extract. Each cell is excited primarily by only one (or, in the case of the glutamate–betaine cells, two) of the eight individual compounds, and the response to this compound is usually similar to the response to the complete mixture or extract. From Derby and Ache (1984a).

spectra are strongly excited by one or a very limited number of the many compounds tested. Several distinct classes of such narrowly tuned receptor cells are now known, including those excited only by a single amino acid (either taurine, glutamine, glycine, arginine, hydroxyproline, or glutamine), by nucleotides (adenosine 5′-monophosphate), by quaternary ammonium compounds (betaine), or by ammonium ions (Fuzessery et al., 1978; Derby and Atema, 1982; Derby and Ache, 1984a; Derby et al., 1984a; Johnson and Atema, 1983; Johnson et al., 1984). Other receptor cells having broader excitatory response spectra have also been identified, but they appear to be less numerous.

If one considers only the narrow excitatory response spectra of specific populations of chemoreceptor cells such as those described above for lobsters, then such populations of cells could theoretically function as labeled-line arrays in the detection of food chemicals. In a labeled-line system, the identity of a specific compound in the chemical environment is made on the basis of the activation of cells specifically sensitive to that compound. Those chemicals for which labeled lines exist presumably elicit specific behaviors.

A labeled-line code would be most advantageous if the labeled-line information is transmitted relatively unchanged to subsequent levels of the chemosensory neural pathway and eventually to motor neurons controlling the relevant behavior. An analysis of responses of olfactory interneurons in the brain of the spiny lobster reveals that this is not the case for most interneurons and for most chemicals that have been examined. Progressing inward from the olfactory receptor cells to successively higher neural levels, cells become increasingly more broadly tuned, with interneurons usually responding to many more than one chemical (Derby and Ache, 1984a; Derby et al., 1984b). This fact strongly suggests that stimulus quality for most chemicals is probably not encoded in the brain of the spiny lobster by labeled-line activity, but rather as the patterns of activity generated across a large population of neurons, a so-called across-neuron pattern code (Erickson, 1974). Indeed, statistical analyses indicate that the across-neuron patterns for different food-related chemicals can be quite distinct, indicating that these chemicals are probably discriminable (see Figure 6). Nonetheless, there exists a smaller number of brain interneurons in the spiny lobster that have response specificities nearly as narrow as those of the specialized receptor cells, suggesting that labeled-line information generated at the receptor level can be retained by some neurons in the brain, at least for some stimulants (Derby and Ache, 1984a; Derby et al., 1984b). The contribution of such narrowly tuned interneurons toward coding of odor quality in this system is presently unknown but is under investigation.

Neural Coding of Mixtures

The selection of compounds to be tested in experiments of quality coding, we believe, should be dictated by the natural history of the animal and not solely

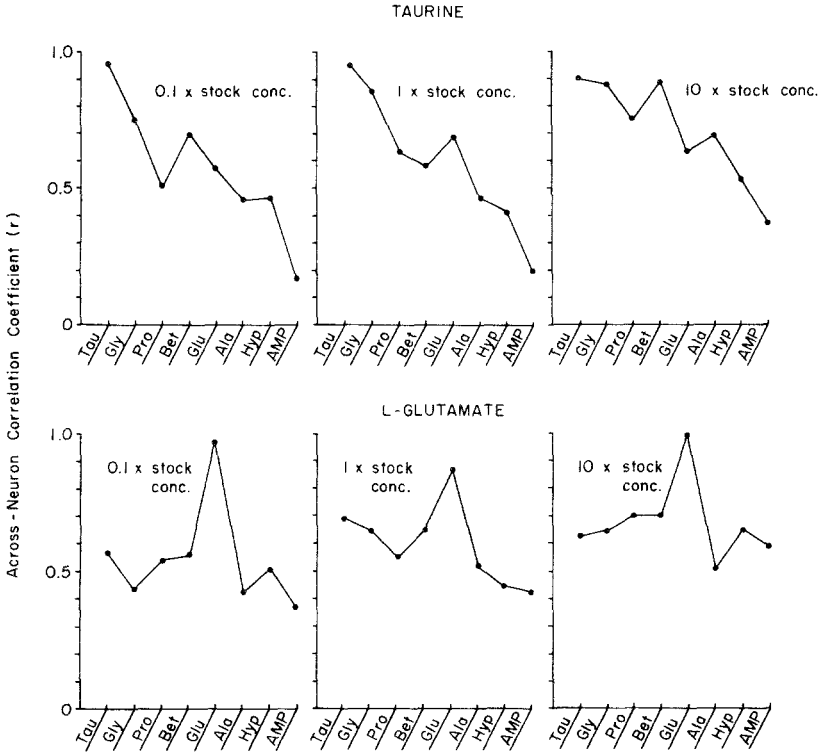


FIG. 6. Across-neuron pattern coding of chemical quality by olfactory neurons in the brain of *P. argus*. Shown are across-neuron correlation profiles for the responses of 45 neurons to taurine and glutamate, each at three concentrations. Each point represents the correlation coefficient (from Pearson's product moment correlation) between the responses of the 45 interneurons to the compound at the top (taurine or glutamate, at one of three concentrations) and the responses of the same neurons to one of the eight compounds tested. "Stock concentration" refers to the concentration that the particular compound is found in the artificial crab mixture used in this experiment (described in legend to Figure 5). The shapes of the six correlation profiles are representative of similarities and differences in across-neuron patterns for the six stimuli. Stimuli eliciting profiles of similar shape are less likely to be differentiated by these neurons; stimuli eliciting profile of different shape are more likely to be differentiated by these neurons. Thus, the three concentrations of taurine, which evoke similar across-neuron correlation profiles, are probably determined by this population of neurons to be of a similar sensory quality. On the other hand, taurine and glutamate, which have different across-neuron correlation profiles, are probably judged by these neurons to be of different sensory qualities. Hence, by using across-neuron patterns as a code for chemical quality, these neurons could differentiate chemical qualities (e.g., taurine vs. glutamate) in spite of changes in chemical quantity.

by the availability of chemicals or by other arbitrary means. Likewise, for studies of the neural coding of mixtures, it is most appropriate to utilize naturally occurring mixtures. The foregoing criteria are satisfied in the studies with lobsters cited below.

Neurophysiological studies reveal that the magnitude of the neural responses to particular mixtures cannot always be predicted from the responses to the individual components in that mixture. In a neurophysiological study of brain interneurons in the spiny lobster (Derby and Ache, 1984b), the observed responses to mixtures were compared to the predicted responses obtained by using additive models similar to those described earlier for behavioral studies with shrimp. Unlike the synergistic interactions described earlier for the shrimp behavioral studies, the lobster physiological study has shown suppressive mixture interactions to be most prevalent (Figure 7). Subsequent studies have shown

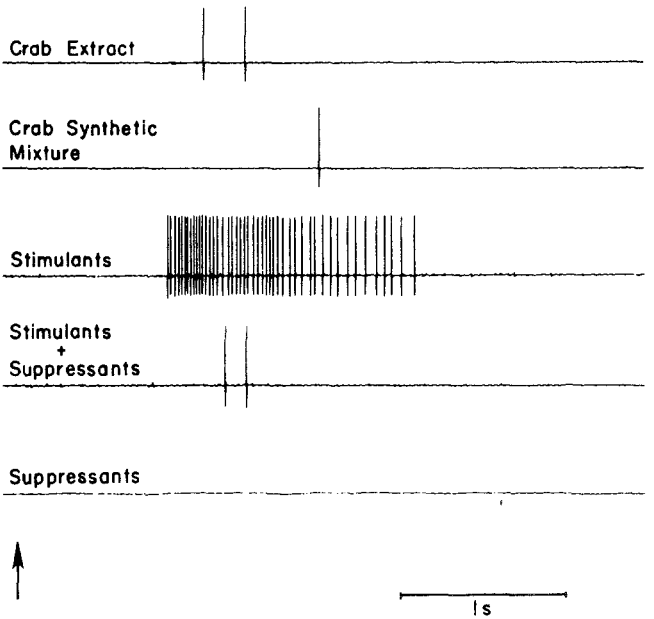


FIG. 7. Mixture suppression in an olfactory interneuron in the brain of *P. argus*. Responses are to the crab extract, the artificial crab mixture (Table 1, with a total concentration of 4.25 mM), a mixture of 12 stimulants in the artificial crab mixture (AMP, ADP, ATP, alanine, betaine, glutamic acid, glutamine, glycine, lactic acid, phenylalanine, taurine, trimethylamine oxide), a mixture of three suppressants in the artificial crab mixture (arginine, cysteine, proline), and a mixture of the 12 stimulants plus the three suppressants. These data show that addition of the suppressants to the stimulants results in a dramatic reduction in the ability of the stimulants to excite this interneuron, i.e., mixture suppression occurs. Arrow indicates stimulus introduction. Modified from Derby and Ache (1984b).

that suppressive mixture interactions also occur in several classes of receptor cells in lobsters (Derby et al., 1985; Gleeson and Ache, 1985; Johnson et al., 1985). Several of the suppressant substances present in natural mixtures have been identified for these receptor cells (Derby et al., 1985; Gleeson and Ache, 1985). However, not all receptor cells show mixture suppression at all mixture concentrations (Derby and Ache, 1984a; Derby et al., 1985; Gleeson and Ache, 1985). In fact, mixture synergism has been reported to occur at low mixture concentrations for some types of receptor cells (Johnson et al., 1985).

In an attempt to identify mechanisms responsible for mixture interactions in one class of olfactory receptor cell excited by taurine, Gleeson and Ache (1985) have performed a kinetic analysis of responses evoked by binary mixtures of stimulants and suppressants identified for the spiny lobster. This analysis requires the assumption that the frequency of action potential output from receptor cells is an accurate monitor of receptor–ligand binding. The results with this class of cells indicate that the mixture suppression is partly, possibly largely, a result of competitive inhibition among the components of the binary mixtures. Mixture interactions in other classes of receptor cells do not appear to conform strictly to either competitive or noncompetitive kinetics (Johnson et al., 1985).

Studies of olfactory interneurons in the brain of the spiny lobster have extended our understanding of the origin of mixture interactions. Not only is mixture suppression of peripheral origin manifested in brain interneurons, but some mixture suppression is even generated within the central nervous system, adding to that originating in the peripheral nervous system. This is supported by the observation that mixture suppression is expressed in some interneurons when stimulatory compounds and suppressive compounds are presented simultaneously to *separate* regions of the receptive field of a particular neuron (Derby et al., 1985).

In evaluating the significance of mixture interactions with respect to the coding of chemical quality, we must be aware of additional complexities inherent in this system. Firstly, although mixture interactions are known to occur, they probably are not expressed in all receptor neurons or in all neurons of higher order. Secondly, those neurons that do express a mixture interaction (e.g., suppression) may do so in a concentration-dependent fashion (Johnston et al., 1985). These complexities imply that coding by receptor cells, even those with narrow excitatory response spectra, may not be as simple as we originally thought. Thus, it is likely that even at the receptor level, chemical quality is coded as an across-neuron pattern. In fact, the added complexity of mixture suppression seen clearly in lobsters may make an important contribution to the coding of different chemical qualities. The superimposed effects of excitation and suppression could theoretically create even more distinct differences among across-neuron patterns for the numerous mixtures of chemicals likely to be encountered by animals in their natural chemical world (Derby, 1986).

ENIGMA OF CONTRASTING RESULTS OF BEHAVIORAL AND PHYSIOLOGICAL STUDIES

The results of the behavioral study described earlier in the section on synergistic interactions show clearly that synergistic interactions were expressed in the behavioral responses given by the shrimp, *P. pugio*, to four artificial chemical mixtures. One of the same artificial mixtures (artificial crab mixture) that expressed synergism in the behavioral study was found to express suppressive interactions when used in physiological studies with the spiny lobster, *P. argus*. In fact, the phyisological studies revealed mixture suppression at three levels of the olfactory system extending from receptor cells to brain-output interneurons (Derby and Ache, 1984a, b; Gleeson and Ache, 1985; Derby et al., 1985).

We do not yet understand why the behavioral studies revealed mixture interactions that were opposite in "direction" to those expressed by the physiological studies. One possible explanation is that the neurons expressing mixture suppression may not be part of the neural pathway mediating feeding behavior. The identity and behavioral function of the individual neurons showing suppression is unknown. However, since mixture suppression is expressed pervasively in several levels of the nervous system, it is very likely that at least some of these neurons influence walking, grasping, or other activities associated with the lobster's feeding behavior. A second possible explanation of the difference in the behavioral and physiological results is that the magnitude of a behavioral response may not be predicted by the magnitude of a physiological response that is generated solely by the olfactory system. The latter is supported by the following observations of lobster behavior.

In a behavioral trapping study done with spiny lobsters, an artificial crab mixture (similar to that used in the behavioral study with shrimp) was very effective at stimulating lobsters to enter a trap. However, two individual components of the mixture, taurine and AMP, each with marked physiological activity, did not stimulate trap entry. Furthermore, tests of submixtures containing either five or ten of the components, identified physiologically to be among the major olfactory stimulants in the complete artificial crab mixture, revealed that each submixture was either ineffective or less effective than the complete mixture in stimulating entry into traps (Carr, Ache and Milstead, unpublished results). The foregoing shows that physiological measurements of events occurring in the antennular chemosensory system are not necessarily reliable predictors of a behavioral response expressed by the whole animal. Behavioral responses of the sort observed in the shrimp or lobster may be evoked by a multimodal process that includes inputs from chemosensory cells in the antennae, legs, and/or mouthparts, in addition to those in the antennules. Synergistic interactions expressed by a multi-modal process may not be expressed when one examines only a single component of that process.

Another factor that may influence the apparent directionality of a mixture

interaction is the particular concentration(s) at which the mixture is tested. Behavioral data presented in Figure 8 show that the apparent direction of a mixture interaction can change dramatically when the mixture is tested over a broad range of concentrations. The figure shows that a biphasic D-R curve is obtained when shrimp are tested behaviorally with crab extract at concentrations higher than those depicted earlier (cf., Figure 4A). Note the convergence and intersection of the D-R curves for the observed behavioral responses and the responses predicted by the additive method of response summation. The extended curves imply that at mixture concentrations greater than ca. 800 μM, which is a saturating concentration, a zone of increasing mixture suppression is encountered in which the concentration predicted to elicit a response of a particular

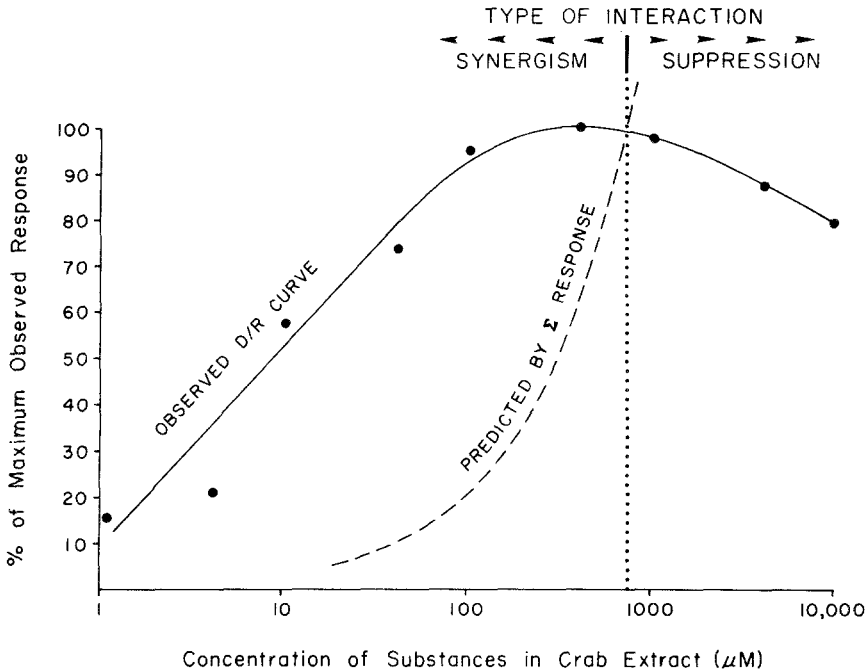


FIG. 8. Effect of concentration on the direction of mixture interactions as shown by behavioral responses of shrimp, *P. pugio,* to a crab extract tested over a wide range of concentrations (N = 756 shrimp). Concentrations given are based on the total concentration of all substances measured in crab extract as shown in Table 2. The predicted D-R curve obtained by the additive method of response summation is the same as that shown in Figure 4A for the artificial crab mixture (ACM). The convergence and intersection of the curves indicate that synergistic interactions occur at concentrations less than about 800 μM, whereas suppressive interactions occur at greater concentrations. The biphasic nature of the observed D-R curve is characteristic of all stimulants we have tested behaviorally over a wide concentration range (e.g., see Carr and Thompson, 1983).

magnitude is a lesser concentration than that actually observed to elicit such a response. In short, at high saturating concentrations the mixture is less effective than predicted by the method of response summation. This relationship at high mixture concentrations is in direct contrast to that of the synergism seen to occur at lower mixture concentrations where the mixture is more effective than predicted. Electrophysiological studies are currently in progress with the spiny lobster to determine if a change in the direction of a mixture interaction, similar to that observed behaviorally, does indeed occur when a mixture is tested over a broader range of concentrations (Ache and Herder, personal communication). It is noteworthy that evidence for synergism at low concentrations and suppression at high concentrations was reported recently by Johnson et al. (1985) in physiological studies of two classes of chemoreceptor cells in the American lobster.

FUNCTIONAL SIGNIFICANCE OF MIXTURE INTERACTIONS

Both synergistic and suppressive mixture interactions can play a significant role in increasing the effectiveness of the chemical senses whenever stimulants occur as mixtures. This is most evident in systems composed of cells with broad excitatory response spectra (see discussion in Derby, 1986). Synergism at low mixture concentrations provides an organism with a capacity to extend its range of sensitivity downward to odor concentrations that are subthreshold for individual components. On the other hand, suppression at high mixture concentrations may extend the range of responsiveness upward while avoiding either an overworking or a saturation of the system. Hence, the resultant effect of suppression could be similar to that noted earlier by Bartoshuk (1975), namely, to encode a broad range of potential concentrations into a much smaller range of potential response intensities.

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MACROMOLECULAR CUES IN MARINE SYSTEMS

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Abstract—A review of the roles of biopolymers as marine chemical cues is presented. The goal of the review is to provide a context within which to view present research and to provide insight into future research potential for macromolecules in marine chemical ecology. The roles of peptides, proteins, glycoproteins, proteoglycans, lectins, and mucopolysaccharides are discussed. Biological events mediated include: larval settlement and metamorphosis, gamete attraction, predator-prey interactions, alarm responses, feeding responses, nonfood resource acquisition, trail following, and larval-release behavior. Molecular origins, transmission, modulation, and multifunctionality of cues are discussed and illustrated with specific examples. The advantages of biopolymers, especially peptides and proteins, as specific cues in marine systems derive from their solubility, specific information content (due to the asymmetric nature of the monomer and the wordlike information content of the primary structure of the polymer), distance transmission in water by bulk flow rather than diffusion, relatively high signal-to-noise ratio, and common occurrence as structural and metabolic components of all living organisms.

Key Words—Marine animals, macromolecules, peptides, glycoproteins, behavior, prey detection, site selection, metamorphosis, biopolymers.

INTRODUCTION

Chemical mediation of behavior is well known in virtually all groups of living organisms. Marine animals are no exception. Most reports describing the chemicals which mediate these behaviors in marine species have been concerned with feeding (reviews: Bardach, 1974; Scheuer, 1977; Dunham, 1978; Atema, 1982). Where known, these chemicals are primarily low-molecular-weight compounds such as amino acids. Only a few reports detail the mediation of marine organism behavior by macromoleculars such as peptides, proteins, gly-

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coproteins, proteoglycans, lectins, and mucopolysaccharides. Although the total number of reports is small, the spectrum of documented macromolecule-induced responses is broad. The phyla of organisms displaying such responses include Polychaeta, Cnidaria, Mollusca, Ascidia, Echinodermata, and Crustacea. Macromolecules mediate aspects of: (1) larval settlement and metamorphosis [Crisp, 1984 (review); Morse, 1984 (review); Mitchell and Kirchman, 1984 (review); Burke, 1984]; (2) gamete attraction (Miller and Tseng, 1974); (3) predator–prey interactions (Rittschof et al., 1984); (4) alarm responses (Atema and Stenzler, 1977); (5) feeding responses (Gurin and Carr, 1971; Magnum and Cox, 1971; Carr et al. 1974; Carr and Gurin, 1975; Collins, 1975; Zimmer-Faust et al., 1984); (6) shell acquisition behavior in hermit crabs (Rittschof, 1980a); (7) trail following (Dimon, 1905; Hewatt, 1940; Crisp, 1969; Hall, 1972; Wells and Buckley, 1972; Cook and Cook, 1975; Trott and Dimmock, 1978) and (8) larval release behavior (Rittschoff et al., 1985).

Thus, macromolecules appear to play major roles in the transmission of information for the mediation of behavior and resource utilization in the marine environment. However, these roles are not the exclusive domain of macromolecules, and for the sake of perspective it is useful to note that other classes of substances function in similar roles and are equally important (for discussion, see Scheuer, 1977).

The nature of aquatic systems enables use of molecules of virtually any size as cues and signals (Wilson, 1970). In aquatic systems, solubility rather than volatility is of major importance (Atema, 1982). From a chemical point of view, macromolecular cues may not have the unique and interesting features that many secondary compounds from plants and animals have. However, they can exert considerable influence on biological activity. The biological activity of macromolecular cues is based upon the information content inherent in their polymeric structure. Polymer building blocks in specific sequences are high-information-containing macromolecules. Transformation and transduction into biological responses comes from polymers which have various levels of organization and structure, ranging from primary to quaternary.

Previous authors have discussed the tenuous distinction between smell and taste in aquatic organisms (Bardach, 1975; Atema, 1982). "Noses" and "tongues" appear either scattered indiscriminately or in highly complex patterns on virtually every conceivable external surface of marine organisms. Virtually every aspect of an organism's life cycle appears to be capable of being modulated through chemoreceptors. A recent and "delightful" complication is the growing need to distinguish between taste, contact chemoreception and touch with respect to the detection of and response to macromolecules adsorbed on surfaces. Thus, the three intuitively and anatomically distinct senses of taste, touch, and smell in terrestrial vertebrates form a continuum in marine sensory systems.

Equally delightful is the increasing evidence [specifically through the work of Morse's group at the University of California at Santa Barbara (Morse, 1984, 1985) and several groups at the University of Florida C.V. Whitney Marine Laboratory (Greenberg and Price, 1983; Derby et al., 1984)] that endocrine and neuroendocrine interactions which operate inside vertebrate bodies have external analogs that function at the interface between marine invertebrates and the environment. These analogs show great promise as model systems for the study of the effector–receptor interactions that are the basis of neuroendocrinology. This is another example of a simple model system shedding light on much more complex ones.

In the sections that follow we discuss the origins, transmission, information content, modulation, mechanisms of action, and the evidence for multifunctionality of marine macromolecular cues. Our goal is to give a brief overview of perspectives, research directions, as we see them, and insight into the complex nontoxic chemical networks that are fundamental to the daily life in the marine environment.

ORIGINS OF CUES

In instances where the origin of the chemical cue is known (Crisp and Meadows, 1962; Atema and Stenzler, 1977; Rittschof, 1980a; Kirchman et al., 1982; Larman et al., 1982; Forward and Lohman, 1983; Rittschof et al., 1983; Morse, 1985), the primary function of the anatomical source of the cue is as a component of the structural or metabolic machinery of the source organism. Sources are diverse: structural proteins, photosynthetic pigments, blood proteins, and exopolymers such as egg capsule proteins and mucopolysaccharides. Thus, interspecific information transmission may be a secondary function for the cue molecule, while the primary function may be structural or metabolic. Some examples of chemicals with secondary cueing functions are: (1) algal products which cue barnacle or gastropod settlement on substrates (Strathmann et al., 1981); (2) predation of a gastropod and potential shell availability to a hermit crab (McClean, 1974); (3) feeding responses (see Introduction for references); and (4) prey or predator location. Molecules with both intra- and interspecific cueing functions will be discussed in more detail in the section on multifunctionality.

Some of the more completely documented examples of origins of intraspecific molecular cues come from studies of larval settlement and feeding. Regarding cues for larval settlement, Kirchman et al. (1982) found that bacterial mucopolysaccharides promote settlement of polychaete larvae, whereas peptides associated with phycoerythrobilin, a photosynthetic pigment in red algae, promote settlement of abalone larvae (Morse et al., 1979; Morse, 1985). In

snail feeding studies, Carr and Gurin (1974, 1975) provided convincing evidence that glycoproteins of over 100,000 daltons derived from oyster flesh are involved in initial events of feeding stimulation.

In the other examples of intraspecific macromolecular cueing mentioned above (gametic attraction, alarm responses, predator–prey interaction, etc.), there is insufficient information to assign the cues a definite molecular type or origin. However, there is documentation that peptides originating from gastropod muscle proteins cue hermit crab shell acquisition behavior (Rittschof, 1980a). Also, there is evidence that heat-stable peptides of unknown origin are slowly released from living barnacles and cue their location to predatory snails (Williams et al., 1983; Rittschof et al., 1984). Likewise, heat-stable peptides may serve as sperm attractants in hydrozoans (Miller and Tseung, 1974).

Among the molecules cueing conspecific responses, the majority that have been described are glycoproteins. Heat-stable, high-molecular-weight substances found in the blood of mud snails function as an alarm substance when released by a catastrophic event (Atema and Stenzler, 1977). Natural catastrophic events include crushing or injury of the snail during predation. Experimentally, release of alarm substances is accomplished by any procedure that promotes bleeding. Over 16 species of gastropods display alarm reactions in response to crushed conspecifics (Snyder, 1967). Heat-stable proteins promoting settlement of conspecific larvae have been extracted from homogenates of adult barnacle tissues (Larman et al., 1982). Heat-stable peptides from living adult barnacles have similar effects on barnacle settlement (Rittschof, 1985). Finally, peptides from extracts of sand surrounding adult sand dollars (Burke, 1984) promote settlement and metamorphosis of larval sand dollars. However, the exact origin of any of the peptides in these examples has not been determined. The adhesive used by a colonial tube-building polychaete, which promotes settlement of conspecific larvae (Jensen and Morse, 1984), is an example of one interspecific larval settlement cue with a known origin. Peptide-cued conspecific larval release behavior is known in female mud crabs (Rittschof et al., 1985). In this case, small peptides released from hatching eggs (Forward and Lohman, 1983) cause female crabs carrying eggs to pump their abdomens. The precise origin of these larval-releasing peptides is uncertain.

Trail following in gastropods has received considerable attention at the ecological and behavioral level, but little attention has been given to this phenomenon at the molecular level. Many gastropods follow mucus trails laid down by conspecifics. The trails are known to have polarity, and in some instances they contain enough information for the discrimination of individuals of the same species from one another (Trott and Dimmock, 1978). The self/nonself recognition component has not been studied chemically, and the polarity appears to be a short-lived (hours) structural property of the mucus trail.

TRANSMISSION OF CUES

Because of the slow rate of diffusion of molecules in water, bulk water flow is important in the transport of chemical cues (Wilson, 1970; Atema, 1982). Three-dimensional odor trails (Atema, 1982) are a consequence of a slow rate of diffusion coupled to bulk water movement. These factors dictate that for most organisms detection of a chemical gradient and gradient search will not be involved in response to distant cue sources. Rheotaxis and searching in response to on–off type availablilty of cues (Van Haften and Verway, 1958; Derby and Atema, 1980; Rittschof, 1980b; Rittschof et al., 1983; Brown and Rittschof, 1984) is a more commonly demonstrated mechanism. In a careful study of chemically mediated rheotatic responses of oyster drills (Brown and Rittschof, 1984) neither the chemical cue nor the water flow alone were effective, while any combination of flow and cue, within a threefold range of each, elicited creeping responses by the drills.

Macromolecular cues function when in solution (Carr, 1967; Atema and Stenzler, 1977; Rittschof, 1980a) much the way that any other chemicals function. A more unique feature of macromolecular cues is that many are known to function when adsorbed to or part of a surface (Morse et al., 1980; Crisp and Meadows, 1982; Kirchmann et al., 1982; Jensen and Morse, 1984; Rittschof, 1985). Often, hydrolysis of the actual source of the cue can lead to extended cueing functions. For example, small molecules like amino acids which cue feeding for fish and blue crabs are released at effective concentrations for about 12 hr from a 15-g flesh source if it is protected from consumption. In contrast, the macromolecules which cue shell acquisition in hermit crabs are generated from that same source for at least three days (Rittschof, 1980b). Macromolecules adsorbed to surfaces after release from the blood of mud snails also function to prolong behavioral responses. It is not clear whether this effect is due to partitioning of the cue between the water and substrate, or a result of partial hydrolysis of the parent compound. Nevertheless, the result is a stimulation of a specific behavior for up to 24 hr (Atema and Stenzler, 1977).

In addition to macromolecules that can function as guides for trail following in snails, macromolecules implicated in larval settlement appear to function when they are adsorbed to or physically a part of a surface (Crisp and Meadows, 1962; Hidu, 1969; Morse et al., 1979; Kirchman et al., 1982; Rittschof, 1985). This particular phenomenon appears to be widespread among settling larvae. Documentation of specific surface contact requirements exists for larvae of mollusks, crustaceans, and polychaetes, ascidians (Woollacott, 1984), and bryozoans (Mihm et al., 1981). A surface mechanism of action is difficult to demonstrate experimentally because of the possibility of slow leaching or hydrolysis of the adsorbed cue. Minimally, the initial steps in the behavioral responses to

adsorbed molecules must be a molecular contact that intitates the response (Crisp and Meadows, 1962; Morse et al., 1979, 1980; Kirchman et al., 1982; Rittschof, 1985). This particular area of marine chemoreception is one which is at the interface between touch contact chemoreception and taste.

MODULATION OF MACROMOLECULAR CUES

Behavioral and physiological responses to macromolecular cues can be positively and negatively modulated by natural compounds. By themselves, modulators do not evoke responses at "natural" concentrations in the absence of the cue. For example, stimulation by the macromolecules associated with both abalone settlement (Morse, 1984, 1986) and location of prey by predatory snails (Williams et al., 1983; Rittschof and Brown, 1986) can be either facilitated or suppressed by modulatory compounds. These modulators appear to be low-molecular-weight compounds that are different in each specific instance.

Morse (1984) reviews information that his group gathered on modulators that facilitate settlement of abalone larvae. Facilitators are components common in "dissolved organic material" which aid all aspects of metamorphosis in response to low concentrations of the inducer [GABA-mimetic peptide (Morse, 1985)]. Facilitators all appear to be structural analogs of GABA, which themselves possess inducing activity only at high concentrations (Morse, 1984; Trapido-Rosenthal et al., 1985). Morse (1984) makes the argument that facilitators of abalone settlement, because of their structural analogy to natural inducers, may function in any of the steps in the normal induction pathways.

Facilitation (Williams et al., 1983) of responses of snails to soluble peptides from barnacles is due to micromolar concentrations of ammonium ion (Rittschof and Brown, 1985). Compounds suppressing chemotactic responses of snails to these peptides occur in the odors from the predatory snail's bivalve prey (Williams et al., 1983). Modulators with suppressant activity are poorly characterized because of the inability at this time to extract and concentrate them from seawater. However, it is known that suppressants are not amino acids or simple sugars, and that they have an apparent molecular size between 500 and 1000 daltons. Thus, chemical modulations of certain snail predator–prey interactions are expressed in several ways, including camouflaging (Fishlyn and Phillips, 1980), facilitation of the attractiveness of the odor of a prey species (Rittschof et al., 1983; Williams et al., 1983), and suppression of chemotactic behavior (Williams et al., 1983; Rittschof and Brown, 1986).

MULTIPLE FUNCTIONS

Evidence for multifunctionality of macromolecular cues in marine organisms is restricted at this time to preliminary investigations of peptides that orig-

inate from barnacles and oysters (Rittschof and Brown, 1986). These peptides were discovered and isolated by virtue of the creeping response evoked in predatory snails (Rittschof et al., 1983, 1984; Rittschof et al., 1986). Peptides originating from barnacles were isolated and purified from seawater containing live intact barnacles. Detection of biological activity was done with an assay which tested the creeping response observed in inexperienced predatory snails. Soluble peptides from water bathing intact oysters were isolated and purified. The bioassay used in this study measured the creeping response in snails that had been conditioned (Wood, 1968) to respond to peptides from oysters. Although the amino acid sequences of the peptides are not yet known, chromatographic, compositional, and biological properties indicate that several distinct peptides may be present (Rittschof and Brown, 1986; Rittschof et al., 1986).

In addition to their biological activity in predatory snails, the peptides isolated from barnacles and oysters have effects on the settling stage of larval barnacles. At concentrations that can be detected in field situations (2.5 μg/liter), barnacle peptides affect the behavior and induce metamorphosis in settling stage larvae. In this respect these peptides are similar to arthropodins (Crisp and Meadows, 1962). As is the case with arthropodins, soluble but "sticky" barnacle peptides affect larval behavior and induce metamorphosis when adsorbed onto surfaces. In contrast, "sticky" oyster peptides, presented at concentrations comparable to barnacle peptides, alter larval barnacle behavior but do not induce metamorphosis. Tests of effects of these oyster peptides on larval oysters are in progress in collaboration with researchers at the University of Maryland.

Thus, there is preliminary evidence that macromolecular cues may perform more than one cueing function. In the case of barnacle peptides, there is evidence for three functions: (1) prey location by predatory snails plus (2) site selection and (3) induction of metamorphosis by barnacle larvae. In the case of oyster peptides, there is evidence for two functions: (1) prey detection by predatory snails and (2) site selection in larval barnacles. It appears likely that future work will show that macromolecules that provide information concerning the presence of specific kinds of organisms are central to the physiological ecology of those organisms.

Whether adsorbed to surfaces or free in solution, macromolecular cues function to transmit specific information in marine environments. The fact that many cues are proteinaceous but heat stable suggests that higher-order macromolecular structure (secondary, tertiary, and quaternary) is less important than the covalent primary structure inherent in all biopolymers. In a peptide, for example, the "R" groups of each amino acid might function unambiguously, similar to strings of letters forming words. While odor bouquets (Bardach, 1975) of smaller molecules can perform the functions of single macromolecules, there is little evidence in marine systems other than in those areas entailing relatively

nonspecific responses (such as feeding) that mixtures of odors have specific cue functions. However, as is the case in insect pheromone systems, mixtures of marine secondary metabolites may have specific functions.

Advantages related to specific cueing functions are inherent in the primary structure of macromolecules, especially peptides. In many environments peptide cues have a high signal-to-noise ratio. This is because background levels of free peptides and amino acids (Mopper and Lindroth, 1982) are low due to the biological demand for and scavenging of organic nitrogen. Concomitantly, rapid biological uptake combined with dilution result in short signal duration. As the size of the peptide increases, the rapid decrease in the rate of diffusion increases the potential for transport of effective cue concentrations over relatively long distances. Should modulating molecules be present, the change in ratio of cue to modulator in the odor plume due to differences in rates of diffusion could be used by organisms to gain information about the distance to the source. The polar, amphoteric structure of peptides provides high solubility in water. The linear structure and polarity (amino terminal and carboxy terminal) of peptides provide potential for unambiguous information transmission. Should the peptide originate from a repeating structure such as a marine adhesive (Waite, 1983) or blood antifreeze protein (Komatzu et al., 1970), there is remarkable potential for extended slow release function. Finally, as proteins are major metabolic and structural components of all organisms, the intact molecules and their specific degradation products are available as raw materials for the evolution of information transmission.

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PLANT AND FUNGAL CELL WALL FRAGMENTS ACTIVATE EXPRESSION OF PROTEINASE INHIBITOR GENES FOR PLANT DEFENSE

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Abstract—Plant and fungal cell wall fragments produced by enzymic degradation during pest attacks are hypothesized to be activators of a universal recognition system for locally and systemically activating genes which control the synthesis of plant defense chemicals such as the antibiotic phytoalexins and antinutritive proteinase inhibitors. Proteinase inhibitor cDNAs have been prepared from wound-induced mRNAs, isolated, and characterized. The cDNAs have been utilized to quantify specific proteinase inhibitor mRNAs in leaves following wounding or simulated insect attacks. The cDNAs have also been utilized as hybridization probes to isolate and characterize proteinase inhibitor genes from tomato and potato genomic DNA. Proteinase inhibitor proteins have been induced in tomato leaves by chewing insects and shown to be highly correlated with a systemically mediated reduction in the nutritional quality of the leaves toward the larval noctuid *Spodoptera exigua*. Thus, the wound-induced proteinase inhibitors, whose genes in tomato leaves can be activated by wounding, insect attacks, and plant and fungal cell wall fragments, can significantly decrease the quality of the leaves for such herbivorous insects. This inducible set of biochemical reactions leading to the de novo biosynthesis of proteinase inhibitors is, therefore, considered to be a potentially important defense of plant leaves that should be considered both in developing general theories on insect-plant interactions and in selecting insect-resistant crop varieties.

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INTRODUCTION

Current hypotheses concerning the chemical bases for plant defenses against predators include the concept that plants can respond to attacks of insects, microorganisms, and viruses by activating genes that produce defensive chemicals. In recent years, studies on the insect-induced proteinase inhibitors in plant leaves and of phytoalexin synthesis in cotyledons and seedlings have provided models to study the biochemical and molecular biological mechanisms that underlie induced defensive responses. Over the past 15 years, evidence has been accumulating to support a model in which plant and fungal cell walls contain, within their polysaccharide structures, informational components that can be released by attacking insects and microorganisms can subsequently act as signals to activate plant genes that produce defensive chemicals.

The first report of a cell wall polysaccharide with phytoalexin elicitor activity came from the laboratory of Dr. Peter Albersheim at the University of Colorado in the mid-1970's. A β-glucan fraction (glucose polymers containing β-1,3 and β-1,6 linkages) derived from fungal cell walls was found to contain powerful elicitors of the synthesis and accumulation of the antibiotic isoflavonoid phytoalexins in legume seeds (Ayres et al., 1976). Phytoalexins induced by such elicitors can eventually comprise several percent of the wet weight of the plant cells in which they accumulate. Elicitation of pyhtoalexins by fungi appears to be localized only in cells adjacent to and near the site of hyphal infection; hence, the effects of such elicitors, whether they be β-glucans or other chemicals, are not systemic. Recently, the structure of an active oligomeric β-glucan fragment from the cell walls of *Phytophthera megasperma* was identified as a hexaglucosyl hexitol (Sharp et al., 1985). Substitution of the hexitol unit with glucose did not diminish its activity in eliciting phytoalexin synthesis in soybean cotyledons. As little as 1 pmol of the elicitor per 1 ml tissue volume of cotyledon elicited phytoalexin synthesis (Sharp et al., 1985).

In 1980, Hadwiger and Beckman (1980) reported that another component of fungal cell walls, chitin (Figure 1, right) harbors structural components within its polymeric structures that, upon degradation and solubilization, can be potent elicitors of both phytoalexin synthesis and proteinase inhibitor synthesis. Chitin, a β-1,4-*N*-acetylglucosamine polymer, when deacylated and chemically or enzymatically fragmented, yields soluble oligo- and polysaccharides that elicit phytoalexin synthesis in tissues of a variety of plant species from diverse families (Hadwiger and Loeschke, 1981; Walker-Simmons and Ryan, 1984).

In the late 1970s, fragments from the cell walls of plants (Figure 1, left) were shown to activate defensive responses in plants. Pectic fragments were

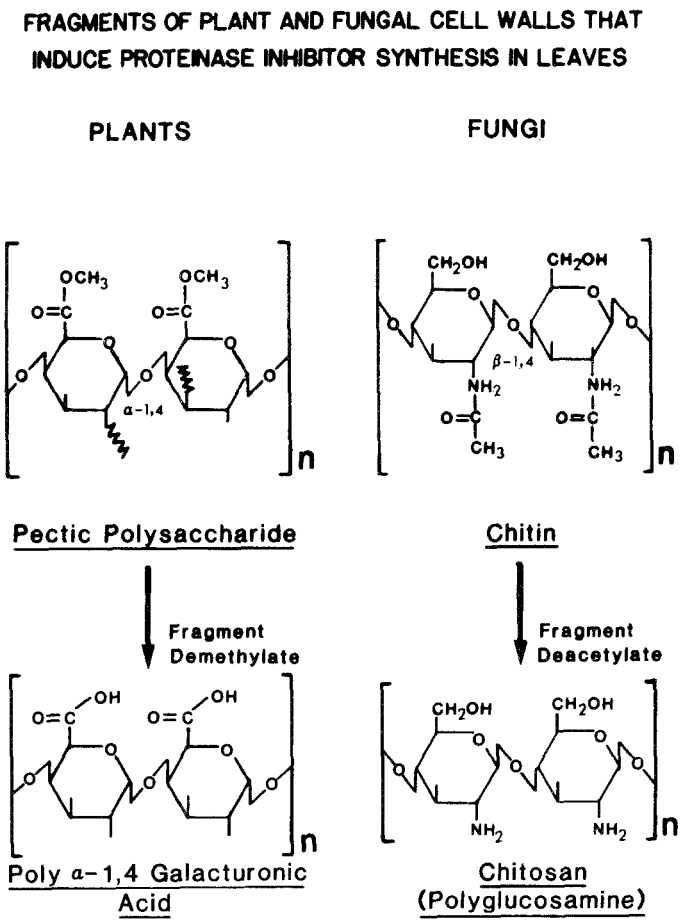

FIG. 1. Structures of the repeating units of fragments that can activate plant defensive responses. The carbons 2 and 3 of the methylated galacturonides of the backbone of plant cell wall pectin (left) are branched with chains of neutral sugars. The fundamental sugar unit of chitin (right) is β-1,4-N-acetylglucosamine.

shown to induce proteinase inhibitor synthesis in plant leaves (Ryan, et al., 1981) and elicit phytoalexin synthesis in legume cotyledons and seedlings (Hahn, et al., 1981). Research on the systemically mediated insect- or wound-induced synthesis of proteinase inhibitors in plant leaves led to the isolation of a component of tomato leaves called the proteinase inhibitor inducing factor, PIIF, that was apparently the active inducer of synthesis of proteinase inhibitors in excised tomato leaves. In 1978, this component was tentatively identified as a pectin-like molecule from tomato leaves (Ryan, 1978), and in 1980, the activity was shown to reside in a well-defined fragment of sycamore cell walls

(Ryan et al., 1981). During this period, the laboratories of Dr. P. Albersheim in Colorado and Dr. C. West at UCLA, reached the same conclusion—that plant cell wall fragments contained biological activity and were elicitors of phytoalexin synthesis in legume seedlings and cotyledons (Hahn, et al., 1981; Jin and West, 1984). Thus, the concept of the potential role for fungal and plant cell wall fragments as initial signals for activating a variety of genes producing defense chemicals has evolved. A summary of the possible origins and actions of these carbohydrate signals in response to insect or microorganism attacks is shown in Figure 2.

As shown in Figure 2, the pectin fragments released by insect attacks, can be simulated in vitro by the mixing of cell walls with plant endopolygalacturonases released from compartments in the cell by the chewing action of attacking insects (or other animals). The fragments, or derivatives or adducts thereof, are thought to be transported throughout the plants where they can activate defensive genes.

On the other hand, microorganisms initiate attacks on plants (Figure 2) by releasing a variety of enzymes, including polygalacturonides that degrade the cell wall (Bateman, 1976). The cell wall fragments are released by the microorganism's endopoloygalacturonases (EPGases) (Lee and West, 1981) or pectic

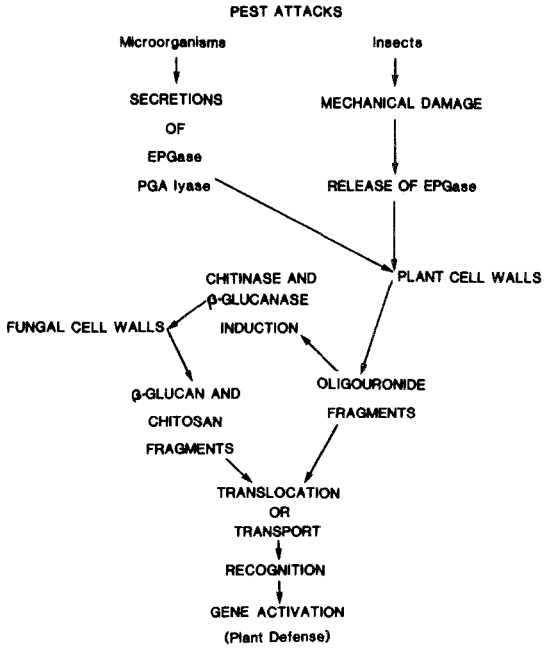

FIG. 2. Proposed scheme for the production and transport of cell wall fragments during attacks by microorganisms and insects.

lyases (PGAlyases) (Davis et al., 1984) and act as localized signals to elicit the synthesis of phytoalexins. Additionally, attack by microrganisms has recently been shown to induce localized synthesis of two plant enzymes, chitinase (Abeles et al., 1970) and β-glucanase (Boller et al., 1983). Since induced plant enzymes would be present in tissues adjacent to the sites of attacks, they could react with the cell walls of invading fungi, causing a release of active chitin and β-glucan fragments which could further amplify the induction of phytoalexin synthesis elicited intitially by the pectic fragments (Figure 2). As an infection proceeds, the fragments (signals) would continue to be released, and therefore cause continued phytoalexin synthesis. Nearly 20 examples of plants producing phytoalexins, proteinase inhibitors, or other defensive chemicals in response to cell wall fragments have now been reported in plants from genera in several plant families including the Solanaceae, Leguminosae, and Pinaceae. This distribution implies that a common recognition-gene activation system may be a fundamental component of all of these plants and may, moreover, serve as a universal regulator of resistance to a variety of detrimental external stimuli.

Based on knowledge of the pathways of pest-induced fragmentation of the plant and fungal cell walls, one can consider a number of potential regulatory processes governing the release of oligo- and polysaccharides. The quantity and specificities of both plant and microorganismal (and perhaps insect) cell wall-degrading enzymes could modulate the temporal and structural features of the released oligo- and polysaccharide fragments. The levels of chitinase and β-glucanase that are induced in plant tissues could be modulated by the promoter strengths of the genes, or by multiple gene copies. Finally, the efficiency of transport mechanisms and receptor responses could come into play in establishing a successful defensive response. Furthermore, both the phytoalexins and proteinase inhibitors are only a part of an array of defensive chemicals, both constitutive and inducible, with which they may act in concert. It is anticipated that a more detailed understanding of the induction of proteinase inhibitors and phytoalexins in response to attack by foreign organisms will emerge as we more incisively depict the identity of the signals that regulate induced defenses, unravel the recognition systems that regulate gene expression in response to these signals, and fully define the structures and organization of the genes and their pest-induced promoters that are regulated by cell wall fragments.

In this article we briefly review the background and status of research concerning the wound signal PIIF and the isolation of proteinase inhibitor genes. We will then describe recent experiments that strongly implicate the proteinase inhibitor proteins as induced defensive chemicals.

INSECT- OR WOUND-INDUCED PROTEINASE INHIBITORS

In 1972, localized wounding of tomato plants by chewing insects was found to induce the systemic accumulation of a proteinase inhibitor in leaves through-

out young tomato and potato plants (Green and Ryan, 1972). For several years, the research focused on the isolation and characterization of the wound-induced proteinase inhibitors and the inducing factor, PIIF. Current research on the response of these plants to wounding has been concerned with (1) and understanding of the intracellular mechanism by which cell wall fragments can activate proteinase inhibitor producing genes in plant leaves, (2) the isolation and characterization the proteinase inhibitor genes, and (3) the regulation of expression of the genes by the cell wall fragments. More recently, the question of the effectiveness of proteinase inhibitors as defensive chemicals against herbivores has also been addressed. Although the antinutritional properties of the inhibitors has indirectly implicated them as potential defensive chemicals, no research has been done until recently to study directly their possible roles in reducing the nutritional quality of plant tissues toward attacking insects.

Many parameters of wound-induction of proteinase inhibitors in tomato plants are now known. This response is easily quantified in young tomato plants (2–3 leaf stage) by crushing the lowest leaf across the midrib with a hemostat. Within 6 hr after wounding, two proteinase inhibitors, called inhibitors I and II (6), can be detected in the wounded leaf and, after an additional 60–90 min, the inhibitors can be detected in upper, unwounded leaves. The inhibitors accumulate in both wounded and unwounded leaves for several hours, although inhibitor I accumulates at about twice the rate of inhibitor II (Ryan, 1978). The inhibitors are compartmentalized in the central vacuole of the cell where they are apparently not degraded as are other intracellular proteins. Thus, even though only a small fraction of total foliar protein synthesis (about 1% of the total protein) is devoted to production of the two inhibitors, they accumulate fairly rapidly.

The pectic fragment, PIIF, was purified from tomato leaves by utilizing an assay system in which young plants, excised at the petioles, were supplied with aqueous extracts of tomato leaves (Ryan et al., 1985) containing tomato leaf cell soluble fractions, separated by conventional analytical techniques. When PIIF was present in a given solution, that solution induced the synthesis of the two inhibitors in leaf cells. The active inducer component was identified as a highly methylated pectic fragment with an average length of 30 residues (Ryan et al., 1981). The fragment, when hydrolyzed and analyzed, contained nearly 70% galacturonic acid, and its general composition closely resembled that of the pectic polysaccharides of plant cell walls (Ryan et al., 1981).

PIIF was partially hydrolized with trifluoric acid to remove neutral side chains, and an active, demethylated polygalacturonic acid polymer with an average chain length of 20 (Bishop et al., 1984) was recovered. Upon analysis, the polymer was found to contain 90% galacturonic acid. Further fragmentation of this polymer by enzymic degradation with tomato endopolygalacturonase and subsequent isolation and assay of the smallest fragments revealed that PIIF activity was present in dimers and in larger oligomers up through the hexamer,

the largest pure polymer tested. However, all of the fragments contained less activity than PIIF itself. A small polymer or oligomer that possesses the full activity of PIIF has not yet been recovered. By extrapolation of the increasing activities of the oligomers, the maximal activity was estimated to be present in about a nonomer. This estimate approximates the size of the polyuronides which are active in the phytoalexin systems (Lee and West, 1981; Nothnagel et al., 1983). However, in the localized phytoalexin response, oligomers less than about seven uronide units are inactive. It is possible that fragments with lengths of seven or less are transported and act systemically, whereas the larger ones may not be transported and act locally. The chemical nature of the factor(s) that is systemically transported and activates proteinase inhibitor synthesis is presently under investigation.

The molecular biology of the wound induction has also been extensively studied. Leaves of wounded tomato plants were found to contain mRNAs coding for both inhibitor I and II (Nelson and Ryan, 1980), identified using an in vitro translational system followed by immunoprecipitation and electrophoresis in polyacrylamide gels containing SDS. Little or no translatable inhibitor I or II mRNA was found in unwounded leaves.

Using partial amino acid sequence data from the *N*-terminal regions of both inhibitors, oligonucleotide sequences were deduced and probes for the two inhibitors were synthesized (Graham et al., 1985a,b). These probes were used in conjunction with hybrid selection techniques using in vitro translations to select cDNAs coding for inhibitor I and II from a library of wound-induced cDNAs prepared in the plasmid pUC9. Several cDNA clones were identified, and full-length cDNAs for both inhibitor I and II were isolated and characterized. These clones have been useful for deducing the entire open reading frames for the two inhibitors. These experiments revealed that both inhibitors are synthesized as preproteins with leader, or transit, sequences of about 20 amino acids at their *N*-terminals that are apparently necessary for posttranslational transport into the central vacuole where inhibitors are stored (Walker, Simmons and Ryan, 1977).

The cDNAs were also used in hybridization experiments to quantify inhibitor mRNA levels in leaves following wounding (J. Graham and C. A. Ryan, in preparation). After a single wound, inhibitor I and II mRNAs first appear at about 4 hr. By 8 hr, the maximum levels are found, the decline with a half-life of about 10 hr. Multiple wounding causes to mRNAs to appear sooner and the levels to increase significantly. For example, four wounds on a leaf, inflicted hourly, cause a doubling of mRNA. Thus, and insect constantly chewing on a leaf would probably produce maximal responses in the leaves. After multiple wounding, the levels of mRNAs for inhibitor I and II have been estimated to cumulatively comprise nearly 1% of the total mRNA of leaf cells.

The cDNAs were also employed to isolate genes for both inhibitors from a tomato genomic library. The characterization and organization of these genes are in progress.

The genes are currently being constructed into a shuttle vector that is a derivative of the Ti plasmid (An et al., 1985) to be used to transform plants to see if the wound-induced promoters are still functional in the isolated genes. If so, the structures of the promoters responsible for reacting to attack by foreign organisms can be resolved. Identification and synthesis of this promoter could be quite useful in further fundamental and applied research to understand and manipulate plant genes to improve natural plant defenses against attacking pests.

EFFECTS OF WOUND-INDUCED PROTEINASE INHIBITORS ON HERBIVORES

The hypothesis that wound-induced plant proteinase inhibitors have a detrimental effect on herbivores was tested by rearing larval *S. exigua* on leaf material from unwounded and wounded tomato plants (Broadway et al., 1985). Two commercial varieties of tomato, Ace 55 (Ace) and Castlemart (CM), were grown to the 7–8 leaf stage in sterilized soil inside fine mesh cages in a greenhouse to prevent wounding of the plant tissue by external agents. The plants were divided into two groups. One group was maintained as a control, and the other group was wounded by enclosing 3–4 fifth-instar larval *S. exigua* on the lower leaf of each plant via a gauze bag. The insects were allowed to feed for 24 hr. After this 24 hr period, the leaves from plants of both groups were removed, immediately frozen at −20°C, lyophilized, and pulverized.

The results of biochemical assays to determine total levels of foliar inhibitor I and II for the four samples of leaf tissue (Ace, Ace/wounded, CM, CM/wounded are shown in Figure 3. Tissues were also assayed for catecholic, phenolic, and protein content. When statistically comparing the four samples of leaf tissue using two-way analyses of variance followed by multiple comparisons of means at 5% level (95% CI), there is no significant relationship between wounding and the levels of total foliar phenolic or protein.

In contrast to the levels of foliar phenol and protein, the combined levels of inhibitor I and II in the tissue samples from both varieties of wounded plants were significantly higher than levels in the samples from both varieties of unwounded plants. In addition, wounding induced the production of a significantly higher level of inhibitor I and II in the tomato variety Castlemart (CM/w) than in Ace 55 (Ace/w). There was no significant difference between the two unwounded varieties, Ace and CM in their total inhibitor I and II content. Thus, among the chemicals investigated, only the levels of inhibitor I and II were elevated in response to wounding.

In order to assess whether this induced level of inhibitor had any effect on herbivorous insects, the following experiment was done. The four samples of lyophilized plant material (Ace, Ace/w, CM and CM/w) were reconstituted to 15% (wet wt) with a 1.6% agar solution and 0.05% sorbic acid and were provided as a diet for second-instar larvae of *S. exigua*. Experiments were termi-

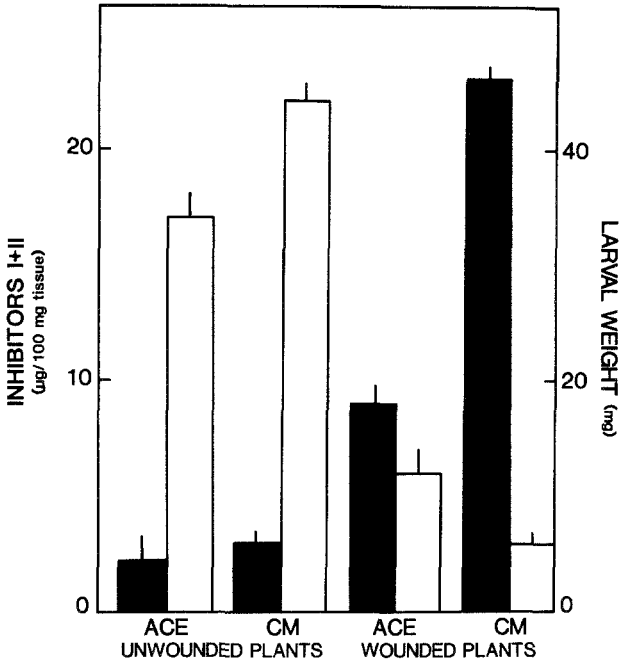

FIG. 3. The levels of proteinase inhibitors I and II in leaves of tomato varieties Ace and Castlemart. Leaves of unwounded and wounded plants (dark columns) are compared with the growth of *Spodoptera exigua* larvae (open columns) that subsequently fed on the leaves. Error bars fefer to 95& CM.

nated and all larvae were weighed when the larvae feeding on the control diets reached fifth (ultimate) instar.

These data indicate that the foliage from wounded palnts is a poorer food source than that from unwounded plants. When larval growth, as measured by weight gain, was examined in relation to the dietary inhibitor I and II, it was clear that there was a significant inverse relationship ($r^2 = 0.81$, $P = 0.05$) between the levels of inhibitor I and II in the leaf tissue and the means of the weight of larval *S. exigua*.

The alternation of the nutritional quality of plants by herbivory resulting in enhanced "resistance" (wound-induced resistance) of the plant, is a subject that has recently received much interest (Rhoades, 1983: Hare, 1983; Ryan, 1983; Kogan and Paxton, 1983). Wounding of a variety of plant species by either artificial or natural means can result in elevated levels of phytochemicals that are potentially strong antibiotic (Ryan, 1983; Kogan and Paxton, 1983; Haukioja and Niemela, 1976, 1977; Niemela and Haukioja, 1979), and artificially defoliated trees can subsequently produce foliage which is of reduced nutritional quality for herbivorous insects (Wallner and Walton, 1979; Werner,

1979). None of the factors that cause the decreased nutritional quality have been determined, and only a few studies have shown that insect herbivory will reduce the nutritional quality of foliage for future herbivores (Karban and Carey, 1984; Benz, 1977; Baltensweiler et al., 1977; Overhulser et al., 1972).

Previous to this research, there have been no studies to show either that insect herbivory induces specific phytochemicals in plants or that these phytochemicals subsequently confer resistance to the plant against pathogens or herbivores. Although the work relating the levels of plant proteinase inhibitors to larval growth and development is indirect, a recent study (Broadway and Duffey, 1986) showed that chronic exposure to purified potato inhibitors related to those induced in tomato leaf has a detrimental effect on the digestive physiology of larval *S. exigua* by inducing a pernicious hyperproduction of gut proteases. It appears that the chronic stress on the digestive enzyme-producing system is a major factor in depressing growth of lepidopterous larvae.

In summary, the systemic insect-induced synthesis of proteinase inhibitors in tomato leaves appears to be regulated by a universal recognition-communication system mediated by cell wall fragments of plants (or fungi) generated during attacks by foreign organisms. The large accumulation of the antinutrient inhibitors from the activation of proteinase inhibitor genes resulted in the synthesis of new mRNA and inhibitor protein. The levels of the inhibitors in the leaves induced by attacks of the larval noctuid *S. exigua* correlated with the reduction of the nutritional quality of the plant tissues as a food source for the insects. Proteinase inhibitors can therefore be seriously considered as potentially important induced defensive chemicals against herbivorous insects.

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COADAPTATION OF *Drosophila* AND YEASTS IN THEIR NATURAL HABITAT

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Abstract—The mutualistic interactions of cactophilic *Drosophila* and their associated yeasts in the Sonoran Desert are studied as a system which has evolved within the framework of their host cactus stem chemistry. Because the *Drosophila*–yeast system is saphrophytic, their responses are not thought to directly influence the evolution of the host. Host cactus stem chemistry appears to play an important role in determining where cactophilic *Drosophila* breed and feed. Several chemicals have been identified as being important. These include sterols and alkaloids of senita as well as fatty acids and sterol diols of agria and organpipe cactus. Cactus chemistry appears to have a limited role in directly determining the distribution of cactus-specific yeasts. Those effects which are known are due to unusual lipids of organpipe cactus and triterpene glycosides of agria and organpipe cactus. *Drosophila*–yeast interactions are viewed as mutualistic and can take the form of (1) benefits to the *Drosophila* by either direct nutritional gains or by detoxification of harmful chemicals produced during decay of the host stem tissue and (2) benefits to the yeast in the form of increased likelihood of transmission to new habitats. Experiments on yeast–yeast interactions in decaying agria cactus provide evidence that the yeast community is coadapted. This coadaptation among yeasts occurs in two manners: (1) mutualistic increases in growth rates (which are independent of the presence of *Drosophila* larvae) and (2) stabilizing competitive interactions when growth reaches carrying capacity. This latter form is dependent on larval activity and results in benefits to the larvae present. In this sense, the coadapted yeast community is probably also coadapted with respect to its *Drosophila* vector.

Key Words—*Drosophila*, Diptera, Drosophilidae, yeasts, cactus, community ecology, mutualism, coadaptation, evolution, alkaloids, fatty acids, sterols.

INTRODUCTION

Coevolution has been defined as an evolutionary change in a trait of the individuals of one population (or species) in response to a trait of the individuals of a second population (or species), followed by an evolutionary response by the second population (or species) to the change in the first (Janzen, 1980). The evolution of secondary compounds in plants, which are generally considered to be defense agents against herbivores and microorganisms (e.g., Whittaker and Feeny, 1971; Levin, 1973; Rosenthal and Janzen, 1979; Futuyma, 1983) and the counterevolutionary response of the herbivores and microorganisms is, therefore, viewed as a process of coevolution. Plant–herbivore or predator–prey relationships can also elicit the evolution of mutualistic interactions between species (Ricklefs, 1979). If the mutualism between species is accompanied by adaptations of both parties which increase the effectiveness of the association, the process is said to involve coadaptation (Roughgarden, 1978). We have been studying a biological system in which species of *Drosophila* feed and breed in the decaying tissue of cacti (Heed, 1978, 1982). The microbial community (various species of bacteria and yeasts) which decays the cactus transforms the tissue into food for these drosophilids. In turn, these drosophilids can vector microorganisms into new habitats (damaged tissue). This is particularly important for the yeasts, which do not disperse by wind and rely on insects such as *Drosophila* for transmission to new habitats (Gilbert, 1980). The nutritional benefits of the yeasts to the fly and the transmission benefits of the fly to the yeasts form an interdependent system, and thus create the potential for coevolution and coadaptation.

One feature of this system which makes it amenable to studies of coadaptation is that, although the secondary chemistry in the host cacti may have involved coevolutionary relationships with herbivores, it is essentially uncoupled from the evolution of the *Drosophila* and associated microorganisms. In essence, the saphrotrophic nature of the system precludes selective forces from the *Drosophila* microbiota on the host plants. Changes in the host chemistry due to selective pressure by pathogenic microorganisms and primary herbivores may exert selective forces on the saphrophytic system, but not vice versa.

In this paper, we will briefly review the chemical constituents of cactus tissue utilized by drosophilids and yeasts in the North American Sonoran Desert. In conjunction with this chemical description, we will focus attention on the yeasts, drosophilids, and yeast–*Drosophila* associations and review evidence which indicates that the chemistry of the host plants has played a role in determining what types of yeasts and drosophilids are present in particular plant species. In conclusion, we will present new data on yeast–yeast interactions in association with their fly vectors. These data will be used to evaluate various factors operating to determine yeast community structure and the coevolutionary or coadaptive features of the *Drosophila*–yeast community association.

CACTUS CHEMISTRY

Kircher (1982) has reviewed and outlined the major chemical constituents of several cacti found in the Sonoran Desert. His analysis (Table 1) includes representatives of two major systematic divisions of the columnar cacti [subtribe Pachycereinae: *Carnegiea gigantea* (saguaro) and *Lophocereus schottii* (senita); subtribe Stenocereinae: *Stenocereus thurberi* (organpipe) and *Stenocereus gummosus* (agria)]. Comparing the approximate chemical composition of the cacti, there is variation in the first two columns, percent H_2O and insoluble residue. Variance in the former, however, may primarily be due to differences in climatic conditions and water availability in the regions where the samples were collected. Although there is variability in amount, the composition of the insoluble residue fraction (cellulose, lignin, polysaccharides, and inorganic compounds) is similar among the four cacti. Major differences among the plants of the two subtribes reside in the lipid and methanol-water soluble fractions.

The major components of the lipid fraction of saguaro and senita are fatty acids (as esters), sterols, and alkaloids. The fatty acids in both species and the sterols in saguaro are considered typical for plants of this type. The sterols in senita are unusual in that this species apparently has an interrupted sterol biosynthetic pathway which leads to the accumulation of intermediate forms of sterols (Campbell and Kircher, 1980). The typical plant sterols, campesterol and sitosterol, are absent. Both species contain isoquinoline alkaloids: carnegine and gigantine in saguaro, lophocereine and its trimer, pilocereine, in senita (Brown et al., 1972; Djerassi et al., 1962). The concentration of alkaloids in senita (3–15% of the dry weight of the plant) is much greater than the concentration in saguaro (1–2% dry weight).

The lipid fractions of the congeners, agria and organpipe cactus, are chemically similar to each other and quite different from the other columnar cacti. The three main components of these fractions are neutral pentacyclic triter-

TABLE 1. APPROXIMATE COMPOSITION OF FRESH CACTUS TISSUE[a]

		Dry weight (%)				
Cactus[b]	H_2O (%)	Insoluble residue	Lipids	Alkaloids	MeOH-H_2O soluble	Triterpene glycosides
Saguaro	87	77	2.5	1.5	21	
Senita	81	71	6.5	3–15	25	
Organpipe	78	61	11		28	+
Agria	80	57	6.5		36	+

[a] Data modified from Kircher, 1982.

[b] Scientific Names: *Carnegiea gigantea* (saguaro), *Lophocereus schottii* (senita), *Stenocereus thurberi* (organpipe), *S. gummosus* (agria).

penes, sterol diols, and unusual fatty acids (Kircher, 1980; Kircher and Bird, 1982). The fatty acids are unusual in that they are predominantly C_8, C_{10}, and C_{12} chain lengths rather than the more typical C_{16}, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$. Most of the triterpenes and sterol diols are monoesterified to the fatty acids.

The methanol–water-soluble fraction of agria and organpipe cactus represents a large portion of the dry weight of these plants (up to 40% dry weight) and is composed mainly of triterpene glycosides (Kircher, 1977). This fraction of both saguaro and senita is a poorly defined mixture of substances, but triterpene glycosides are not present in either species. These compounds then, like alkaloids, are subtribe characteristics since this pattern of mutually exclusive distribution of the compounds extends to other members of the two subtribes (Gibson and Horak, 1978). Stem chemistry of this unusual nature is likely to have evolved as a result of various selection pressures from hervibores and pathogenic microorganisms, as well as adaptations to survive the extremes of desert climates (Gibson, 1982).

YEASTS

The distribution of yeasts in necrotic cactus tissue has been studied from both taxonomic viewpoints (Phaff et al., 1974, 1978, 1980, 1985; Miller et al., 1976; Starmer et al., 1978a, b, 1979, 1984; Miranda et al., 1982; Holzschu et al., 1983) and ecological and evolutionary analyses (Heed et al., 1976; Starmer et al., 1976, 1980, 1982; Starmer, 1980, 1981a, b, 1982a, b; Fogleman et al., 1981, 1982; Starmer and Phaff, 1983; Fogleman and Starmer, 1985). The distribution of yeast species over the four cacti is summarized in Table 2. A correlation analysis of these data shows that yeast communities of plants within subtribes have significantly higher correlations than yeast communities between plants of different subtribes (average correlation within is 0.76, average correlation between is 0.28). The extent to which the cactus stem chemistry has directly influenced the distribution of yeasts within the systematic hierarchy has been determined by studies of yeast growth on media (complete and minimal) which has been supplemented with chemical constituents of the cacti.

The general result has some explanatory power but is far from complete. First, the triterpene glycosides of the *Stenocereus* species do inhibit growth of some yeast taxa (i.e., *P. heedii*, *P. amethionina* var. *pachycereana*, and some strains of *Cr. cereanus*) and, thus, help explain why these species are relatively rare in *Stenocereus* stem necroses (Starmer et al., 1980). Several yeast species (e.g., *P. fluxuum*) not found in cacti are also sensitive to the triterpene glycosides (Starmer et al., 1980) and indicate that these chemicals may be functioning to restrict related yeasts that grow outside of the cactus yeast community from the *Stenocereus* habitat.

The medium chain fatty acids found as esters in agria and organpipe cactus tissue can inhibit yeast growth if they occur in the free undissociated form

TABLE 2. YEAST DISTRIBUTION IN CACTI OF SONORAN DESERT[a]

	Tribe Pachycereeae			
	Subtribe Pachycereinae		Subtribe Stenocereinae	
	C. gigantea	*L. schottii*	*S. thurberi*	*S. gummosus*
No. plants sampled	33	71	56	117
Yeast species[b]				
Pichia cactophila	0.515	0.113	0.571	0.752
P. heedii	0.455	0.676	0.036	0.009
P. deserticola	0.061	0.0	0.304	0.137
P. amethionina var a	0.0	0.014	0.089	0.239
P. amethionina var p	0.273	0.028	0.0	0.009
P. thermotolerans	0.333	0.028	0.0	0.0
P. mexicana	0.0	0.0	0.232	0.051
P. kluyveri (anamorphs)	0.0	0.0	0.036	0.060
P. pseudocactophila	0.121	0.0	0.036	0.0
P. farinosa	0.030	0.0	0.0	0.0
Candida sonorensis	0.121	0.042	0.464	0.444
C. ingens	0.212	0.224	0.321	0.094
C. mucilagina	0.0	0.0	0.071	0.085
C. species "cas"	0.0	0.0	0.089	0.0
C. species "tm"	0.0	0.0	0.071	0.0
C. guilliermondii	0.0	0.0	0.018	0.009
Cryptococcus cereanus	0.243	0.127	0.232	0.094
Cr. albidus	0.061	0.014	0.036	0.026
Cr. laurentii	0.061	0.0	0.0	0.0
Cr. skinneri	0.030	0.0	0.0	0.0
Kluyveromyces marxianus	0.060	0.0	0.018	0.0
Rhodotorula minuta var t	0.0	0.014	0.018	0.0
Clavispora species "o"	0.0	0.0	0.0	0.009
Trichosporon cutaneum	0.0	0.0	0.018	0.0
Prototheca zopfii	0.091	0.0	0.018	0.0

[a] Values are proportion of yeast in given number of plants sampled.
[b] Species designated in quotations are undescribed. *P. amethionina* var a and p correspond to varieties *amethionina* and *pachycereana*, respectively. *Rh. minuta* var t corresponds to variety *texensis*.

(Suomalainen and Oura, 1958). The relative amount of yeast growth in the presence of the free undissociated fatty acids is dependent on pH and yeast taxa. The ability to grow in the presence of these compounds is apparently associated with the ability to utilize them as a source of carbon. *Candida ingens* is unique in this regard, as it can utilize the medium chain fatty acids as the sole source of carbon as well as tolerate them at concentrations which are inhibitory to other yeasts (Henry et al., 1976; Starmer, 1982a). Thus, the lipolytic activity of *C. ingens* might give this yeast an advantage in agria or organpipe cactus tissue

when the unusual lipids are hydrolyzed to free the medium chain fatty acids. The relatively high frequency of *C. ingens* in organpipe rots, where these compounds are in relatively high concentration, is in accord with this notion.

The alkaloids of saguaro and senita apparently have neither inhibitory nor stimulatory effects on yeast growth (Fogleman and Kircher, 1983) and are not likely to be directly responsible for the distribution of yeasts in cactus necroses. In fact, complete medium supplemented with the water-soluble extracts, whole tissue, or filter sterilized rot juices of senita or saguaro does not appear to cause qualitative differences in the growth of cactus yeasts.

In summary, there is some evidence that the chemistry of *Stenocereus* species does function to inhibit some yeasts and thus partially determine which yeast can grow in decaying stems of these cacti. However, there are no indications that chemicals of cacti in the subtribe Pachycereinae directly determine yeast distributions.

DROSOPHILA

The ecology and evolutionary biology of the *Drosophila* species of the Sonoran Desert have been the subject of several recent reviews (Heed, 1978, 1982; Heed and Mangan, 1985). The four endemic species are: *D. mojavensis*, *D. nigrospiracula*, *D. mettleri*, and *D. pachea*. Two major points have been put forth as a result of evolutionary studies of these four drosophilids. First, each species is thought to have evolved its own set of adaptations to desert conditions independently. This statement is supported by the observation that the geographic distribution of the closest relatives of each endemic species either lies entirely outside the desert or overlaps the desert only slightly. Second, in regard to phylogenetic trends, *D. pachea* and *D. mojavensis* are obviously derived species, and they show evidence of origin from the south along the coast of Mexico. On the other hand, neither *D. nigrospiracula* nor *D. mettleri* are clearly derived forms, compared to their relatives, and the route to their place of origin is not known with any certainty.

Twenty-five years of extensive collection data by W.B. Heed and his students and colleagues have firmly established the *Drosophila*–host plant relationship in the Sonoran Desert. Essentially, there is a one-to-one relationship between the flies and the cacti, in that each cactus has only one main resident fly species. The host plant relationships are as follows: *D. mojavensis*—agria and organpipe, *D. nigrospiracula*—saguaro and cardón, *D. pachea*—senita, and *D. mettleri*—soaked soils (mainly from saguaro and cardón).

There is ample evidence that the secondary chemistry of the cacti limits host plant utilization. For example, *D. pachea* has evolved a strict dependency on the unusual sterols present in senita, so that the more typical plant sterols are not adequate for female fertility or larval development (Heed and Kircher,

1965). Since senita is the only cactus that contains these sterols, *D. pachea* is effectively limited to this host plant. In addition, the alkaloids in senita have been shown to be toxic to *D. mojavensis* and *D. nigrospiracula*. Senita, therefore, cannot be used as a host plant by these two species (Kircher et al., 1967; Fogleman et al., 1982).

Another chemical interaction between cacti and *Drosophila* involves the medium chain fatty acids and sterol diols present in both agria and organpipe. The presence of either of these compounds in ecologically realistic concentrations produces a drastic reduction in the viability of *D. nigrospiracula* larvae (Fogleman et al., 1986). Thus, agria and organpipe necroses are not suitable substrates for this species.

Investigations into the chemical interactions between the cacti and the *Drosophila* have clearly demonstrated the important role that stem chemistry plays in host plant utilization. Approximately 60% of all possible combinations of cactus species and *Drosophila* which do not occur in nature are exclusions due primarily to chemistry. Despite the fact that the endemic *Drosophila* exhibit high degrees of behavioral specificity for their host plants, the chemical factors in host plant utilization are still important since they indicate the selection pressures which currently act to maintain the specificities or which historically acted to produce them.

YEAST-*DROSOPHILA* INTERACTIONS

It is well known that *Drosophila* larvae derive nutritional benefits from yeasts (Sang, 1956, 1978). However, almost any yeast is sufficient for growth and development of *Drosophila* larvae in the laboratory (Wagner, 1944, 1949), and in several studies, bacteria growing in the natural substrate are nutritionally sufficient (Robertson et al., 1968; Starmer, 1982a; Vacek, 1982). If coadaptations between *Drosophila* and associated yeasts have occurred, it must therefore be manifested in terms other than nutritional sufficiency.

Investigations on the effects of organpipe cactus chemical constituents on the larvae of *D. mojavensis* have shown the medium chain fatty acid and sterol diol components to be either lethal (Fogleman et al., 1985) or to slow development and reduce adult size (Starmer, 1982a). Because the medium-chain-length fatty acids of organpipe cactus are bound as esters, the realization of this detrimental effect requires hydrolysis during the decay process. Experiments (Starmer, 1982a) have shown that by inoculating fresh organpipe tissue with a bacterium (*Erwinia carnegieana*) and various cactus yeasts, *D. mojavensis* larvae develop faster and attain larger sizes if the yeast present has lipolytic activity (i.e., *Candida ingens* or *Pichia mexicana*). If the bacterium is not included and only crude lipids (with fatty acids as monoesters) are added to a complete medium, there are no differences in viability, development rate, or adult size

of *D. mojavensis* among yeast treatments. Furthermore, if the medium chain fatty acids are added into a complete medium with the yeast *C. ingens*, the fly is partially relieved of the toxic effect.

In summary, the experimental evidence indicates that the lipolytic yeast *C. ingens* can provide benefits to *D. mojavensis* larvae when the unusual lipids of organpipe tissue are hydrolyzed by other microorganisms. The faster developing and larger animals are then more likely to distribute *C. ingens* to other habitats, increasing the frequency of *C. ingens* in the organpipe yeast community. However, *C. ingens* is also found commonly in other cactus rots which do not contain unusual or toxic lipids. It is therefore possible that the production of extracellular lipases by *C. ingens* is irrelevant or may serve other functions in the cactus–*Drosophila*–microorganism system.

Other compounds which are not present in fresh cactus but appear during the decay process may also play a role in the coadaptive process. Decaying *Stenocereus* species are somewhat unusual in that they often produce volatiles such as 2-propanol and acetone (Heed, 1978; Vacek, 1979; Fogleman, 1982). These volatiles at moderate to high concentrations can be toxic to *Drosophila* adults and larvae of several species (David and Bocquet, 1975; Argues and Duarte, 1980; Daggard, 1981; Batterham et al., 1982). However, at lower concentrations, adults can benefit in terms of longevity when the volatile 2-propanol is present in the atmosphere (Batterham, et al., 1982; Sampsell and Latham, 1984). This benefit appears to be diet dependent in *D. mojavensis* since nonaxenic adults do not show comparable longevity as when tested under axenic conditions (Batterham et al., 1982). Controlled experiments utilizing known yeasts or other microorganisms have not yet been conducted with adults. However, experiments with larvae have domonstrated that common cactus yeasts, *Candida sonorensis* and *Cryptococcus cereanus*, which can tolerate and metabolize 2-propanol, provide benefits to three *Drosophila* species in the presence of 2-propanol as compared to another common cactus-associated yeast, *Pichia cactophila*, which has less tolerance and cannot metabolize 2-propanol (Starmer et al., 1986).

YEAST-YEAST INTERACTIONS AND YEAST COMMUNITY-*DROSOPHILA* INTERACTIONS

One perplexing observation in work on nutritional benefits of particular yeast to larvae is that sometimes the yeast of choice does not provide the best nutrition (Lindsay, 1958; Cooper, 1959; Ali and El-Helw, 1974). Given that yeasts rarely occur as monocultures in their natural habitats, it is thus reasonable to postulate that yeast–yeast interactions may be important in the nutrition of the *Drosophila* which might vector them.

Benjamin Metcalf (Syracuse University) has carried out a series of exper-

iments designed to answer the question: are yeast–yeast interactions important to the yeasts and flies? These experiments were conducted by seeding various numbers (0, 25, 50, 75) of axenic *D. mojavensis* first-instar larvae on sterile agria cactus tissue (10 g) inoculated with the bacterium *Erwinia carnegieana* in conjunction with given numbers (10^4 cells) of monocultures and most possible bicultures of five common agria cactus yeasts (*P. cactophila*, *C. sonorensis*, *Cr. cereanus*, *P. amethionina* var. *amethionina*, and *C. ingens*). Observations were then made on numbers of viable yeasts present at sequential time periods (0.5, 1, 3, 5, 7, and 11 days) as well as the percent emergence (viability) and size (thorax length) of adult *D. mojavensis* from treatment vials. Viable yeasts numbers were determined by removing small (approximately 0.1 g) amounts of decaying tissue and then dilution plating on complete or selective media (for bicultures) and growing at 25°C until colonies appeared. The initial growth rate (r) and carrying capacity (K) of each yeast was determined by fitting the observations (using nonlinear least squares) to the logistic growth curve. Coefficients of determination for these fits exceeded 0.90, and thus the logistic equation was a food predictor of yeast growth dynamics.

This experiment was replicated three times so that statistical comparisons could be conducted on yeast growth rates, yeast carrying capacity, fly viability, and size. These comparisons allow for determination of yeast–yeast interactions with and without *Drosophila* larvae present and also allow for comparisons of monoculture versus biculture effects on the fly. Tables 3 and 4 summarize the results for yeast effects on yeasts at various larval densities. It appears that the presence of larvae does not change the pattern of yeast effects on the initial growth rate of other yeasts (Table 3). The pattern is clear; yeasts of this community, in general, increase one another's growth rates, regardless of the presence of larvae.

The pattern for carrying capacity is qualitatively different (Table 4). When no larvae are present all yeast–yeast effects are positive, whereas when larvae are present various negative and positive–negative interactions appear. If one utilizes May's (1974) criteria for qualitative stability of communities, those yeast communities with larvae are qualitatively stable while those without larvae are unstable. These results are consistent with the findings of Heithaus et al. (1980), that the addition of a predator can lead to stable equilibrium for systems involving mutualism. Furthermore, analysis of several models of mutualism (Wolin and Lawlor, 1984; Addicott, 1979) indicates that mutualism that increases r, enhances stability. Because some of the bicultures were not carried out, the validity of this conclusion is not without question.

The results for yeast effects (monoculture vs. biculture) on thorax size and viability are depicted in Figures 1 and 2. The points on the graph in Figure 1 represent the average size of adults (sex and density separate) reared from each monoculture plotted against the size of adults from the corresponding biculture. It is apparent from Figure 1 that adults reared from bicultures are about the

TABLE. 3. ESTIMATES OF INTRINSIC GROWTH RATE (r) IN CELLS PER HOUR UNDER FOUR DENSITIES OF *D. mojavensis* LARVAE (0, 25, 50, and 75) PER 10 g OF CACTUS TISSUE[a]

	Yeast species[b]				
	PC	CS	PAA	CC	CI
No Larvae					
PC	0.23	0.26	0.30	0.25	0.34[c]
CS	0.27[c]	0.14	0.31[c]	0.16	0.21
PAA	0.28[c]	0.28[c]	0.12	—	—
CC	0.18[c]	0.09	—	0.10	—
CI	0.11	0.09	—	—	0.10
25 larvae					
PC	0.24	0.27	0.30	0.29	0.26
CS	0.30[c]	0.14	0.32[c]	0.18	0.15
PAA	0.25[c]	0.33[c]	0.18	—	—
CC	0.14	0.11	—	0.10	—
CI	0.11	0.08	—	—	0.09
50 larvae					
PC	0.25	0.24	0.24	0.25	0.26
CS	0.30[c]	0.13	0.31[c]	0.17	0.15
PAA	0.25[c]	0.28[c]	0.13	—	—
CC	0.14[c]	0.10	—	0.07	—
CI	0.12	0.06	—	—	0.13
75 larvae					
PC	0.23	0.22	0.27	0.23	—
CS	0.30[c]	0.13	0.33[c]	0.17	—
PAA	0.25[c]	0.30[c]	0.13	—	—
CC	0.12	0.06	—	0.07	—
CI	—	—	—	—	—

[a] The comparison is with the response to self on the diagonal and is made with an LSD derived from a separate 2-way (competitor × density) ANOVA for each responding species (i.e, row).
[b] Scientific names: *Pichia cactophila* (PC), *Candida sonorensis* (CS), *P. amethionina* var. *amethionina* (PAA), *Cryptococcus cereanus* (CC), and *Candida ingens* (CI).
[c] Statistically significant differences (increases) of the estimates within each larval treatment group.

same size (scattered about the line of equivalence) as adults taken from the corresponding monocultures. The linearity of the points is due to sex (females are bigger) and larval density (higher density results in smaller adults).

The points on the graph in Figure 2 represents the average emergence percent of larvae (density separate) reared from each monoculture plotted against the emergence of larvae of the corresponding biculture. This figure demonstrates that larvae receive viability benefits from bicultures and, in general, do better when two yeasts are present.

Taken together, the yeast–yeast interactions and monoculture versus bi-

TABLE 4. ESTIMATES OF CARRYING CAPACITIES (K) IN ln(CELLS PER GRAM) UNDER FOUR DENSITIES OF *D. mojavensis* LARVAE PER 10 g OF CACTUS TISSUE[a]

	Yeast species[b]				
	PC	CS	PAA	CC	CI
No larvae					
PC	18.69	19.68	20.02[c]	18.84	20.06[c]
CS	20.00[c]	19.46	19.68	19.15	19.19
PAA	19.37	19.25	18.97	—	—
CC	18.23	19.89	—	18.69	—
CI	18.03	18.60[c]	—	—	17.71
25 larvae					
PC	19.78	19.15	19.21	20.05	19.83
CS	18.91	19.10	19.93[c]	18.93	18.12[d]
PAA	18.64[d]	18.68[d]	19.27	—	—
CC	18.09	18.23	—	18.10	—
CI	17.52	17.37	—	—	17.15
50 larvae					
PC	19.05	18.87	18.79	20.13[c]	19.55
CS	19.81	19.21	19.50	19.00	17.89[d]
PAA	17.93[d]	17.91[c]	19.23	—	—
CC	16.59[d]	16.21[d]	—	18.27	—
CI	15.89	17.64	—	—	17.69
75 larvae					
PC	18.82	18.64	18.80	20.29	—
CS	17.55	18.64	18.96	18.49	—
PAA	17.15[d]	17.86	18.65	—	—
CC	16.81	16.86	—	17.89	—
CI	—	—	—	—	—

[a] The comparison is with the response to self on the diagonal and is made with an LSD derived from a separate 2-way (competitor × density) ANOVA for each responding species (i.e, row).
[b] Scientific names: see Table 3.
[c] Statistically significant differences (increases) of the estimates within each larval treatment group.
[d] Statistically significant differences (decreases) of the estimates within each larval treatment group.

culture yeast effects on *Drosophila* are compelling evidence for community coadaptation of the yeasts with respect to their fly vector. Members of the yeast community of agria cactus tend to facilitate one another's initial growth and, in the presence of larvae, experience competitive interactions which are stabilizing. In turn, the mixed cultures of yeasts provide viability benefits to the *Drosophila* and thus increase the likelihood of being transported to a new habitat.

The effects of monocultures and bicultures of yeast on *D. mojavensis* larvae growing in decaying agria cactus tissue has been demonstrated in another cactus–fly system. In this case, *D. buzzatii*, which breeds in decaying *Opuntia stricta* cladodes, where these species have been introduced in Australia, were

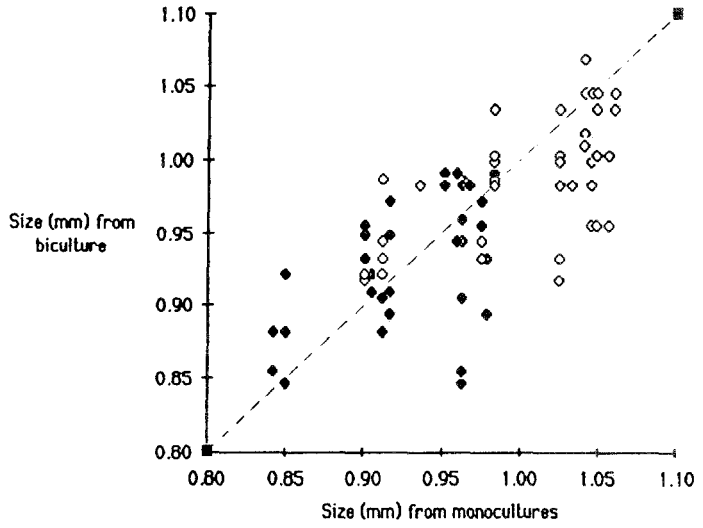

FIG. 1. Average thorax size (mm) of adult *D. mojavensis* reared from bicultures of yeasts plotted against the size of adults reared from each corresponding monoculture. Males are filled diamonds, while females are open.

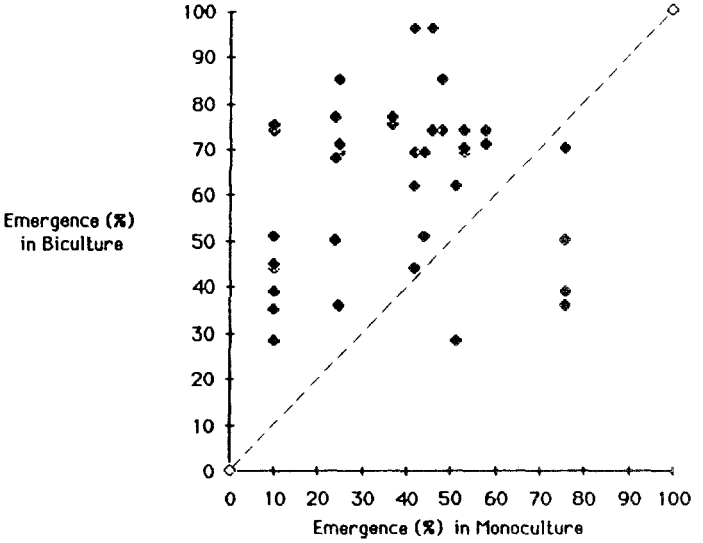

FIG. 2. Average emergence (%) of *D. mojavensis* larvae grown on bicultures of yeasts plotted against the emergence of larvae from each corresponding monoculture.

utilized. The yeasts associated with *O. stricta* rots (Barker et al., 1983) are similar to those found in columnar cacti. However, some species (e.g., *Clavispora* sp. "O") are more common in *O. stricta* necroses. Experiments were designed to measure the effects of monocultures and bicultures of four common yeasts (*P. cactophila*, *C. sonorensis*, *Clavispora* sp. "O," and *P. amethionina* var. *amethionina*) on the growth and development of three *Adh*-1 genotypes of *D. buzzatii*. Experiments were conducted by seeding 40 axenic first-instar larvae (10 *Adh*-1^b/*Adh*-1^b:20 *Adh*-1^b/*Adh*-1^c:10 *Adh*-1^c/*Adh*-1^c) onto 5 g of sterile, homogenized *O. stricta* cladode tissue in 30-ml vials. Ten yeast treatments (four monocultures and all six possible bicultures) were applied by inoculating 50 μl of an approximately 10^5 cells/ml suspension of each yeast. Bicultures received 25 μl of each suspension. Each treatment was replicated five times and incubated at 25°C. Adult sex, genotype, thorax size, and development time

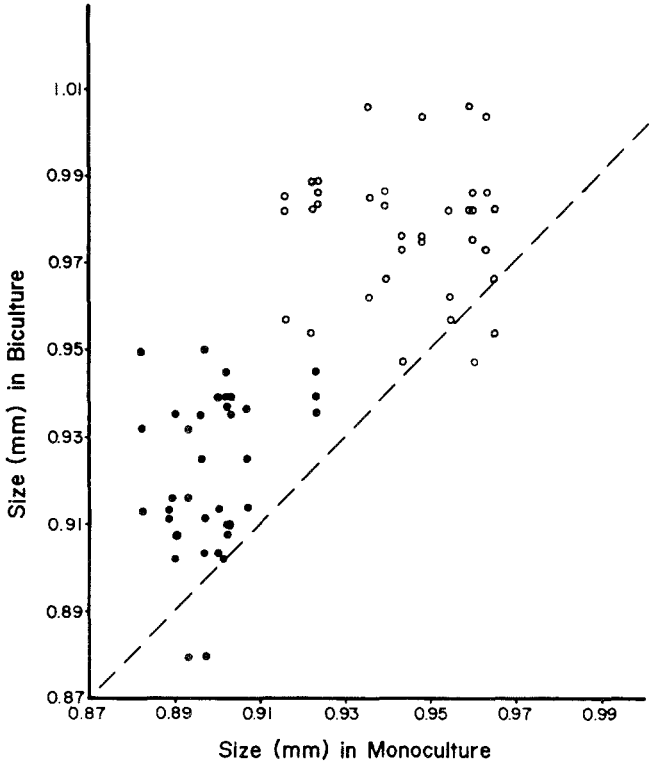

FIG. 3. Thorax length (mm) of *D. buzzatii* adults which grew as larvae on monocultures and all possible bicultures of four yeast species. Each point represents a monoculture against each biculture it is a member of for each *Adh*-1 genotype and each sex (female = ○) (male = ●) averaged over all animals of five replicate vials.

were observed. Because *Adh*-1 genotypic effects are not of interest here and genotype by yeast interactions were not statistically significant, we will only present the results for flies irrespective of genotype.

A priori contrasts of yeast monocultures versus bicultures showed highly significant effects for development time ($F = 44.8$, df = 237,6) and thorax length ($F = 14.1$, df = 237,6) but no significant effect on viability ($F = 1.7$, df = 120,6). These contrasts are depicted in Figures 3–5. It is apparent from these figures that larvae develop faster and reach larger sizes on bicultures as compared to monocultures. Once again, although for different reasons, mixed cultures of yeasts provide benefits to the *Drosophila* and thus increase the likelihood of being transported to a new habitat.

SUMMARY

The mutualistic interactions of cactophilic *Drosophila* and their associated yeasts are viewed as a system which has evolved within the framework of the host cactus stem chemistry. Because the *Drosophila*-yeast system is saphro-

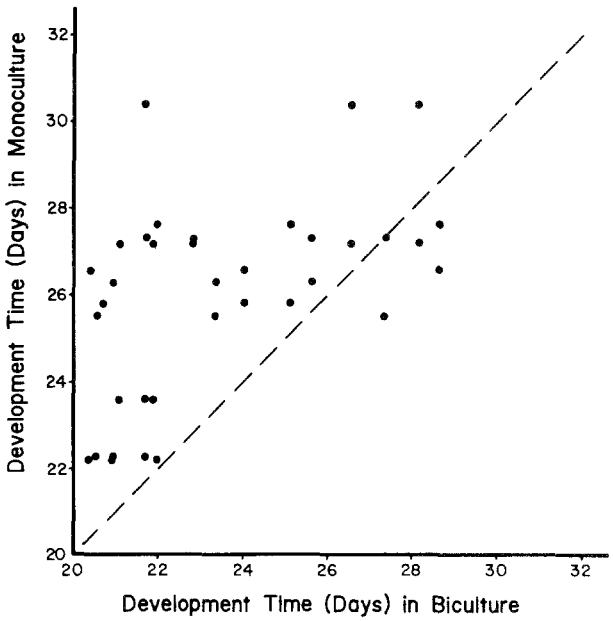

FIG. 4. Development time (days) of *D. buzzatii* larvae grown in monoculture and all possible bicultures of four yeast species. Each point represents a monoculture and the biculture combination it is a member of for each *Adh*-1 genotype averaged over five replicate vials.

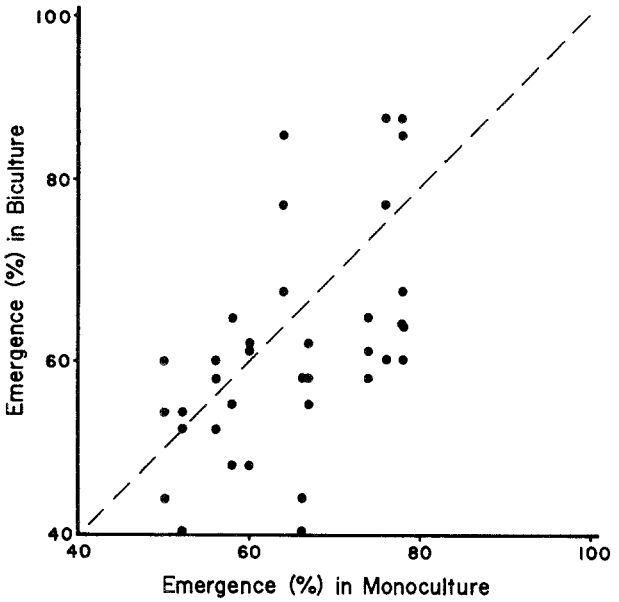

FIG. 5. Percent viability for *D. buzzatii* larvae grown in monoculture and all possible bicultures of four yeast species. Each point represents a monoculture against each biculture it is a member of for each *Adh*-1 genotype averaged over five replicate vials.

phytic, their responses are not thought to directly influence the evolution of the host.

Host cactus stem chemistry appears to play an important role in determining where cactophilic *Drosophila* breed and feed. Several chemicals have been identified as being important: (1) sterols of senita cactus are required in the diet of *D. pachea*, (2) alkaloids of senita cactus are toxic to other *Drosophila* species, and (3) medium chain fatty acids and sterol diols of *Stenocereus* species are detrimental to *D. nigrospiracula* and thus restrict this species from utilizing organpipe and agria cactus.

Cactus chemistry appears to have a limited role in directly determining the distribution of cactus-specific yeasts, even though the yeast community composition does correspond to chemical groupings of the host cacti. Those effects which are known are due to triterpene glycosides and unusual lipids of *Stenocereus* species. These compounds can restrict yeast growth and thus eliminate some species of cactus yeasts from the *Stenocereus* habitats.

Drosophila–yeast interactions are viewed as mutualistic and can take the form of (1) benefits to the *Drosophila* by either direct nutritional gains (viability, increased size, or longevity) or indirect gains due to detoxification of harmful chemicals produced during decay of the host stem tissue (e.g., *C. ingens* can metabolize toxic medium chain fatty acids of organpipe cactus) and (2)

benefits to the yeast in the form of increased likelihood of transmission to new habitats.

Experiments on yeast–yeast interactions in decaying agria cactus provide compelling evidence that the yeast community is coadapted. This coadaptation takes the form of (1) mutualistic increases in growth rates, which is independent of the presence of *Drosophila* larvae, and (2) stabilizing competitive interactions when growth reaches carrying capacity. This latter form is dependent on larval activity and results in benefits to the viability (*D. mojavensis* on agria cactus) or increases in size and faster development (*D. buzzatii* on *Opuntia stricta*) when fed bicultures or resident yeasts.

Given that *Drosophila* may be the primary vector of the yeasts, any yeast or combination of yeasts which serve to benefit the *Drosophila* (greater viability, larger size, or faster development) will also benefit the yeast. In this sense the yeast community is probably coadapted with respect to its *Drosophila* vector.

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CELLULOSE DIGESTION IN *Monochamus marmorator* Kby. (COLEOPTERA: CERAMBYCIDAE): Role of Acquired Fungal Enzymes

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Abstract—Larvae of the balsam fir sawyer, *Monochamus marmorator* Kby. (Coleoptera, Cerambycidae), contain midgut digestive enzymes active against hemicellulose and cellulose. Cellulases from larvae fed on balsam fir wood infected with the fungus, *Trichoderma harzianum* Rifai (Deuteromycetes, Moniliales, Moniliaceae), were found to be identical to those of the cellulase complex produced by this fungus when compared using chromatography, electrophoresis, and isofocusing. When larvae are maintained on a fungus-free diet, their midgut fluids lack cellulolytic activity, and they are unable to digest cellulose. Cellulolytic capacity can be restored by feeding the larvae wood permeated by fungi. We conclude that the enzymes which enable *M. marmorator* larvae to digest cellulose are not produced by the larvae. Instead, the larvae acquire the capacity to digest cellulose by ingesting active fungal cellulases while feeding in fungus-infected wood.

Key Words—Cellulases, xylophagy, cellulolysis, acquired enzymes, nutritional ecology, *Monochamus marmorator*, *Trichoderma harzianum*, Coleoptera, Cerambycidae, Moniliaceae.

INTRODUCTION

The ability of wood-boring larvae of cerambycid beetles to utilize the structural polysaccharides in their food has been the subject of many investigations dating back to the early 1900s (Linsley, 1959). Cellulolytic enzymes have been detected in the gut fluids of many species (Dajoz, 1968; Chararas et al., 1971; Chararas, 1981), and approximate digestibility measurements have demonstrated that between 12 and 57% of ingested cellulose may be assimilated (Martin, 1983). At one time it was believed that intracellular symbionts might play

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a role in cellulose digestion in these beetles (Buchner, 1928; Campbell, 1929; Uvarov, 1929), but this possibility has failed to receive confirmation (Mansour and Mansour-Bek, 1934a, b;, Parkin, 1940). The inability to demonstrate symbiont-mediated cellulolysis has led to the assumption that cerambycid larvae produce all of the enzymes necessary for cellulose digestion, although there is little evidence to support this assumption.

Cellulose digestion is generally accomplished by a collection of enzymes (the cellulase complex) consisting of three major classes of hydrolases: endoglucanases (C_x-cellulases), cellobiohydrolases (C_1-cellulases), and β-glucosidases (e.g., cellobiases) (Martin, 1983). In no study of any species of cerambycid beetle has compelling evidence been presented that all of the requisite enzymes of the cellulase complex are produced by the larvae themselves.

We have studied cellulose digestion in the balsam fir sawyer, *Monochamus marmorator* Kby. (Cerambycidae, Lamiinae). Larvae of this species tunnel extensively through the wood of moribund balsam fir (*Abies balsamea*) stems, frequently passing through patches of wood decayed by white rot fungi. The objective of this study was to determine whether the capacity of *M. marmorator* larvae to digest cellulose is due to fungal enzymes ingested by the larvae during feeding. Our research on *M. marmorator* is part of ongoing investigations into the roles of acquired fungal enzymes in the digestive processes and nutritional ecology of xylophagous insects (Martin, 1984).

METHODS AND MATERIALS

Collection and Maintenance of Larvae. M. marmorator larvae were collected from moribund stems of balsam fir at the University of Michigan Biological Station, Cheboygan County, Michigan. Larvae were maintained in the laboratory at 22°C in 150-ml wide-mouth polyethylene jars containing decayed balsam fir wood chips (BFWC), kept at a 30–50% moisture content. In experiments that involved larvae reared on a fungus-free medium, 40 *M. marmorator* larvae were reared for two to four weeks in 25-ml plastic cups containing small blocks of a wheat germ–casein artificial diet (Tobacco Hornworm Diet, Bio-Serv). Larvae have been successfully maintained on this diet for over nine months.

Isolation and Maintenance of Fungi. Fungi associated with the galleries of *M. marmorator* larvae in balsam fir stems were isolated by placing small wood chips, removed aseptically from the wood adjacent to larval galleries, onto plates of 1.5% malt extract agar, 4% potato dextrose agar, and 5% Czapek agar (Difco).

For the production and harvest of cellulase, *Trichoderma harzianum* was grown in 7-liter batches on a medium of the following composition (ingredients given in grams per liter): microcrystalline cellulose (Polysciences), 5.0; $(NH_4)_2SO_4$, 2.0; KH_2PO_4, 1.7; $K_2HPO_4 \cdot 3H_2O$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5;

$CaCl_2 \cdot 2H_2O$, 0.05; H_3BO_3, 2×10^{-4}; $CuSO_4 \cdot 5H_2O$, 3×10^{-4}; $ZnSO_4 \cdot 7H_2O$, 4×10^{-3}; $MnCl_2 \cdot 4H_2O$, 1×10^{-3}; $FeSO_4 \cdot 7H_2O$, 1×10^{-3}; $CoCl_2 \cdot 6H_2O$, 3×10^{-4}; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 2×10^{-4}. Incubation was on a rotary shaker at 22°C for two weeks.

Extract Preparation and Enzyme Assays. Midguts were dissected from *M. marmorator* larvae as described by Martin et al. (1980). Since the pH of the midgut fluid ranged between 6.2 and 6.9, extracts were prepared in 50 m*M* Bis-Tris-HCl buffer [bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane] (Sigma), pH 6.5. For enzyme assays, the pooled midguts from one to three larvae were prepared as described in Martin et al. (1981). For purification of the cellulase enzyme complex from *M. marmorator* gut fluid, the midguts from 30 larvae were pooled.

Total activity of the cellulase complex was determined by incubating 0.5 ml of suitably diluted extract with 0.5 ml of 0.5% microcrystalline cellulose suspended in 0.1 M sodium acetate buffer, pH 5.0, at 45°C for 2 hr. Carboxymethylcellulase and xylanase activity were determined with 0.5% carboxymethylcellulose, sodium salt (Sigma), or 0.5% larchwood xylan (United States Biochemical), in sodium acetate buffer, with incubation at 45°C for 1 hr. The incubation was terminated by the addition of 1.0 ml of alkaline dinitrosalicylic acid (DNSA) reagent (Bernfeld, 1955). Insoluble cellulose and xylan were removed by centrifugation at 3500*g* for 1 min. The supernatant solution was then heated at 110°C for 8 min, diluted with 1.5 ml of water, and the absorbance at 540 nm determined. Enzyme/reagent blanks were prepared using heat-denatured aliquots of extract.

Cellulase Purification and Analysis. The cellulase enzyme complex from *T. harzianum* was purified from 28 liters of two-week-old culture fluid as previously described (Kukor and Martin, 1983). The cellulase complex from *M. marmorator* larvae reared on *T. harzianum*-infected BFWC was prepared from the pooled midguts of 30 larvae, and the cellulase from artificial diet-reared larvae was prepared from the pooled midguts of 12 animals.

M. marmorator cellulase purification was achieved by an initial ammonium sulfate fractionation, with all of the cellulase activity contained in the 26–50% S ammonium sulfate fraction. Precipitated proteins were collected by centrifugation, dissolved in Bis-Tris buffer (pH 6.5), and the residual ammonium sulfate was removed by diafiltration in a stirred cell. Total protein was estimated by the dye-binding method of Bradford (1976).

The *Trichoderma* and *Monochamus* enzyme preparations were fractionated by high-resolution anion-exchange chromatography using a fast protein liquid chromatography (FPLC) system (Pharmacia). FPLC separations were carried out at a flow rate of 1.0 ml/min with an HR5/5 MonoQ column equilibrated with 50 mM Bis-Tris buffer (pH 6.5) containing 10 mM KCl. Proteins were eluted with a 10–500 mM linear KCl gradient, and 1.0-ml fractions were collected.

Discontinuous-SDS-gradient gel electrophoresis was performed on 7.5–15% polyacrylamide gels using the system of Neville, as modified by Piccioni et al. (1982). Gels were stained with brilliant blue R250 (Sigma). Analytical isoelectric focusing was performed on ultrathin polyacrylamide gels (Servalyt Precotes, Serva Fine Biochemicals), as previously described (Kukor and Martin, 1983).

[^{14}C]*Cellulose Utilization.* For [^{14}C]cellulose utilization experiments, individual larvae were placed in stoppered 25-ml scintillation vials. The bottom of each vial was coated with a thin layer of 2% agar in 0.1 N HCl, which provided moisture for the larva while it was feeding on the labeled cellulose. The headspace in each vial was periodically flushed with air at a flow rate of 30 ml/min for 15 min, and the $^{14}CO_2$ in the exit gas was bubbled through phenethylamine (1 : 1 v/v in MeOH) in ACS counting scintillant (Amersham). The trapping efficiency of this system was 90%.

Each animal was provided with a 100-mg food tablet made from α-cellulose fiber (Sigma) amended with 5% (w/w) [U-^{14}C]cellulose (ICN). The labeled cellulose was repurified before use according to the procedure of Rapson (1963), to ensure removal of any water- or alkali-soluble noncellulosic contaminants that might have been present. The specific activity of labeled cellulose in each food tablet was calculated from the amount of radioactivity in weighed portions of the tablets, which were digested in 67% sulfuric acid and diluted 10-fold with water before mixing with counting scintillant.

Counting efficiency was estimated by the H-number method or by internal standards, as appropriate.

Assimilation Efficiencies. For approximate digestibility determinations, larvae from two groups (those reared for four weeks on *T. harzianum*-infected BFWC and those reared for two weeks on artificial diet followed by two weeks on autoclaved BFWC) were placed in individual 150-ml polyethylene bottles containing 4–5 g (wet weight) of autoclaved BFWC. The jars, covered with Saran wrap, were incubated at 22°C, and the diet was kept at 30–50% moisture content by periodic addition of sterile distilled water. After seven days, uneaten food and fecal pellets were separated using a series of standard mesh sieves. Food fragments were retained on a 1-mm mesh screen, whereas the fecal pellets were collected on 0.5- and 0.4-mm mesh screens. The few small food fragments that passed onto the 0.5-mm and 0.4-mm mesh screens could be separated from the spherical fecal pellets by allowing the pellets to roll down an inclined metal pan subjected to moderate vibration by tapping with a pencil. Approximate digestibility was determined on a dry weight basis as AD (%) = 100 (food ingested − feces)/food ingested.

The cellulose content of BFWC and *M. marmorator* fecal pellets was estimated by the procedure of Updegraff (1969).

RESULTS

Digestive Enzymes of M. marmorator. The digestive fluids from the midguts of *M. marmorator* larvae, either collected from their natural galleries in balsam fir stems or cultured in the laboratory on balsam fir wood chips permeated by mycelium of the fungus, *T. harzianum*, contain enzymes that enable them to digest both cellulose and hemicellulose (Table 1). Activity toward microcrystalline cellulose indicates the presence of the entire cellulase complex required to degrade native cellulose, while activity toward carboxymethylcellulose (CMC) indicates the presence of endoglucanases (C_x-cellulases) which attack soluble degradation products of cellulose or amorphous regions of the predominantly crystalline structure of native cellulose. Activity toward larchwood xylan indicates the presence of enzymes required to degrade arabino-4-O-methylglucuronoxylans, which are a major class of hemicelluloses present in both hardwoods and softwoods. Although the assays were routinely conducted at a pH of 5.5, the cellulolytic and hemicellulolytic enzymes exhibit significant activity in the pH range 6.2–6.9 observed in the midgut fluids of these beetle larvae. Since cellulose and arabino-4-O-methylglucuronoxylans make up 42 and 9%, respectively, of the dry weight of extractive-free balsam fir wood (Côté, 1977), the presence of cellulases and xylanases in the gut fluid of *M. marmorator* larvae is of obvious adaptive value.

In contrast to larvae from natural galleries or reared on fungus-infected wood, larvae fed for two weeks on a fungus-free artificial diet have gut fluid in which activity toward microcrystalline cellulose is barely detectable (Table 1). CMCase and xylanase activity is still evident, albeit at somewhat reduced levels, in the larvae from the fungus-free diet. When cellulase-free larvae are transferred from the artificial diet to autoclaved fungus-infected balsam fir wood chips, they do not regain the enzymes of the cellulase complex. This finding demonstrates that the cellulolytic activity of the gut fluid of larvae reared on normal fungus-infected wood is not due to enzymes of insect origin, the secretion of which is induced by heat-stable components of the normal diet, but instead suggests that cellulolytic activity might be due to ingested fungal enzymes. CMCase and xylanase activity is increased in larvae transferred to autoclaved, fungus-infected wood.

Fungi Associated with M. marmorator *Galleries.* The galleries of middle- to late-instar *M. marmorator* larvae often pass through regions of wood decayed by white rot fungi. Although we have not conducted an exhaustive study to characterize all of the fungi associated with *M. marmorator* galleries, we did establish that *Hirschioporus abietinus*, *Stereum sanguinolentum*, *Amylostereum chailletii*, and *Trichoderma harzianum* are readily isolated from the wood adjacent to larval galleries. *T. harzianum* was the species most frequently isolated.

TABLE 1. ENZYMATIC ACTIVITY TOWARD CELLULOSE, CARBOXYMETHYLCELLULOSE, AND XYLAN OF EXTRACTS OF MIDGUTS OF *Monochamus marmorator* LARVAE, BALSAM FIR WOOD CHIPS (BFWC) AND THE FUNGUS, *Trichoderma harzianum*

Source of extract	Enzymatic activity [units/mg (dry weight) of dissolved solids in extract][a]		
	Cellulase complex	CMCase	Xylanase
M. marmorator larvae			
Collected from natural galleries in balsam fir	0.55 ± 0.04 (5)	10.8 ± 0.7 (5)	22.5 ± 2.4 (5)
Cultured for 2 weeks on BFWC permeated by *T. harzianum*	0.45 ± 0.08 (10)	14.7 ± 0.7 (12)	17.7 ± 1.5 (12)
Cultured for 2 weeks on artificial diet	0.01 ± 0.005 (6)	8.0 ± 2.8 (6)	9.3 ± 3.4 (6)
Cultured for 2 weeks on artificial diet, then for 2 weeks on autoclaved, *Trichoderma*-infected BFWC	0.01 ± 0.005 (3)	16.4 ± 4.9 (3)	15.2 ± 5.1 (3)
BFWC			
Sterile	0.0 (1)	0.0 (1)	0.0 (1)
Permeated by *T. harzianum*	0.40 ± 0.01 (3)	1.5 ± 0.5 (3)	6.8 ± 2.4 (3)
Culture fluid from 2-week-old axenic culture of *T. harzianum*	2.3 ± 0.7 (3)	13.3 ± 6.6 (2)	8.2 (1)

[a] A unit of activity is the amount of enzyme required to liberate 1 μmol of maltose equivalents per hour under the conditions of the assay (45°C, pH 5.0, incubation volume 1.0 ml). Each value is the mean ± standard error of the mean for the number of replicates in parenthesis.

All of these fungi are cellulose digesters and hence are potential sources of cellulolytic enzymes. In agreement with the hypothesis that the beetle larvae are acquiring a capacity to digest cellulose through the ingestion of fungal cellulases, we demonstrated activity toward microcrystalline cellulose, carboxymethylcellulose, and larchwood xylan in an extract of balsam fir wood permeated by the mycelium of *T. harzianum*, as well as in the culture fluid of *T. harzianum* grown on microcrystalline cellulose in a defined medium (Table 1).

Purification and Comparison of Monochamus *and* Trichoderma *Cellulases.* When subjected to high-resolution anion-exchange chromatography (FPLC), 84% of the total activity toward microcrystalline cellulose in the gut fluid of larvae fed *T. harzianum*-infected wood eluted at 0.15 M KCl in a single large protein peak (Figure 1A). This peak was absent in the gut fluid of larvae cultured sequentially on an artificial diet and then on autoclaved *T. harzianum*-infected balsam fir wood chips (Figure 1B), although there was some activity toward carboxymethylcellulose and xylan associated with the various protein peaks in the elution profile. When the culture fluid from *T. harzianum* was subjected to a similar separation, a large protein peak eluted at 0.15 M KCl which accounted for 67% of the total activity toward microcrystalline cellulose (Figure 1C).

A comparison of the major cellulase peak from the insect and fungal preparations was made by discontinuous, denaturing-gel electrophoresis and isoelectric focusing. On an SDS-gradient gel, the FPLC cellulase fractions from *T. harzianum* culture fluid and from the gut extract of *M. marmorator* reared on *T. harzianum*-infected balsam fir wood chips show identical patterns of protein

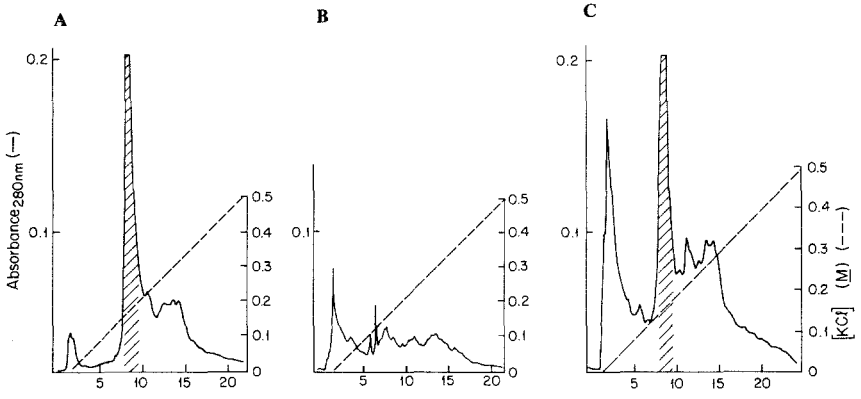

FIG. 1. FPLC elution profiles of *M. marmorator* midgut extracts and *T. harzianum* culture fluid. (A) *M. marmorator* reared on *T. harzianum*-infected BFWC. (B) *M. marmorator* reared on artificial diet and then on autoclaved *T. harzianum*-infected BFWC. (C) Fluid from an axenic culture of *T. harzianum* grown on a defined medium. Crosshatching indicates fractions with most of the activity against microcrystalline cellulose.

FIG. 2. SDS-gradient gel (7.5–15%) of the major FPLC cellulase peaks from *M. marmorator* reared on *T. harzianum*-infected BFWC (M), and culture fluid of *T. harzianum* grown on a defined medium (T). Apparent molecular masses of the major bands: 83.9, 80.0, 70.0, and 17.9 kD.

bands (Figure 2). The four major bands have apparent molecular masses of 83.9, 80.0, 70.0, and 17.9 kilodaltons. In an analytical isoelectric focusing gel (Figure 3), the fungal and insect FPLC cellulase fractions also have identical patterns, appearing as a broad band focused between pH 4.73 and 5.07, with the highest density of protein at pH 5.0. These comparisons of fungal and insect cellulases demonstrate that the enzymes responsible for activity toward microcrystalline cellulose in the digestive fluids of *M. marmorator* larvae reared on *T. harzianum*-infected wood are identical to the enzymes produced by this same fungus grown in a defined medium, and they confirm our hypothesis that the insects acquire essential digestive enzymes by the ingestion of fungi or fungal enzymes present in wood they consume.

[^{14}C]*Cellulose Utilization.* In order to demonstrate the importance of ingested fungal enzymes to cellulose digestion, we fed larvae [U-^{14}C]cellulose

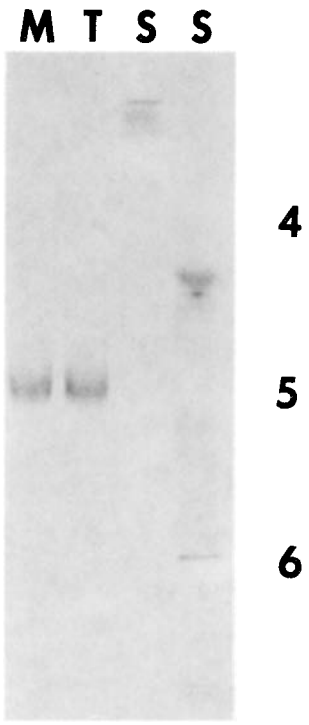

FIG. 3. Analytical isoelectric focusing on an ultrathin polyacrylamide gel (Serva Precote, nominal pH range 4–6) of the major FPLC cellulase peaks from *M. marmorator* reared on *T. harzianum*-infected BFWC (M), culture fluid of *T. harzianum* grown on a defined medium (T), and isoelectric point marker proteins (S). The marker proteins used and their corresponding pI values are pepsinogen, 2.80; amyloglucosidase, 3.50; glucose oxidase, 4.15; β-lactoglobulin, 5.34; conalbumin, 5.9.

and determined the percentage of ^{14}C from the food incorporated into respired carbon dioxide and body tissue (Table 2). The production of significant amounts of ^{14}C-labeled carbon dioxide by larvae reared on a fungus-containing diet (larvae 1, 2, 3) and the incorporation of ^{14}C into the fat body and integument (larva 1) clearly reveal the ability of these insects to digest cellulose, while the absence of any labeled carbon dioxide in the respiratory gases from larvae reared on a fungus-free diet (larvae 4, 5, 6, 7) shows that they are unable to digest cellulose. In this set of experiments it was also demonstrated that a larva that had lost its acquired cellulases by maintenance on a fungus-free diet for two weeks (larva 5) regained cellulolytic capacity after only 24 hr on a diet of fungus-infected wood. A second larva (larva 6) did not regain its cellulolytic capacity after feeding for 24 hr on fungus-infected wood that had been autoclaved in order to kill the fungus and deactivate any fungal enzymes that had been secreted into

TABLE 2. UTILIZATION OF [^{14}C]CELLULOSE BY *Monochamus marmorator* LARVAE CULTURED ON FUNGUS-CONTAINING AND FUNGUS-FREE DIETS

Larva	Treatment prior to transfer to [^{14}C]cellulose-containing diet	Time on [^{14}C]cellulose-containing diet (hr)	Label from [^{14}C]cellulose in diet released as $^{14}CO_2$ (%)	Label from [^{14}C]cellulose in diet incorporated into body tissue (%)
1	Cultured for 2 weeks on *Trichoderma*-infected BFWC	24	17	3.8
2	Cultured for 2 weeks on *Trichoderma*-infected BFWC	48	31	ND[a]
3	Cultured for 2 weeks on *Trichoderma*-infected BFWC	48	19	ND
4	Cultured for 2 weeks on fungus-free artificial diet	24	0	ND
5	Cultured for 2 weeks on fungus-free artificial diet	48	0	ND
6	Cultured for 2 weeks on fungus-free artificial diet	48	0	ND
7	Cultured for 2 weeks on fungus-free artificial diet	96	0	ND
5	After 48 hr on the [^{14}C]cellulose diet, larva was transferred to a diet of *Trichoderma*-infected BFWC for 24 hr, then back to the [^{14}C]cellulose diet	24	3	ND
6	After 48 hr on the [^{14}C]cellulose diet, larva was transferred to a diet of autoclaved, *Trichoderma*-infected BFWC for 24 hr, then back to the [^{14}C]cellulose diet	24	0	ND

[a]ND = not determined.

TABLE 3. APPROXIMATE DIGESTIBILITY OF AUTOCLAVED, FUNGUS-INFECTED BALSAM FIR WOOD CHIPS (BFWC) AND CELLULOSE IN BFWC BY *Monochamus marmorator* LARVAE

Larval diet immediately prior to experiment	Approximate digestibility (%)[a]	
	BFWC	Cellulose in BFWC
Four weeks on BFWC permeated by live mycelium of *Trichoderma harzianum*	52.2 ± 1.9 (4)	26.7 ± 4.0 (4)
Two weeks on artificial diet followed by 2 weeks on autoclaved, *Trichoderma*-infected BFWC	20.8 ± 5.4 (3)	3.7 ± 2.6 (2)

[a] Each value is the mean ± standard error of the mean for the number of replicates given in parenthesis. Duration of experiment was one week.

the substrate. These experiments confirm that larvae can digest cellulose only after they have fed on a substrate that contains active fungal enzymes.

Assimilation Efficiencies. When reared on fungus-infected wood, *M. marmorator* larvae assimilated over 50% of the dry matter and 25% of the cellulose in their food (Table 3). By contrast, larvae reared on a fungus-free artificial diet assimilated only 21% of the dry matter of balsam fir wood chips and a negligible portion of the cellulose in balsam fir wood (Table 3). These results clearly demonstrate that acquired fungal cellulases make a significant quantitative contribution to the ability of *M. marmorator* larvae to utilize their natural food.

DISCUSSION

In this study we have demonstrated that the ability of the larvae of *M. marmorator* to digest cellulose is due to fungal enzymes ingested by the larvae during the consumption of fungus-infected wood. The larvae do not secrete the full complement of enzymes required for cellulose digestion, and in the absence of a dietary source of fungal cellulases, they are unable to digest cellulose. The demonstration that the capacity for cellulose digestion by the larvae of this cerambycid beetle is due to the acquisition of fungal enzymes calls into question the often-stated but untested hypothesis that other xylophagous cerambycid larvae, as well as anobiid and buprestid larvae, secrete all of their own cellulases. While there is convincing evidence that many of these beetles do produce endoglucanases active against carboxymethylcellulose (Martin, 1983), there is no compelling evidence that any species secretes the entire cellulase complex, and

no evidence that rules out the possibility that cellulose digestion is mediated by ingested fungal enzymes.

The dependence upon ingested fungal enzymes to digest refractile polysaccharides has been demonstrated previously in the macrotermitine termites (Martin and Martin, 1978, 1979) and siricid woodwasps (Kukor and Martin, 1983). However, unlike the fungus-growing termites and siricid woodwasps, both of which have highly coevolved mutualistic associations with specific fungal symbionts, the cerambycid beetles are not known to maintain specific associations with particular species or strains of fungi, although cellulolytic fungi have been reported as surface contaminants on the eggs of *M. scutellatus* (Leach et al., 1937). Species of the genus *Monochamus* generally oviposit under the bark of sound timber, and the early larval instars feed in sound phloem and sapwood. It is only the later instars that expand their digestive capabilities by ingesting the enzymes of wood-rotting fungi that subsequently colonize the substrate. Finding that ingested enzymes derived from casual fungal associates play a vital role in the digestive processes of this wood-feeding beetle underscores the potential generality of a strategy for resource exploitation based upon the ingestion of microbial enzymes.

Fungal tissue plays an important role in the nutrition of a number of wood- and detritus-feeding invertebrates. Because of the chemical alteration of substrate brought about by fungi and because of the ability of fungi to concentrate nutrients, fungus-infected wood is a resource of higher nutritive quality than uninfected wood. Numerous studies of xylophagous anobiid and cerambycid beetles have shown improved performance (decreased larval mortality, increased growth rate, increased pupal weight, increased adult body size, and increased fecundity) of animals reared on fungus-infected substrate compared to fungus-free substrate (Campbell and Bryant, 1940; Fisher, 1940, 1941; Campbell, 1941, Becker, 1942, 1968; Bletchly, 1953). It is reasonable that the exploitation of ingested fungal enzymes for the digestion of refractile polysaccharides would evolve in a species with a preference for fungus-infected wood and gut conditions compatible with enzyme function. We predict that additional examples of the acquisition of digestive enzymes will be provided by further studies of the nutritional ecology of xylophagous insects.

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BEHAVIORAL RESPONSES TO CHEMICAL CUES BY BACTERIA

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Abstract—Bacterial chemotaxis presents a model sensory system in which cells modulate the direction of rotation of their flagella in response to gradients of certain chemicals. The chemotactic machinery of *Escherichia coli* is currently being systematically reduced to its individual components through the accomplishments of behavioral, physical, genetic, molecular genetic, and biochemical analyses. Thirteen of the so called "MCP-related" class of chemotaxis gene products are known. Transmembrane methyl-accepting chemotaxis proteins (MCPs) are important for the chemical sensing, signal generation, and sensory adaptation processes. Soluble chemotaxis proteins relay information from these MCPs to structural components of the flagella referred to as switch proteins. Emphasis here is on the separate roles each of these groups of chemotaxis proteins perform, as well as their protein–protein relationships.

Key Words—Bacteria, chemotaxis, methyl-accepting chemotaxis proteins, molecular biology, signal transduction, switch proteins.

INTRODUCTION

Prokaryotic organisms have evolved a plethora of methods to sense and respond to each other and to their environment. For example, luminescent marine bacteria secrete small autoinducer molecules into their surroundings which, when present in sufficient concentration, induce the cells to produce light (Engebrecht and Silverman, 1984). Individual cells of the soil bacterium *Myxococcus xanthus* respond to chemical signals from one another and come together to form a fruiting body structure (Zusman, 1984). Blue-green algae respond to nitrogen

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deprivation by developing specialized cellular compartments for nitrogen fixation (Golden et al., 1985). Many other prokaryotic chemical response systems also exist.

One of the most extensively studied bacterial response systems is that of chemotaxis. The chemotactic characteristics of a bacterial community often affect an entire ecosystem. Bacterial chemotaxis to oxygen in pond water may shield methane-producing bacteria from oxygen's toxic affects as well as provide carbon dioxide for the anerobic production of methane (Rosen, 1984). Soil bacteria chemotax to a wide variety of potential energy sources such as fungal propagules and plant exudates (Arora et al., 1983; Gitte et al., 1978). Marine bacteria may be attracted by coral and algal extracellular products and even rum distillery wastewater (Chet and Mitchell, 1976; Fuentes et al., 1983.). Chemotaxis is also believed to play an important role in certain human diseases, such as cholera wherein *Vibrio cholerae* must colonize the intestinal mucosa (Freter et al., 1979).

The focus for this review concerns a class of chemotaxis in *Escherichia coli* (*E. coli*) which is governed by transmembrane methylatable chemotaxis proteins (MCPs). The bulk of bacterial chemotaxis research has been performed on *E. coli* or *Salmonella typhimurium* (*S. typhimurium*). There is virtually complete functional homology among the flagellar, motility, and chemotaxis genes between these two enteric gram-negative species (Defranco et al., 1979; Kutsukake et al., 1980). Chemotaxis research on these organisms has provided an impressive array of behavioral, physical, genetic, molecular, and biochemical data. For information concerning the related subject areas of bacterial flagellar formation, energy transduction for motility, as well as additional taxis reviews, other sources are available (Silverman, 1980; Berg and Khan, 1982; Parkinson and Hazelbauer, 1983; Taylor, 1983; Macnab and Aizawa, 1984; Mizuno et al., 1984; Ordal, 1985).

BEHAVIOR

E. coli cells are 1–2 μm in length, peritrichous, and may, depending on environmental conditions, contain up to 10 flagella per cell, each approximately 10–15 μm in length (Hilmen and Simon, 1976). The regulation of the motion of the flagella governs the cells' chemotactic responsiveness. It has been conclusively demonstrated that the flagellum operates as a rotary engine with the flagellar filaments playing a passive role much like the propeller for a ship (Berg and Anderson, 1973; Silverman and Simon, 1974). This bacterial motor may rotate in either the counterclockwise or clockwise direction. Counterclockwise rotation (as viewed along the flagellum towards the cell) is typically associated with positive wave propagation of the filaments and straight swimming. In this mode the filaments are all in a stable left-handed helical conformation, and

together form a bundle of flagella which rotate in concert. During clockwise rotation, however, hydrodynamic forces induce the formation of right-handed filaments which cause the unwinding of the bundle (Macnab and Ornston, 1977). The net result is that the cell jerks about ("twiddles") until counterclockwise rotation is resumed and the flagellar bundle restored. In an isotropic environment, bacterial motility has been described as possessing a "random three-dimensional walk" which is characterized by a series of runs (the product of counterclockwise rotation), which are interrupted every 1–2 sec by twiddles (the product of clockwise rotation) (Berg and Brown, 1972).

Chemotactic behavior in *E. coli* consists of the ability to alter migration in response to gradients of chemicals. The response to attractant stimuli is a decreased probability of switching to tumbling, as well as an increased probability of switching from tumbling to smooth swimming (Khan and Macnab, 1980). The converse is true for repellent responses. These actions promote the net movement of a population of cells in a favorable direction. A diagram representing chemotactic behavior is shown in Figure 1. Because the cells do not actually modulate the angle of their turns, this is not a true taxis. Their responses have been more precisely described as klinokinesis, a biased random walk (Taylor, 1983). In addition to the movements of the cells, *E. coli* may also respond to certain attractants or repellents by increasing or decreasing the rate of its cell division, respectively (Sherman and Vorobyeva, 1983).

After the initial perception of an attractant or repellent (also called a chemoeffector), cells respond along two distinct pathways, beginning with a rapid sensory transduction or excitation phase, and followed by the adaptation phase during which the cells return to normal motility. Excitation requires approximately 0.2 sec (Segall et al., 1982; Block et al., 1982), while adaptation can be observed to last for several minutes.

E. coli can sense chemical differences of only one part in 10^4 over the length of its entire body (Dahlquist et al., 1972). How is such sensitivity achieved? Rapid mixing experiments with known chemoattractants or repellents and bacteria have shown that the chemotactic system operates as a time averaging system rather than a spatial sensory system (Macnab and Koshland, 1972). Chemoeffector information is integrated while the bacteria swim through their environment. Implied in this system is therefore some type of rudimentary memory.

che GENES, PRODUCTS, AND PRODUCT LOCATIONS

One of the advantages of studying sensory transduction in *E. coli*, especially as compared with higher organisms, has been the wealth of techniques available for genetic analysis. The study of mutants deficient in various aspects of chemotaxis has greatly aided the dissection of the individual processes in-

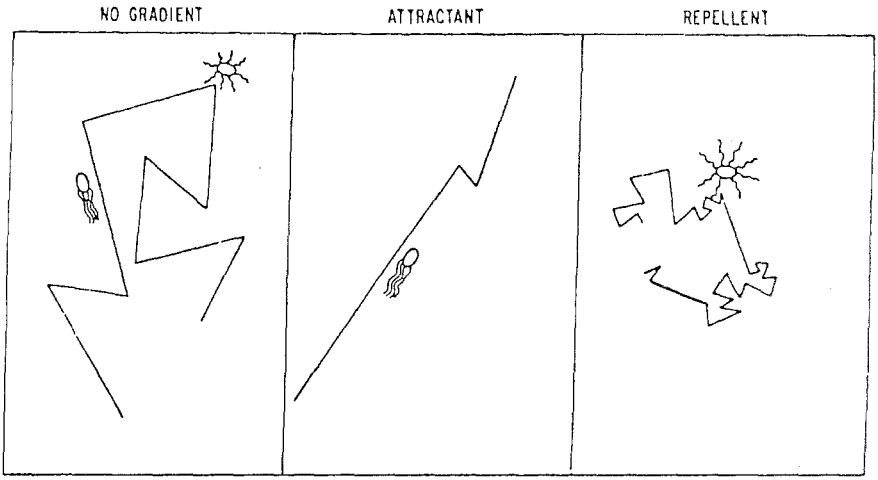

FIG. 1. Effect of chemoeffectors on bacterial motility. In the absence of an attractant or repellent, a cell exhibits smooth swimming interrupted every 1–2 sec with a tumble (left). Cells presented with an attractant spend a greater percentage of their time smooth swimming (middle), while those stimulated with a repellent bias their motility towards tumbling (right). Taken with permission from Silverman (1980).

volved. Furthermore, the availability of such mutants has provided the foundation for the recombinant DNA analyses which have since taken place. Nonchemotactic mutants (*Che*$^-$) have been isolated by a variety of techniques involving such characteristics of *Che*$^-$ mutants as their smaller region of growth on 0.4% agar plates (Parkinson, 1976) and their migration characteristics on preformed attractant gradients (Armstrong et al., 1967; Aswad and Koshland, 1975). These mutants contain flagella which rotate but exhibit altered swimming patterns. Some of these mutants tumble constantly, while others rarely tumble, and some will respond to no chemotactic stimuli, while others are only defective in responding to a particular class of chemicals. Thus, mutants blocked at various points along the flow of sensory information processing have been obtained.

The mapping of *che* (chemotaxis) genes in *E. coli* has been accomplished through Hfr × F$^-$ matings (Armstrong and Adler, 1969; Harayama et al., 1979) and deletion analysis in λ *che* transducing phage (Silverman and Simon, 1977b; Parkinson, 1978; Slocum and Parkinson, 1983). All but two *che* genes have mapped to areas near 42 or 43 minutes on the *E. coli* genetic map. Hand in hand with the advent of molecular biology, techniques were developed which allowed the definition of *che* operon structure, as well as *che* gene product identification. λ *che* transducing phage were used to direct the expression cloned *che* genes' products (Silverman et al., 1977). Also, *che* gene products have been identified using the minicell technique for selectively programming the *de*

novo expression of cloned *che* genes on plasmids (Matsumura et al., 1977). Altogether, 12 genes and gene products required for chemotaxis have been identified (Silverman and Simon, 1976; Silverman et al., 1977; Matsumura et al., 1977; Hazelbauer et al., 1981; Bartlett and Matsumura, 1984; Clegg and Koshland, 1985; Malakooti and Matsumura, in preparation). Most of these gene products have also been localized to the outer membrane, inner membrane, or soluble subcellular compartments (Ridgeway et al., 1977; D. Bartlett; PhD thesis, University of Illinois, Chicago; 1985; Clegg and Koshland, 1985). Table 1 lists the *E. coli che* genes, their map positions, product molecular weights, and product locations.

METHYL-ACCEPTING CHEMOTAXIS PROTEINS

A group of randomly situated transmembrane proteins, termed transducers, or methyl-accepting chemotaxis proteins (MCPs), play key roles in both the excitation and adaptation phases of certain chemotactic responses. MCPs have been found in enteric bacteria, *Caulobacter*, photosynthetic bacteria, *Bacillus*, and *Spirochaeta* (Kort et al., 1975; Goldman et al., 1982; Kathariou and Greenberg, 1983; Shaw et al., 1983). Each MCP is responsible for integrating infor-

TABLE 1. MCP-RELATED CHEMOTAXIS GENES, GENE PRODUCTS, PRODUCT LOCATIONS, AND PROPOSED FUNCTIONS

Map position	Gene	Product molecular weight (kd)	Product location[a]	Function
31	*trg*	55	IM	MCP III transducer
42	*cheA*	76, 66[b]	IM/CYT	regulates demethylation
42	*cheW*	12	CYT	?
42	*tar*	65	IM	MCP II transducer
42	*tap*	65	IM	transducer ?
42	*cheR*	28	CYT/IM	methyltransferase
42	*cheB*	38	CYT/IM	methylesterase
42	*cheY*	11	CYT	tumble regulator, inhibits methylation
42	*cheZ*	24	CYT/IM	?
43	*flaBII*	38	basal body (?)	switch protein
43	*flaAII*	37	basal body (?)	switch protein
43	*motD*	16	basal body (?)	switch protein
99	*tsr*	65	IM	MCP I transducer

[a] IM = innermembrane localization, CYT = cytoplasmic localization.
[b] The *cheA* locus contains two overlapping genes whose products are 76 and 66 kd. The smaller cheA product is found exclusively in the cytoplasm, while the larger product is found in both the cytoplasm and the inner membrane.

mation pertaining to one or a few chemoeffectors. In the case of *E. coli* this is accomplished through direct interactions on the outer face of the inner membrane with specific amino acids or with occupied periplasmic binding proteins (Anraku, 1968; Adler, 1969; Hazelbauer and Adler, 1971; Kellerman and Szmelcman, 1974; Willis and Furlong, 1974; Hazelbauer, 1975; Clarke and Koshland, 1979; Kondoh et al., 1979; Hedblom and Adler, 1980; Wang and Koshland, 1980). After signaling a flagellar response, the MCPs undergo changes in methylation that correlate with sensory adaptation (Silverman and Simon, 1977b; Springer et al., 1977). The degree of occupancy of an MCP by one of its chemoeffectors is a measure of that cell's current environment. The level of methylation of the MCPs appears to reflect the cell's chemoeffector environment in the past and may participate in the memory process. Curiously, in *E. coli*, the MCPs become more methylated in response to an appropriate attractant stimulus, while in the gram-positive *Bacillus subtilis*, the MCPs become less methylated (Goy et al., 1977; Goldman et al., 1982). The methylation–demethylation reactions occur at several γ-glutamyl residues along each MCP (Kleene et al., 1977; Van der Werf and Koshland, 1977) and are catalyzed by chemotaxis-specific enzymes. The *cheR* protein is the methyltransferase and the *cheB* protein is the methylesterase (Springer and Koshland, 1977; Stock and Koshland, 1978). *S*-Adenosylmethionine (SAM) provides the methyl groups (Armstrong, 1972). Methanol is the hydrolysis product (Toews and Adler, 1979).

The *cheB* protein also performs a second modification: the irreversible deamidation of glutamine to glutamic acid residues on the MCPs that are then capable of being methylated (Rollins and Dahlquist, 1981; Sherris and Parkinson, 1981). The function of this process is unknown.

Four MCP genes are known to exist: *tar*, *tap*, *tsr*, and *trg* (Ordal and Adler, 1974; Silverman and Simon, 1977a; Springer et al., 1977; Hazelbauer et al., 1981; Krikos et al., 1983). The *tar* gene product mediates responses to the attractants aspartate and maltose and to the repellents Co^{2+} and Ni^{2+} (Clarke and Koshland, 1979). The *tsr* gene product mediates responses to the attractant serine and to the repellents acetate, leucine, benzoate, and indole (Hedblom and Adler, 1980). The *trg* gene product is responsible for attraction to ribose and galactose (Kondoh et al., 1979). As yet no known function exists for the *tap* gene product. MCP-mediated chemotaxis does not require transport (Adler, 1969). The *tar* product is methylated four times, *tsr* product six times, and the *trg* gene product more than five (Kehry et al., 1983).

MCP methylation is particularly interesting in view of its analogy with mammalian sensory systems. Both neurosecretion and macrophage chemotaxis have been found to involve reversible methylation (Odea et al., 1981; Aksamit and Backlund, 1983). Furthermore, these multiple MCP methylations are similar to the multiple phosphorylations of rhodopsin, a covalent modification associated with adaptation to light in vertebrates (Schichi and Sommers, 1978).

All of the *E. coli* MCP genes have been sequenced, as well as the *S. typhimurium tar* gene (Boyd et al., 1981; Krikos et al., 1983; Russo and Koshland, 1983; Bollinger et al., 1984). This has allowed the determination of their primary amino acid sequence as well as their predicted hydrophilic and hydrophobic domains. All of the MCPs appear to follow the same general configuration. At the *N*-terminal end is a very hydrophobic region which has been proposed to serve as a signal sequence that spans the membrane. This is followed by the receptor binding site which extends into the periplasmic space, followed by a second membrane-spanning domain and, finally, the bulk of the protein resides at the cytoplasmic face. Biochemical analysis of the *tar* and *tsr* products has determined two tryptic peptides which localize to the cytoplasmic regions of both proteins and which are methylated and show similarities in amino acid sequence (Wang and Koshland, 1980; Kehry and Dahlquist, 1982).

The extensive regions of nucleic acid sequence homology between *tsr*, *tar*, and *tap* genes have suggested that they comprise a gene family whose products might contain some functionally similar and some functionally different domains (Boyd et al., 1981). Indeed, two chimeric genes containing the 5′ end of the *tar* gene fused to the 3′ coding region of the *tsr* gene have been found to be chemotactically functional. In cells lacking both *tar* and *tsr* wild-type functions, the chimeric gene products were capable of triggering tactic responses to *tar* chemoeffectors (Krikos et al., 1985).

There have been two possibilities proposed for MCP mediation of initiation of signal transduction (Krikos et al., 1983). It may occur through the MCP polypeptide backbone, pushing or pulling its transmembrane segments, thereby altering the structure of the MCP carboxyterminal portion present in the cytoplasm. Alternatively, chemoeffector binding may influence the aggregation–disaggregation properties of monomeric MCP molecules.

ADDITIONAL CHEMOTAXIS PROTEINS

In addition to the receptors, transducers, methyltransferase, and methylesterase, additional chemotaxis proteins exist which are known to play a key role in the signal transduction and/or adaptation process. They do so by interacting between the transducer molecules and the flagella. These are the products of the *cheY*, *cheZ*, *cheA*, and *cheW* genes (Silverman and Simon, 1976; Parkinson, 1978). Mutations in any one of these genes, as well as the *cheR* and *cheB* genes result in a generally nonchemotactic mutant. By transcriptionally fusing the above genes to high level promoters, all of their gene products have been overexpressed to the level of detectability on SDS-polyacrylamide gels (Matsumura et al., 1984; Matsumura et al., unpublished data).

Complementation tests between *S. typhimurium* and *E. coli* have suggested that the *cheY* gene product and *cheR* product (methyltransferase) interact (De-

franco et al., 1979). Like *cheR* mutants, *cheY* mutants show a counterclockwise rotational bias. Reversion analysis of *cheY* and switch mutants (discussed in the section on switch proteins) suggest that they too interact (Parkinson and Parker, 1979; Parkinson et al., 1983). Hence, *cheY* appears to serve in some capacity to bridge the sensory and/or adaptation aparatus between the MCP–methyltransferase complex and the flagellar aparatus. The *cheY* protein is the first chemotaxis protein which has been purified to greater than 99% homogeneity and for which X-ray crystallographic data may soon be ready (Matsumura et al., 1984). *In vitro* it is eluted from cibacron blue columns with SAM (the methyl donor for MCP methylation) and SAM analogs in such a manner as to suggest that it is a noncatalytic SAM-binding protein rather than a catalytic methyltransferase. The *cheY* protein *in vitro* also inhibits EcoRI methyltransferase activity. Based on these results, it has been proposed (Matsumura et al., 1984) that a possible function for the *cheY* protein in chemotaxis is to negatively regulate *cheR* methyltransferase activity, either by interaction with the *cheR* product or *cheR* product–substrate complex. Since then, *cheY* protein has been shown to inhibit *cheR*-mediated methylation of MCPs, an effect which can be reversed if a chemoeffector such as 5 mM aspartate is present (Vacante and Matsumura; unpublished results). This is shown in Figure 2. Flagella from cell wall-containing cell envelopes, when energized, rotate exclusively counterclockwise; however, addition to *cheY* protein induces clockwise rotation (Eisenbach; personal communication). Likewise, overexpression of *cheY* protein has been noted to produce a clockwise rotational bias (Clegg and Koshland, 1984). For these reasons *cheY* has been designated the tumble regulator.

The *cheZ* product appears to close the circle of *che* protein interactions. Complementation tests between *S. typhimurium* and *E. coli* suggest that the *cheZ* and *cheB* (methylesterase) products interact (Defranco et al., 1979). Like *cheB* mutants, *cheZ* mutants possess a clockwise rotational bias. Revertants of *cheZ* often map to the same flagellar switch genes as *cheY*, which also suggests that the *cheZ* product interacts at the flagella (Parkinson and Parker, 1979; Parkinson et al., 1983). Because both *cheY* and *cheZ* interact with the methylation machinery as well as the motor, they may both serve as second messengers which convey information to the motors about the methylation state of the MCPs (Parkinson et al., 1983). For example, enhanced interaction of *cheY* protein with the switch might reflect an increased rate of dissociation of a *cheR–cheY* complex from the MCPs, resulting in a decrease in the rate of MCP methylation. Interestingly, the *cheZ* protein is methylated, but no function has yet been assigned to this modification (Silverman and Simon, 1977b).

The *cheA* gene has gained some notoriety due to the fact that it contains two overlapping genes: one which codes for a membrane-bound product, and a shorter cytoplasmic product formed by a later ribosome binding site (Matsumura et al., 1977; Smith and Parkinson, 1980). Both *cheA* products have been implicated with increasing demethylation of the MCP molecules (Springer and

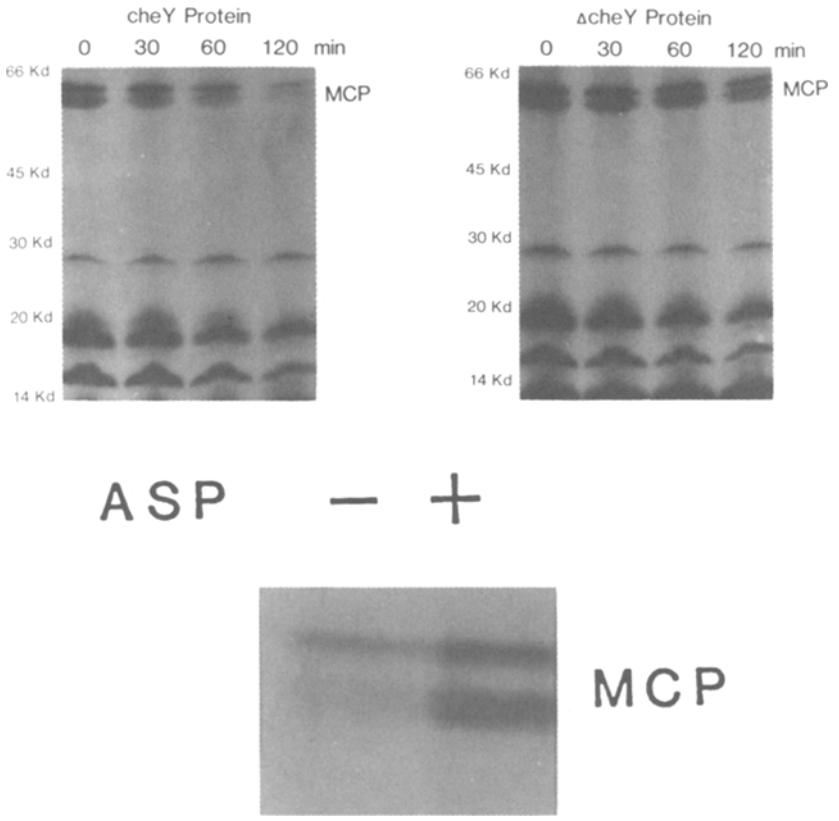

FIG. 2. Effects of *cheY* protein overexpression on MCP methylation in situ. The *cheY* gene has been placed under the transcriptional control of the high level *trp* promoter. The MCPs of cells which have been permeabilized to tritiated methyl SAM show a decrease in radioactive methyl group incorporation following the induction and overexpression of the *cheY* gene product (upper left). This effect is not observed when an internal deletion within *cheY* is introduced (upper right). This inhibition of methylation may be reversed if a chemoeffector such as 5mM aspartate is added (lower panel). A complete description of the effect of *cheY* protein on MCP methylation will be presented elsewhere (Vacante and Matsumura, in preparation).

Zanolari, 1984). However, reversion analysis of flagellar component genes implies that these gene products may associate with the flagellar motor (Parkinson, 1977). Because both the *cheA* and *cheW* genes are part of a known motility operon, and because related genes are often clustered, both *cheA* and *cheW* may act at the motor. This implies that the *cheA* role in demethylation may be an indirect one. The *cheA* gene product has also been demonstrated *in vitro* to bind to the *cheW* product. In the presence of *cheA* protein, *cheW* protein selectively

binds to affinity columns prepared with antibody directed against the *cheA* protein (MacNally and Matsumura, in preparation). Both *cheA* and *cheW* mutants possess a counterclockwise rotational bias, implying that they both have roles in tumbling (Parkinson, 1978).

SWITCH PROTEINS

In *E. coli* there exist three particular flagellar genes, each of which may be altered to impart Fla$^-$ (flagellar defect), Mot$^-$ (no flagellar rotation), or Che$^-$ phenotypes. These are the *flaBII*, *flaAII*, and *motD* genes (no *fla* designation for the *E. coli motD* gene currently exists). The fact that genetic defects within the same gene may express themselves so differently underlines the obviously close relationships of flagellar assembly, rotation, and chemotaxis. These genes' products are believed to be at the interface of the chemotactic machinery and the flagellar structure. Because of the central role these proteins play in modulating flagellar rotation in all types of taxes, they have been termed the switch proteins (Macnab and Aizawa, 1984). Most likely these products are structural components of the motor which are also essential for energization function and sensory reception.

These conclusions are based upon the facts that a class of chemotactic mutants, designated *scyB* (suppressors of *cheY* mutations) map to the *flaBII* gene (Parkinson et al., 1983; Bartlett and Matsumura, 1984), because the *cheC* gene maps within the *flaAII* cistron (Silverman and Simon, 1973; Clegg and Koshland, 1985), and by analogy with experiments in *S. typhimurium*, the *flaBII*, *flaAII*, and *motD* genes may acquire Mot$^-$ or Che$^-$ phenotypes in addition to their Fla$^-$ null phenotypes (Collins and Stocker, 1976; Warrick et al., 1977; Dean et al., 1983). Although no flagellar mutants mapping to the *E. coli flaBII* gene have been obtained through random mutagenic techniques, reversed genetic methodology (in which recombination between a cloned mutant gene and the corresponding wild-type locus occurs) has demonstrated that the null phenotype of the *flaBII* gene is indeed Fla$^-$ (Bartlett, PhD thesis).

All the switch genes are located within two flagellar operons mapping at about 43 minutes. The *flaBII* gene is located in an operon whose genes, transcribed from left to right, are *flaBI*, *flaBII*, *flaBIII*, *flaC*, *flaO*, and *flaE*. The *flaAII* and *motD* genes comprise part of the next downstream operon which is transcribed in the order *flaAI*, *flaAII*, *motD*, *flbD*, *flaR*, *flaR*, *flaQ*, and *flaP* (Silverman and Simon, 1973; Bartlett and Matsumura, 1984; Malakooti and Matsumura, in preparation). The *flaB* locus was divided into three complementary groups based upon the ability of various deletions and insertions with *flaB*-locus-containing plasmids to complement specific flagellar and chemotaxis mu-

tants, as well as code for polypeptides (Bartlett and Matsumura, 1984). The existence of three *flaB* genes has been confirmed through DNA sequence analysis (Frantz et al., unpublished data). Similar approaches have been used to subdivide the *flaA* locus into the *flaAI* and *flaAII* genes (Malakooti and Matsumura, in preparation). Although the phenotype of a *flaAI* mutant is unknown, it has been given a *fla* designation because of the previous *flaA* designation to this locus.

The switch proteins along with other flagellar components may comprise a portion of a structure located beneath flagellar basal bodies. Such a location would be consistent with their proposed roles of interacting with soluble *che* proteins as well as affecting flagellar rotation. They do not appear to be soluble proteins, as Ravid and Eisenbach (1984) have found that *E. coli* ghosts which contain energized flagella retain their rotational biases when prepared from strains bearing *che* mutations within the *flaAII* or *flaBII* genes. Similar ghosts prepared from cells with defects in soluble chemotactic products (such as *cheY* protein) do not retain such rotational biases.

A subflagellar basal body structure has never been observed during morphological examinations of *E. coli* flagella. However, these techniques have involved lysozyme digestion of the cell wall, and detergent solubilization of the cell membrane, in which case many components of the basal body or a subbasal body structure may have been lost (Depamphilis and Adler, 1971; Hilmen and Simon, 1976). Isolated flagellar organelles from the gram-negative *Aquaspirillum rubrum* reveal adherent fibrils emanating from the basal bodies (Coulton and Murray, 1978). Analyses in *Pseudomonas aeruginosa* and *Vibrio metchnikoviic* have revealed similar results. Thin-section electron micrographs in these organisms have demonstrated a dense cluster of "ribosome-like" granules at the cytoplasmic face of the flagellum, approximately 60 nm in diameter (Vaituzis and Doetsch, 1969).

Overexpression of *flaBII*, *flaAII*, and *motD* genes results in most of the switch proteins partitioning to the soluble cell fraction (Clegg and Koshland, 1985; Bartlett, PhD thesis; Malakooti and Matsumura, in preparation). This may reflect either a loose, or a site-limiting cytoplasmic membrane association. A glimpse into the tendency of flagella-associated proteins to associate with the membrane has been investigated from their overall hydrophobicity determined from their amino acid composition (Kyte and Doolittle, 1982). We have found a complete correlation between these hydrophobicity values determined for the switch/basal body proteins, and their overexpressed products' subcellular localization. The more hydrophobic *motA* and *flaAI* products do not show site-limited incorporation into the membrane fraction, while the less hydrophobic *flaBI*, *flaBII*, *flaBIII*, *motB*, and *motD* products do (Table 2). The derived amino acid sequence of the three *flaB* genes and *motD* gene provides no membrane spanning domain for the *flaBII* protein, while the *flaBI* protein possesses a pos-

TABLE 2. RELATIONSHIP BETWEEN HYDROPHOBICITY AND SITE-LIMITED INCORPORATION INTO INNER MEMBRANE AMONG MOTILITY AND BASAL BODY PROTEINS

	Average hydrophobicity	Site-limited membrane incorporation
motA	+0.353	No[a]
flaAI	+0.032	No
motD	−0.036	Yes
flaBIII	−0.402	Yes
flaBII	−0.429	Yes
motB	−0.434	Yes
flaBI	−0.474	Yes

[a] Data of Wilson and Macnab (personal communication).

sible membrane spanning region near its amino terminus, and the *flaBIII* and *motD* products contain possible membrane spanning sites near their carboxy termini (Frantz et al., unpublished results). One or both of these flagellar components, as well as others, may help to anchor the *flaBII*, *flaAII*, or *motD* proteins into their positions. It is noteworthy that in *S. typhimurium* the analogous gene to the *E. coli flaBI* gene appears to comprise the cytoplasmic membrane-bound M-ring of the basal bodies. This is based upon examinations of basal body proteins from temperature-sensitive flagellar mutants (Aizawa et al., 1985). Intergenic suppression analysis using the *mot* alleles of the switch genes suggests that all three of their proteins may interact together (Macnab and Aizawa, 1984). Figure 3 includes possible protein–protein relationships among the sensory transducers, additional *che* proteins, and switch/basal body complex.

In addition to the requirements for the above-mentioned chemotaxis proteins, there is also a role of phosphate in MCP-mediated chemotaxis. Cells depleted of methionine or ATP do not synthesize SAM, the methylation donor for MCP methylation. Tumbly *cheC* mutants which possess defects in the flagellar switch will not swim smoothly when depleted of methionine, but will when depleted of ATP, as do all cells (even when their proton motive force remains high enough for the flagella to switch to tumbly swimming) (Arai, 1980; Galloway and Taylor, 1980; Shioi et al., 1982). This indicates a role for ATP outside of SAM synthesis. In the presence of 5 mM phosphate, even with low ATP levels, smooth swimming may be restored (Arai, 1980). Therefore some other phosphorylated compound besides ATP appears to be involved in chemotaxis. Ordal (1985) has proposed that this compound may serve as a co-factor for the *cheY*, *cheA*, or *cheW* proteins for their flagellar interactions, or that kinase activity exists for one of the switch components.

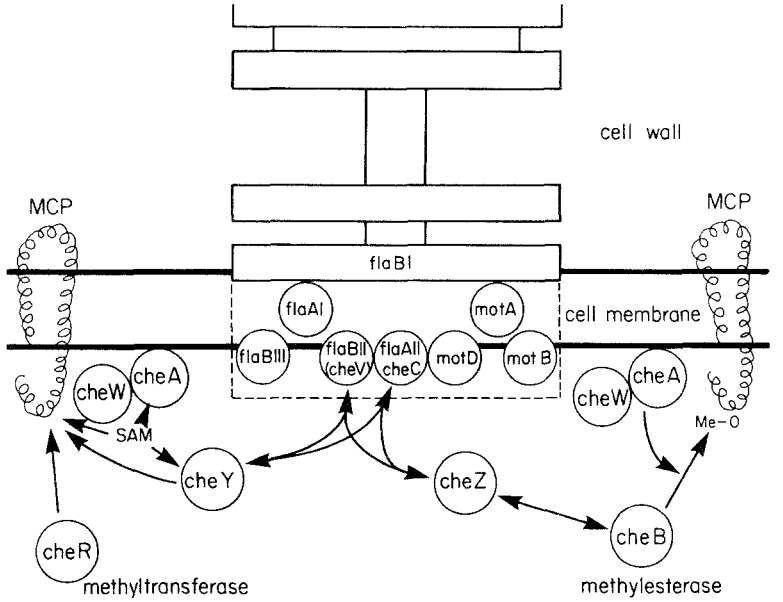

FIG. 3. Schematic representation of sensory flow in *E. coli* MCP-dependent chemotaxis.

A COMMON TAXIS SIGNAL?

Virtually nothing is known about the nature of the excitatory signal in chemotaxis or in any other taxis. The response system may simply involve a series of protein–protein, or protein–small molecule (such as Ca^{2+}) binding steps (Parkinson, 1977; Ordal and Fields, 1977; Ordal, 1985). In addition, there may be regulation of enzymes involved with the generation and degradation of this signal, such that magnitude amplification, allosteric cooperativity; or the newly discovered zero-order ultrasensitivity might be involved in a kinetic pathway (Koshland, 1984). There is some evidence for the role of both the *cheB* and *cheZ* gene products in signal transduction for a few different types of taxis. Thus it is possible that these different sensory systems may interface at one point along their signaling pathways. Impulse response studies in MCP-mediated chemotactic mutants have indicated that only *cheZ* mutants have an altered impulse response time (Block et al., 1982; Segall et al., 1982). $CheD^-$ mutants which map to the *tsr* gene and which may possess MCPI molecules locked into the signaling mode show defective *cheB* product activity (Callahan and Parkinson, 1985; Kehry et al., 1985). $CheB^-$ mutants appear to be defective in thermosensory excitation (Imae et al., 1984.). Furthermore, both *cheB* and *cheZ* mutants are defective in aerotaxis and the phosphotransferase-system-

mediated taxis, both of which proceed via MCP methylation-independent adaptation pathways (Niwano and Taylor, 1982).

FUTURE PROSPECTS

All of the *E. coli* MCP-related *che* genes have been cloned, and their products' primary amino acid sequence have either been determined, or are in the process of being ascertained (Boyd et al., 1981; Krikos et al., 1983; Bollinger et al., 1984 Matsumura et al., 1984; Frantz et al., unpublished data; Malakooti and Matsumura, in preparation; N. Mutoh and M. Simon, personal communication; J.S. Parkinson; personal communication). In the near future, site-directed mutagenesis and the determination of the nucleic acid sequences containing various *che* mutations will become important for more clearly defining structure and function relationships among these genes' products. Concerning the MCPs, such analyses may help the determination of chemoeffector binding sites, as well as domains on the proteins necessary for methylation, methylase binding, methylesterase binding, and signal transduction. Similar analyses may be directed towards discovering what moieties of the *cheY* protein bind SAM, *cheR* protein, or the flagellar switch. Furthermore, structure and function studies among the switch proteins may yield valuable information concerning their association with *cheY* protein, *cheZ* protein, and each other.

Does an elaborate submembrane basal body complex exist? A structure analogous to the *E. coli* ATPase can be envisioned (Downie et al., 1979; Cox et al., 1981). This analogy is strengthened by the fact that Mitchell (1985) has offered the fascinating ATPase rotating subunit hypothesis in which proton motive force drives rotation of the ATPase enzyme in a manner similar to that proposed for the bacterial flagellar motor. By examining the ability of switch or basal body products to fractionate into the membrane fraction in various switch or basel body mutants, a hierarchy of assembly into the basal body complex may be determined. In this way information concerning protein-protein interactions for these membrane-associated proteins may be determined.

A wealth of information relating to chemotaxis is *E. coli* already exists, and the future purification of all the *che* proteins promises to elucidate new biochemical properties.

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CHEMICALLY MEDIATED BEHAVIOR IN ACARI: Adapations for Finding Hosts and Mates[1]

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Abstract—Ticks and mites respond to a limited spectrum of stimuli in their search for hosts and mates. Airborne chemical signals include carbon dioxide, ammonia, organic acids, terpenoids, 2,6-dichlorophenol, and other phenolic compounds. These are detected primarily by sensilla in and adjacent to Haller's organ. Most ixodid species examined have one or more multiporose sensilla that detect such volatiles. These olfactoreceptors enable the ticks to respond to remote volatile chemicals from hosts and from the other ticks, e.g., sex pheromones. Other sensilla, probably mechanogustatory in function, also occur on the tarsi. Gustatory sensilla on the palps detect assembly pheromones that enable ticks and mites to respond to conspecific or heterospecific chemical stimuli in their environment. Responses to those stimuli in ticks result in clustering, i.e., arrestant behavior. Arrestant behavior also occurs in certain mites. Finally, cheliceral chemosensilla enable ticks to recognize specific phagostimulants in host blood, e.g., ATP and glutathione, that stimulate feeding. In *Dermacentor variabilis* and *D. andersoni*, these same cheliceral chemosensilla recognize species-specific genital sex pheromones in the vulvae of conspecific mates, without which they do not copulate.

Key Words—*Dermacentor variabilis*, *Dermacentor andersoni*, *Amblyomma* spp., semiochemicals, behavior, sensilla, Haller's organ, phenols, genital sex pheromone, 2,6-dichlorophenol.

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INTRODUCTION

Acarines are a remarkably diverse group of animals, comprising tens of thousands of species that are found in an immense variety of terrestrial and freshwater habitats. Mites are major components of soils, where they feed on fungi, dead and decaying leaves and, occasionally, one another. Others are parasitic, attacking numerous mammals, birds, reptiles, and even insects. Many infest nests, burrows, and human habitations. Plant mites are notoriously destructive pests, affecting many of our most valuable fruits and ornamentals. As vectors of animal diseases, the ticks are without peer among the Acari in terms of the variety and sheer destructive potential of the pathogenic agents that they transmit.

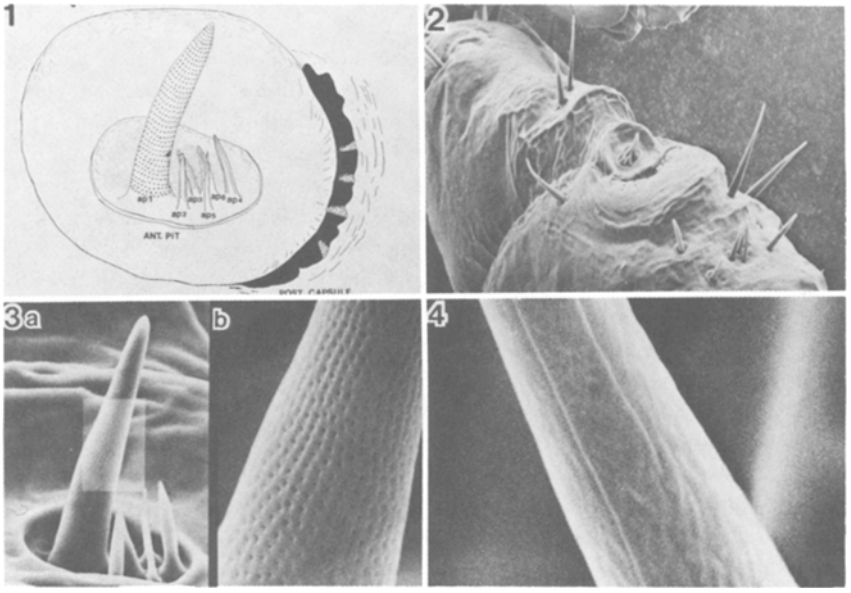
An assemblage of such diversity may be expected to have numerous semiochemicals and behavioral adaptations. However, relatively few such behavior-modifying compounds have been found to date, a fact which may reflect the paucity of our knowledge rather than a dearth of chemical communication in nature. Acarines produce a variety of arrestant pheromones, a limited spectrum of sex pheromones, several dispersal pheromones, and an unusual pheromone or pheromones, specific for the genus *Amblyomma*, for regulating attachment and clustering of feeding ticks. Absent are trail pheromones, food-finding pheromones, the innumerable defensive compounds (Staddon, 1979) and the great variety of androconia, hair pencils, and other devices for sexual display and pheromone dispersal. As Blum (1981) has noted, the insects "are remarkable chemists" and have evolved an incredible cornucopia of compounds to deal with the need to communicate information.

This review will examine the variety of acarine semiochemicals and the behavioral adaptations regulated by them. Special attention will be given to ticks, where the greatest amount of new knowledge has accumulated. We will limit ourselves almost entirely to chemoreceptors and the semiochemicals that they perceive, although the potential role of other receptor types can hardly be excluded.

PERCEPTION OF PHEROMONES AND HOST ODORS

Three organs are used for detection of chemical cues in ticks, (1) the Haller's organ on the first leg tarsi, (2) the terminal segment of the palps, and (3) the cheliceral digits. Little is known of the organs used for pheromone perception in mites.

Haller's Organ. This complex organ consists of an anterior trough with six or seven setiform sensilla, completely exposed to the atmosphere, and a posterior capsule with both setiform and pleomorphic sensilla, exposed only by a small pore or narrow slit (Figure 1). The anterior trough (Figure 2) in most



FIGS. 1–4. Haller's organ complex in ixodid ticks. Figure 1. Diagrammatic reconstruction of the Haller's organ illustrating the different types of sensilla. The open, anterior pit usually contains six sensilla that are visible when examined by SEM. Other sensilla on dorsal surface of the tarsus may also serve as chemosensilla, but are not physically located in the Haller's organ complex in most ixodid species that have been examined. The posterior capsule is represented by a narrow slit, shown in black, which serves as the opening to the underlying chamber, or posterior capsule. Four multiporose sensilla are depicted as protruding from the capsular cavity for purposes of illustration. Composite illustration based on study of the organ in *Dermacentor variabilis* and *D. andersoni*, as well as studies of other authors. ANT. PIT = anterior pit; POST. CAPSULE = posterior capsule; ap-1 through ap-6 = anterior pit sensilla. Figure 2. SEM view of the dorsal surface of the tarsus of leg I of a *D. andersoni* male illustrating the Haller's organ complex and other sensilla nearby. 213×. Figure 3. Split-screen view illustrating (a) the anterior pit sensilla, ap-1 through ap-6 and (b) high-magnification detail of the multiporose sensilla ap-1; 1349× for (a), 5396× for (b). Figure 4. Enlargement of one of the grooved sensilla, ap-2, 8410×.

ixodid species that have been studied (Foelix and Axtell, 1971, 1972; Hess and Vlimant, 1982a, b; Leonovich, 1977; Waladde and Rice, 1982) has at least four types of setiform sensilla: (1) a large, broadly based multiporose seta (ap-1) serving as a sensillum basiconica (Figure 2) (two such sensilla occur in *A. americanum*); (2) three grooved sensilla with perforations in the grooves (ap-2, 3, 4); (3) one smooth-walled sensillum (ap-5); and (4) a grooved sensillum without perforations (ap-6) (Figure 4). In addition, a group of setae on a ridge

or hump anterior to the trough contain one or more multiporose setae similar to ap-1 (=MD-3 of Waladde, 1982).

In *Amblyomma variegatum*, only one such seta occurs. In both cases, the multiporose sensilla have characteristic internal profiles. The pores are actually plugged, and contain branching dendrites with branches believed to be in physical contact with the plugged pores. In *A. variegatum*, the pores communicate directly with the interior lumen of the seta (single walled type). The pores are believed to be permeable to airborne odor chemicals, since dye molecules penetrate through the pores into the sensillum liquor (Foelix and Axtell, 1972). These sensilla are clearly olfactoreceptors, the functional capabilities of which will be discussed below. Olfactoreceptors with plugged pores have been identified in several mesostigmatid mites (e.g., *Dermanyssus & Mesonyssus*) (Davis and Camin, 1976; Moritsch et al., 1974). Thus, this type of receptor is probably widespread throughout the Acari. The grooved sensilla, with perforations in their grooves, are also olfactoreceptors. However, these sensilla typically have three or four unbranched dendrites in a central lumen that opens to the exterior via fine canals, providing the characteristic "spoke wheel" arrangement. In *A. variegatum*, they are described as double-walled sensilla, with radiating canals connecting the central lumen to the grooves. Their precise chemical sensitivities are unknown. The other sensilla in the anterior trough are probably not olfactoreceptors. Tip-pore sensilla also occur on the tarsus outside of Haller's organ as well as on other leg tarsi. They are probably mechanogustatory receptors, responding to water and salts (Hess and Vlimant, 1982a).

More is known about the function of the Haller's organ sensilla than any other tick or other acarine sensory system. Electrophysiological recordings from microelectrodes implanted below the anterior trough demonstrate that this structure will respond to crude animal odors, e.g., human breath, cattle body washings, etc. This type of study is useful as a sort of "electrotarsorgram," comparable to the electroantennograms used with insects. Precise measurements were initiated by Haggart and Davis (1979). These workers implanted uninsulated tungsten microelectrodes into the hemolymph space below the anterior trough and a group of tarsal seta anterior to Haller's organ to record extracellular action potentials in *Rhipicephalus sanguineus*. Using this method, they were able to demonstrate relatively specific responses of certain neurons in each test location to NH_3. The magnitude of the responses were similar to stimulus intensities. The responses were characteristic of sensory neurons, with an initial burst of impulses reaching 40 impulses/sec (phasic response), followed by a slow decline in impulse frequency (tonic response), with no significant decrement over the duration of stimulation. Removal and restimulation revealed the same pattern, indicating rapid recovery. The NH_3-sensitive neurons were relatively specific for this compound, with high sensitivities to NH_3 and slight sensitivity to several other acids. The authors suggested that the response to am-

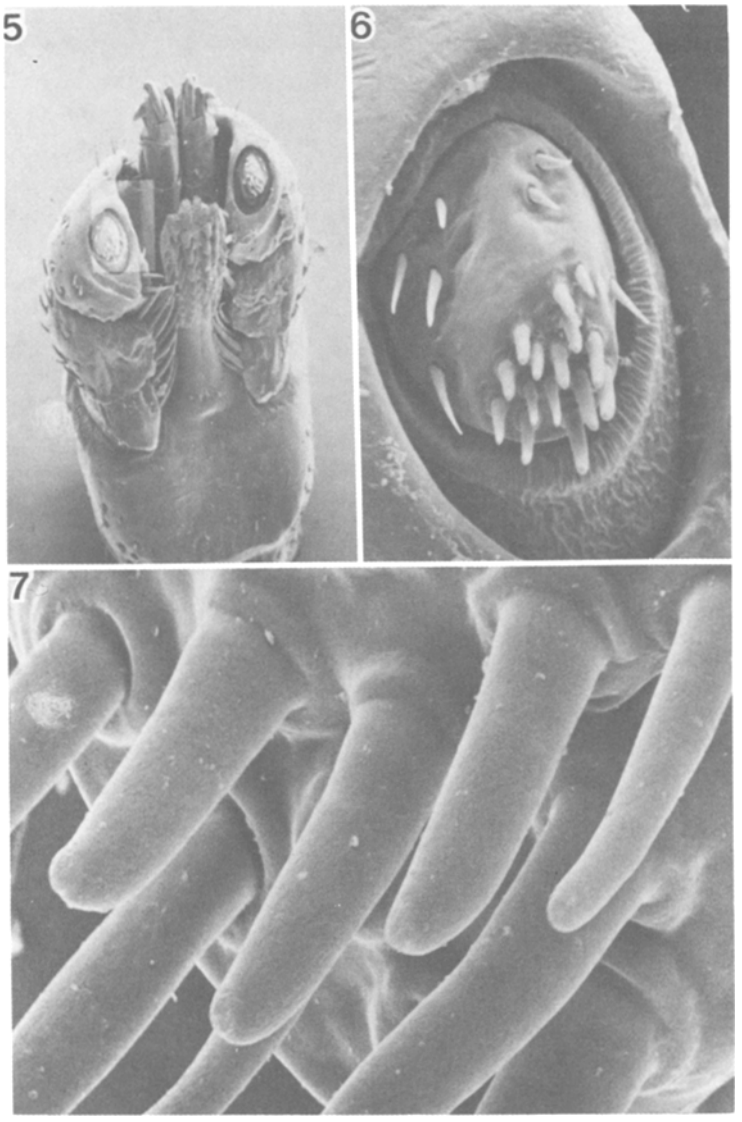
monia may be useful in host-locating behavior, e.g., along well used animal trails.

In further studies by these authors (Haggart and Davis, 1981) using the same methods, highly significant responses to 2,6-dichlorophenol (2,6-DCP) were found when the microelectrode was positioned below the ap-1 sensillum in *A. americanum*. They concluded that only one sensillum, ap-1, is the 2,6-DCP receptor. Unfed males, fed males, and unfed females responded to this pheromone. The response characteristics were similar to those described above for ammonia, but the tonic phase declined gradually with time. The response intensity was proportional to stimulus intensity over a relatively broad range. The 2,6-DCP-sensitive neurons were also found to respond to different cresol isomers; responses to NH_3 were also found, but this was believed to be due to action potentials from neighboring neurons. In view of the fact that unfed males and females as well as fed males detect the pheromone, Haggart and Davis concluded that the behavior responses to 2,6-DCP are based on integration of information in the central nervous system rather than at the level of peripheral neuron sensitivies.

A significant advance in selective recording of sensory responses was made by Waladde (1982), who used the tip recording technique to test the responses of individual sensilla in *A. variegatum* and *R. appendiculatus*. With this method, Waladde demonstreted that the two multiporose sensilla present in these species, ap-1 and MD-3, were able to detect 2,6-DCP in amounts as low as 0.04 ng/μl. The same sensilla were relatively insensitive to *p*-cresol or salicylaldehyde, while phenol was actually inhibitory. The sensilla were sensitive to hexane washings of larval and female ticks; moreover, ap-1 was capable of detecting NH_3. In contrast to *A. americanum*, two sensilla are used to detect the pheromone in this species, but the stimulus-response characteristics were otherwise very similar (although no decline in response intensity was seen in this species). These findings are remarkable because neither species has been found to contain 2,6-DCP. Moreover, these findings raise serious doubts about the role of phenol and *p*-cresol, reported to serve as the sex pheromone of *R. appendiculatus* by Wood et al. (1975).

The Haller's organ sensilla are also used to detect the male originated aggregation/attachment pheromone, but the specific sensilla used for this purpose are unknown. In view of the fact that two of the three components of this pheromone are also phenols (Schoni et al., 1984), it is probable the detection is accomplished by the same multiporose sensilla used to detect 2,6-DCP. Future research should be directed to determining this activity.

Palpal Organ. The fourth segment of the palp of ixodid ticks contains nine or more tip-pore sensilla at its distal margin that serve as mechanogustatory receptors, as well as mechanoreceptor sensilla along its lateral edges (Figures 5–7). None are known to serve as thermoreceptors. These are high-threshold



FIGS. 5–7. Scanning electron micrographs illustrating the palpal receptors in a *Dermacentor andersoni* male. Figure 5. Ventral view of the entire capitulum. High-lighted area enlarged (Figure 6), 38×. Figure 6. Enlargement illustrating the fourth article (segment) of the right palp, partially recessed in a cavity of the preceding third article. A total of 13 short, thick, and blunt sensilla, all similar in appearance to one another, occur at the tip of the fourth article (=palpal organ); nine others, including seven thinner, tapering sensilla, occur along the margins. Porelike depressions that appear at the tips of some of the sensilla represent contrast-related artifacts, as shown in the enlargement below, 306×. Figure 7. High-magnification view illustrating the blunt sensilla at the tip of the palpal organ. No pores are evident, 2490×.

receptors, requiring many times higher concentrations of each specific chemical to be stimulated than with olfactoreceptors. Two types of distal margin sensilla are known, the long, thin type A sensilla, and the much shorter and thicker type B sensilla. Both types exhibit only minute wall pores near the seta tip (Foelix and Chu-Wang, 1972; Ivanov and Leonovich, 1983) which are not evident when the sensilla are examined by scanning EM [(although Waladde and Rice (1982) illustrate what they believe to be much larger terminal pores in *B. microplus*]. The type A sensilla detect salts, glucose, and sucrose (Balashov et al., 1976); they probably detect compounds in sweat and other skin secretions and are believed to facilitate the selection of feeding sites. The type B sensilla do not respond to the same compounds as the type A sensilla. Rather, they are believed to perceive pheromones (Ivanov and Leonovich, 1983). The palpal organ is known to be used in the perception of assembly pheromones (Leahy et al., 1975; Graf, 1975) and may even participate in courtship behavior. Feldman-Muhsam and Borut (1971) reported that males of ixodid ticks would not copulate when their palps were amputated. However, *Dermacentor variabilis* are apparently unaffected by the loss of their palps and copulate readily. Observations of courting males indicate that they apply their palps to the female's body cuticle and may obtain unknown chemical signals which guide them to the female's venter and genital pore (Sonenshine, unpublished). The extent to which the palps are used in this role is unknown. Electrophysiological studies also suggest a generalized sensitivity to steroids (Taylor, unpublished). Further study of the palpal organ sensilla and their capabilities for detecting specific chemicals should be encouraged and may provide needed new knowledge of tick feeding behavior.

Cheliceral Digit Sensilla. Chemoreceptive, mechanoreceptive, and, most probably, even thermoreceptive sensilla occur on the digits of the chelicerae (Waladde and Rice, 1977, 1982; Sonenshine et al., 1984). In *Boophilus microplus*, two pores and a papilla occur on one of the denticles of the inner digit. Only one of these sensilla, the proximal pore, is a chemoreceptor, innervated by 11 chemosensory dendrites. Chemoreceptor dendrites also occur in the lumen of the outer digit, but the precise sensillum innervated is uncertain. Similar structures occur on the digits of *D. variabilis*, but the papilla is replaced by a cone-shaped cavity that probably serves as a proprioceptive sensillum. In *B. microplus*, electrophysiological records reveal selective responses to host blood, saline solutions, and the phagostimulants ATP and reduced GSH. Evidently, the cheliceral digit sensilla provided the final pieces of information needed to complete the food acquisition process, namely, the evaluation of the contents of fluids that are being sucked. Presumably, failure to detect the appropriate phagostimulants leads to detaching and reattachment elsewhere.

In *D. variabilis*, the cheliceral digit sensilla also respond to extracts of the female reproductive tract and 20-hydroxyecdysone, as well as 10^{-3} M ATP, 10^{-3} M reduced GSH, and physiological saline. In summary, these ticks have

the necessary morphological and physiological capabilities to respond to sexual excitants in the female genital pore, as well as to specific phagostimulants in host blood. This is not surprising, in view of the widespread use of the chelicerae in different acarines as sperm transfer devices, e.g., the spermatodactyls of parasitic mesostigmatid mites, in addition to their role in penetration of the host integument.

ARRESTANT BEHAVIOR

In ticks, this behavior is induced by assembly pheromones. Ticks encountering the pheromone cease ambulatory activity and become akinetic. Studies of Gothe and Kraiss (1982) implicate substances passed via the anus and, possibly, the coxal glands, as the excitant. Assembly pheromones are widespread among species of both Argasidae and Ixodidae. Moreover, interspecific and even interstadial responses to these chemical signals are common. This evidence strongly suggests a single class of compounds, perhaps even a single molecule, as the common assembly pheromone of ticks. Obenchain (personal communication) suggested that guanine and another, unidentified purine voided in tick feces probably serves this role (cited by Sonenshine et al., 1982a). Detection of this pheromone is accomplished by the palpal organ sensilla.

Assembly pheromones occur most frequently in species that inhabit arid environments, e.g., most Argasidae, rhipicephalids of tropical Africa, etc. Taylor and Sonenshine (unpublished), using a laboratory simulated "meadow environment" (Figure 8, Table 1), were unable to find evidence of assembly pheromones in *Dermacentor variabilis* and only weak responses to saline washings in *D. andersoni*. *D. variabilis* is a tick of the humid eastern deciduous forest biome of North America. These findings are consistent with the chemical characteristics of the pheromone, which is highly soluble in saline and other dilute aqueous salt solutions. Such solubility characteristics would favor its persistence in arid environments but not under the conditions of frequent rainfall, high humidity, and dew-covered vegetation that is characteristic of eastern North America. In Switzerland, however, an area with high rainfall, *Ixodes ricinus* assemble on vegetation in response to other ticks or tick extracts (F. Jigand, personal communication). Comparative evidence suggests that differences in perceptive abilities (i.e., chemoreception) rather than the actual presence of the pheromone may account for the differences in response to these compounds noted above.

Arrestant behavior has also been reported in mites, but for entirely different reasons. In the Tetranychidae and Phytoseidae, developing female nymphs (pharate females) emit the sesquiterpene alcohols farnesol, nerolidol, and geraniol, and the monoterpene citronellol. Mate-seeking males encountering such sexually active nymphs stop, palpate them, and remain akinetic nearby, a pose

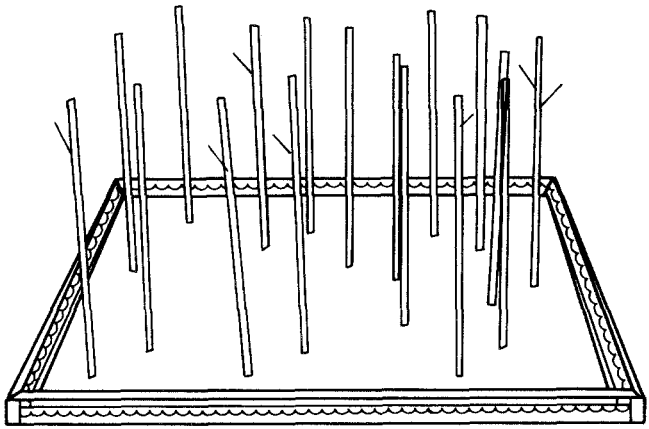


FIG. 8. Simulated "meadow" environment in which experiments on adult tick clustering behavior was done. Two identical environments were used, one of which served as the control. Each consisted of a 1.9-cm-thick acrylic (Plexiglas) base, 61 × 61 cm square, surrounded by a water filled moat, 1.8 cm wide. Acrylic rods, 1.3 cm. diam × 30 cm high, were placed at random throughout the acrylic base and cemented in place. Small secondary acrylic rods, 0.2 cm diam and 4 cm long, were attached at random heights to the upright rods to simulate branches. Ticks, *D. variabilis* adults, were released on the base and allowed to climb the rods.

TABLE 1. RESULTS OF SIMULATED "MEADOW" BIOASSAYS OF *Dermacentor variabilis* TO DETECT PRESENCE OF ASSEMBLY PHEROMONE[a]

Treatment	No. ticks in test	× No. ticks/rod[b]	× No. clusters[c]
1. Filter paper + tick extract	360	0.84	0.0a
2. Filter paper contaminated by female ticks	216	0.69	0.19b
3. Filter paper contaminated by male ticks	576	0.98	0.29
4. Rods contaminated by live ticks	864	0.94	0.36
5. Rods with other live ticks during test	576	1.02	0.42
6. Controls	2736	0.92	0.35

[a] Done in a controlled environment chamber at 27° ± 1°C, 80 ± 5% relative humidity, and uniform daylight fluorescent light.

[b] No significant difference in the mean number of ticks/rod due to the different treatments, $F = 2.169$, $P > 0.1$, *df*, 6, 13. There was significant added variance due to the position of the "meadow" environment probably related to air currents in only one of the positions tested, $F = 16.765$, $P = 0.001$, *df*, 2, 5737.

[c] Significant differences in the mean number of clusters/rod were observed, $F = 4.913$, $P < 0.001$, *df*, 6, 5737. However, those treatments that were significant were less than the controls. Using the least significant difference (LSD) test, a = significantly different from all other treatments except No. 2, b = significantly different from treatment No. 3.

known as "guarding" (Regev and Cone, 1980). Although akinetic, the males are not insensitive. Molting of the female deutonymphs arouses the male and copulation ensues. The nature of the pheromone and its mode of detection provide worthwhile problems for future study.

HOST-SEEKING BEHAVIOR

Despite the obvious importance of this behavior, little is known about the specific stimuli to which ticks respond, and even less about the receptors that perceive them. CO_2 and NH_3 are known host-originated stimuli to which ticks respond (Wilson et al., 1972; Haggart and Davis, 1980, 1981), and butyric acid is reputed to be an excitant (Waladde & Rice, 1982). No other host-originated chemical stimuli are known. Questing ticks also respond to vibrations. In *B. microplus*, questing larvae respond best to 80–800 Hz, the frequency range associated with sound made by cattle (Waladde and Rice, 1982). Radiant heat is another important stimulus. Few of these signals are likely to be important independently. The integrative value of different types of stimuli, demonstrated so effectively by Lees (1948), remains to be explored. Finally, while olfactoreceptors and thermoreceptors provide information that confirm the presence of the host's body, gustatory receptors are especially important in determining skin characteristics and selection of the feeding site. An extensive literature exists on this subject, reviewed most recently by Waladde and Rice (1982).

MATE-FINDING BEHAVIOR: PHEROMONES USED IN COURTSHIP

Courtship is the term used to describe a ritualistic sequence or hierarchy of behavioral events used by members of a species to identify and accept their prospective partners for mating. The complexity of this process varies greatly. In most acarines in which the process has been subjected to intensive study, a characteristic mating behavior is observed with one or more of these events regulated by pheromones. The best known examples are the ticks, especially the metastriate Ixodidae. Species of this group of ticks, comprising more than 600 species, occur in all continents except Antartica and infest various reptiles, birds and mammals. Despite this exceptional diversity of hosts and habitats, these ixodids are remarkably similar with regard to procedures used in their mating behavior. In all cases, the adults become sexually active only while feeding on a host, and the males must detach and search for females that have achieved sexual readiness. Males encountering receptive females mount their partners dorsally, then turn to the ventral surface, probe the body cuticle with their palps and chelicerae until they locate the gonopore, and commence cop-

ulation. Pheromones guide some, probably all, of these discrete behavioral events. (Sonenshine et al., 1982b; Sonenshine, 1985).

In *Amblyomma maculatum*, *A. variegatum*, and possibly three other African *Amblyomma* species, a male-originated, species-specific aggregation–attachment pheromone guides the attachment process, so that species-specific clusters are formed before the ticks are ready for mating. Males of these species attach readily even without the presence of other males, but they will also respond to the pheromone secreted by other males and attach beside them. Females, however, will attach only if conspecific males are present and have matured to the point that they are secreting this pheromone. In *A. variegatum*, a three-component blend of compounds guides different events in this process. *O*-Nitrophenol induces searching behavior and the aggregation response. The other two components, methyl salicylate and pelargonic acid, induce the attachment and clasping behavior that is characteristic of this species. These pheromone-regulated behavioral events explain the clustering observed on the hosts. However, it is not known how the males recognize when the females are sexually ready or even if such distinctions are necessary. *A. variegatum* males can detect 2,6-DCP (Waladde, 1982), a compound which is present in females of this species (Wood et al., 1975). In the Gulf Coast tick, *A. maculatum*, both males and females are found to contain 2,6-DCP. However, males respond to this compound, seeking out, mounting, and copulating with females emitting the pheromone. Sonenshine et al. (1982a) suggest that 2,6-DCP functions in these situations as a generalized excitant, stimulating males to detach and mate with any adjacent, sexually ready females already present in the conspecific feeding cluster. Thus, mate finding in these *Amblyomma* species of large ungulates commences with the attachment process, leading to the formation of conspecific clusters, followed by the secretion of 2,6-DCP one or two days later, exciting the males to detach, locate, and copulate with the pheromone-secreting females. No other pheromones are known in these large ungulate parasites and none appears necessary to ensure that mating will be accomplished only with partners of the same species.

In most other ixodid species that have been studied, males and females attach to hosts independently. Feeding females secrete 2,6-DCP, exciting males to detach and commence mate-seeking behavior. 2,6-DCP has been found in 14 species of ixodid ticks, representing five genera (Sonenshine et al., 1986). Although perhaps lacking in some species not thoroughly studied it is unquestionably the most common of the several ixodid sex pheromones. Many of these species are sympatric and often share the same hosts. This problem of species-specificity in the courtship behavior of ticks and the use of pheromones to regulate it was discussed in a previous review (Sonenshine, 1985). Consequently, the remainder of this paper will be devoted to examining examples of this process in the genera *Hyalomma* and *Dermacentor* in order to appreciate the dif-

ferent chemical and physical signals and their role in maintaining reproductive isolation.

In the camel ticks, *H. dromedarii* and *H. anatolicum excavatum*, the males exhibit a unique ability to discriminate differences in the concentration of 2,6-DCP (Khalil et al., 1983). *H. anatolicum excavatum* males respond readily to concentrations of this pheromone that are near threshold for *H. dromedarii*, while those concentrations that are highly attractive to *H. dromedarii* males actually repel *H. anatolicum excavatum* males. In a series of experiments with authentic 2,6-DCP administered to females (killed and washed, but still attached), the optimal concentration for attraction of *H. anatolicum excavatum* males was 15 ng/tick. Increasing the concentration to 30 ng repelled these males. In contrast, 15 ng elicited weak responses by *H. dromedarii* males whereas 30 ng was highly attractive to these ticks. Clearly, a marked difference in sensitivity is exhibited in responses of these two species. This ability to discriminate differences in concentration is consistent with natural differences in the occurrence of 2,6-DCP in the two species (Silverstein et al., 1983). In effect, the (relatively) high concentration of the compound emitted by *H. dromedarii* acts as a distinct pheromone, attractive to conspecific males, but repellent to *H. anatolicum excavatum* males, a sympatric species with a similar host range. The natural differences in the amounts of 2,6-DCP secreted by the two species and the differences in male response to these differences enable the same molecule to act as two separate pheromones, minimizing the need to generate separate molecules for this purpose.

Recent research suggests that 2,6-DCP may be the only sex pheromone required to elicit species-specific mating in *Hyalomma*, at least in *H. dromedarii*. Males of this camel tick readily respond to female *D. andersoni* and *D. variabilis*, orienting to and mounting them in almost all cases, resulting in numerous copulations; 40% of the trials with *D. andersoni* and 20% with *D. variabilis* were completed in this manner. When 13 *H. dromedarii* females were neutered and cleansed of surface chemicals, virtually all (78%) of *H. dromedarii* males deposited spermatophores in these females despite the neutering treatment (Sonenshine and Taylor, unpublished)! Several males even failed to distinguish the female genital aperture and deposited their spermatophore in the wound scar posterior to it. Coating the female genital pore with lacquer reduced copulation, but the males merely left without attempting to pierce this barrier, quite the opposite of the male response to similar challenges in *D. variabilis*. However, Khalil et al. (1983) did observe reduced mating success after severing the vagina from the gonopore.

In the North American *D. andersoni* and *D. variabilis*, a genital sex pheromone not only guides the courting males to the female gonopore, but provides species-specific information that enables the males to identify and copulate only with conspecific partners. *D. variabilis* males, excited by the general, interspecific attractant 2,6-DCP will court all pheromone-secreting females in a mixed

species population, mount them, and probe their genitalia; however, they will copulate only with conspecific females (Sonenshine et al., 1982b). Physical and chemical characteristics of the female body surface may also provide information which enables the males to confirm the females as prospective mating partners. Inanimate objects of the same general shape and size as partially engorged female tick, "pseudoticks," are ignored by sexually excited males, as are females that lack pheromone. However, adminstration of 2,6-DCP to the authentic female induces a vigorous courtship response, even if the treated female is dead. The males palpate these treated tick remnants, mount them, probe their genital pores, and many even copulate. However, males exposed to the 2,6-DCP "pseudoticks" orient to the pheromone source but fail to mount or copulate with these inanimate objects. When the texture and chemical characteristics of the cuticle are preserved, the males mount and probe these "pseudoticks" in the normal manner, and several males even copulate with these objects. Experiments are planned to determine whether physical characteristics, e.g., texture of the superficial folds on the female body, or chemical characteristics, e.g. cuticle waxes, or some combination of both physical and chemical features are essential to the mounting response.

Regardless of how they guide themselves to the female's ventral body surface, male *D. variabilis* and *D. andersoni* appear to require a genital sex pheromone to identify the gonopore and commence copulation. At least one component of this pheromone provides species-specific information which must be perceived or the male will not copulate. As has been discussed previously, the males bear gustatory receptive sensilla on their cheliceral digits. Males that have had the digits of the chelicerae removed are unable to form spermatophores, apparently because they are unable to identify the species-specific component of the genital sex pheromone. Thus, a multicomponent pheromone system appears to have evolved in the American *Dermacentor* species to provide precise mate-identifying information. As expected, *D. variabilis* males will not mate with females of any other species, since they appear to require the presence of the conspecific genital pheromone in order to form and release the spermatophore.

Further study of the genital sex pheromone was done by preparing an extract (in methanol) of the anterior reproductive tract (ART) tissues, fractionating it chemically, and assaying the different fractions to determine which ones were most active. Preliminary tests demonstrated that thorough washing of the female genital surface and cutting the vulva, thereby severing the reproductive tract from the gonopore, would prevent mating. Males were observed to mount and probe these "neutered" females, but few males would copulate, even though the appearance of the gonopore remained unaltered by this treatment. However, when an aliquot of the crude extract of the female ART was placed in the genital pore of these "neutered" females, the male response was restored; males copulated readily. These findings appear to meet the essential criteria for demon-

stration of chemical communication, i.e., a pheromone: removal of the material resulted in loss or at least extreme reduction of the response, while implanting the crude extract restored the response. Using gel permeation chromatography to separate the extract components by molecular size, Sonenshine et al (in press) demonstrated activity with at least two groups of fractions containing compounds of widely differing molecular weights. Their evidence suggests that the pheromone contains one or more compounds with a molecular weight of 600–700 atomic mass units (amus), and one or more compounds with molecular weights in the range of 400–500 amus. Further studies of the chemical identity of the genital sex pheromone are in progress.

The ability of the males to detect the genital sex pheromone was confirmed by electrophysiological methods. Davis and Sonenshine applied micropipets filled with solutions of the extract to the cheliceral digits of fed, sexually active male ticks (Sonenshine et al., in press). Rapid bursts of spikes indicating strong responses by the excited neurons were observed. The cheliceral digits were also responsive to physiological solutions of ATP, glutathione, and sodium chloride, i.e., similar to the responses observed by Waladde and Rice (1977) in *B. microplus*. Washings of the female vulva and genital pore were also stimulatory, although such extracts failed to elicit copulations in the bioassay. These studies were repeated by Taylor and Sonenshine (unpublished), with similar results (Figure 9).

Additional evidence of the existence of the genital sex pheromone in *D. variabilis* is demonstrated by the success of interspecific matings when *D. variabilis* were exposed to heterospecific females treated with *D. variabilis* female extract. Normally, *D. variabilis* males rarely if ever copulate with females of the camel tick, *H. dromedarii*. However, when the *H. dromedarii* females were neutered and extracts of *D. variabilis* females (anterior reproductive tract) were placed in their vulvas, the male response was restored. Although magnitude of the response (43%) was considerably less than in the conspecific assay (78%), such copulations between species are unusual; the frequency of this occurrence in the controls was only 10.6%.

These findings, summarizing evidence obtained by two different assay methods, argues persuasively for the existence of a genital sex pheromone in *D. variabilis* and *D. andersoni*, originating in the female reproductive tract and dispersed onto the external genital surface.

Although the chemical identity of the genital sex pheromone is unknown at this time, considerable evidence has accumulated implicating 20-OH ecdysone as one of the components of the pheromone in *D. variabilis*. Extracts of the anterior reproductive tract tissues gave a strong positive response when assayed by radioimmunoassay, 180 pg/tick (4.7 ng/mg) for *D. variabilis* as compared to only 12.5 pg/tick (0.3 ng/mg) in *H. dromedarii*. Bioassays also show a high proportion of successful mating responses when 20-OH ecdysone is implanted in the vulval remnant of neutered *D. variabilis*, or in the vulvae of

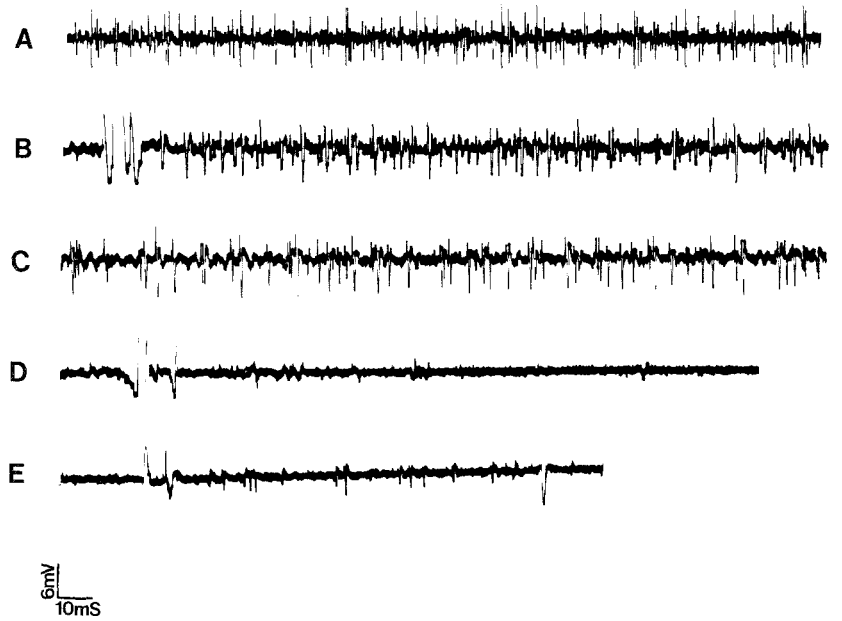


FIG. 9 Oscilloscope tracings illustrating the responses of *D. variabilis* chelicerae and palps to various chemical stimuli administered by capillary microelectrodes. (A) Response of the inner cheliceral digit sensilla to a 0.15 M NaCl solution. (B) Response of the inner cheliceral digit sensilla to 20-OH ecdysone (1×10^{-3} M). (C) Response of the inner cheliceral digit sensilla to ecdysone (1×10^{-3} M). (D) Absence of a response of the palpal sensilla to 20-OH ecdysone (1×10^{-3} M). (E) Absence of a response of the palpal sensilla to ecdysone (1×10^{-3} M).

"pseudoticks," i.e., remnants of conspecific females in which the interior contents have been replaced with wax. In contrast, similar tests with *D. andersoni* were unsuccessful. The natural hormone was also demonstrated in the *D. variabilis* ART extracts by HPLC (2.2 ng/tick), but not in the ART of *H. dromedarii*. Although 20-OH ecdysone is generally present in many species of ticks (Solomen et al., 1982), its occurrence in the ART of *D. variabilis* appears to be unusually concentrated. Soneshine et al. (in press) suggest, albeit with considerable caution, that 20-OH ecdysone plays a unique role in *D. variabilis*, perhaps by elevating the level of nervous excitation and, therefore, is one of the active components of the genital sex pheromone in that species.

SUMMARY

This review has emphasized the importance of acquiring and integrating knowledge of sensory organ structure, functional capabilities of the sensilla, and behavioral observations to assess the biological value of the incoming in-

formation. Special stress has been accorded to the chemical stimuli, although these are not the only stimuli important in tick activities, nor are they necessarily paramount in the performance of all necessary behavioral functions. Nevertheless, study and correlation of the ultrastructure, neurophysiology of acarine sensory systems, and behavioral responses to specific chemical cues have enabled biologists and chemists, working in concert, to make important advances in understanding host-finding, feeding, and mating behavior in these remarkably diverse arthropods. These include such important discoveries as the identification of 2,6-dichlorophenol as the common sex attractant of numerous ixodid species, the identification of a blend of *O*-nitrophenol, methyl salicylate, and pelargonic acid as the aggregation attachment pheromone of *Amblyomma variegatum*, the response of tarsal olfactoreceptors to various substituted phenols, the discovery of genital sex pheromones, and the detection of these secondary sex pheromones by olfactoreceptors on the cheliceral digits. New advances and increasing improvements in the sophistication of such tools as electron microscopy, electrophysiology, and gas chromatography offer promise of an even greater pace of discovery, leading to recognition of new or poorly known semiochemicals and a much better understanding of how these compounds influence acarine behavior.

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COEVOLUTIONARY ADAPTATIONS OF ROOTWORM BEETLES (COLEOPTERA: CHRYSOMELIDAE) TO CUCURBITACINS

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Abstract—The cucurbitacins are oxygenated tetracyclic triterpenoids produced as secondary plant compounds by nearly all genera of Cucurbitaceae. The very bitter and toxic cucurbitacins are effective semiochemicals acting ecologically as allomones to protect the Cucurbitaceae from attack by a variety of invertebrate and vertebrate herbivores. For the Luperini (Coleoptera: Chrysomelidae: Galerucinae) the cucurbitacins have become kairomones for host selection, affecting the behavior of this large group of 1500 species of Aulacophorina (Old World) and Diabroticina (New World) by arrest and compulsive feeding. When feeding on bitter cucurbits these beetles sequester large amounts of cucurbitacins in their blood and tissues, and these act as allomones to deter predation. Specific detoxification and excretory mechanisms of the Diabroticina enable these beetles to avoid the toxic effects of the cucurbitacins.

Key Words—Rootworm beetle, *Diabrotica* spp., *Aulacophora* spp., Coleoptera, Chrysomelidae, *Cucurbita* spp., *Cucumis* spp., cucurbitacin, allomone, kairomone, Chinese mantis, *Tenodera aridifolia sinensis*, predator, Orthoptera, Mantidae.

INTRODUCTION

Coevolutionary adaptations between terrestrial plants and insect herbivores must have originated in the Carboniferous Period about 300 million years (BP) when land plants were diversifying and insects had begun to evolve from primitive segmented ancestors (Riek, 1970; Taylor, 1982). By the Permian Period 270 million years BP insects had segregated into modern orders, and the first fossil records of insect-damaged leaves are recorded. The first flowering plants oc-

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curred in the Triassic Period about 225 million years BP (Smart and Hughes, 1973).

Over this vast stretch of evolutionary time, many of the estimated 100,000 secondary plant compounds appeared in response to evolutionary pressures for protection of plants from herbivore attack. Semiochemical communication has become the dominant force in coevolutionary relationships between plants and animals, by allomones that benefit the producer, kairomones that benefit the receiver, and synomones where both producer and receiver benefit. Fraenkel (1969) stated the profundity of the case for host plant selection by herbivores: "evidence for the importance of secondary plant substances in host selection is so overwhelming as to need no further proof."

The roles of the cucurbitacins of Cucurbitaceae as allomones and kairomones regulating herbivore attack provide a compelling example of the ecological and behavioral effects of secondary plant compounds (Chambliss and Jones, 1966; Sharma and Hall, 1973, Metcalf et al., 1980). The oxygenated tetracylic triterpenoid cucurbitacins (Cucs) are extremely bitter and toxic substances characteristic of the Cucurbitaceae (Metcalf et al., 1980). An appropriate scenario for the evolutionary and behavioral interactions between Cucurbitaceae and herbivores can be portrayed as follows (DaCosta and Jones, 1971a; Price, 1975; Metcalf, 1979): (1) ancestral Cucurbitaceae with *bi bi* genes for Cuc synthesis are heavily preyed upon by herbivores; (2) mutation in Cucurbitaceae to *Bi bi* forms bitter and toxic Cuc that deters herbivore attack; (3) strong selection pressure spreads *Bi bi* genes throughout evolving Cucurbitaceae species; (4) mutant Cucurbitaceae flourish in absence of herbivore attacks; (5) mutant ancestral Luperini rootworm beetles develop detoxification and excretion pathways to neutralize harmful effects of Cucs; (6) Luperini beetles expand into new ecological niches developing specific receptors for Cuc detection; and (7) Luperini beetles develop high blood and tissue levels of Cuc conjugates for defense against predators.

CUCURBITACINS AS ALLOMONES FOR CUCURBITACEAE

There is no ambiguity about the role of the Cucs as protective semiochemicals of the Cucurbitaceae and allied families against the attacks of herbivores. This is immediately apparent to anyone who has tasted a bitter squash, cucumber, or melon. The Cucs are the bitterest substances yet identified and can be detected by humans at dilutions as great as 1 ppb (Metcalf et al., 1980). The merest trace produces an almost paralytic response in lips and mouth and a persistent aftertaste. Moreover, the Cucs are extremely toxic to mammals with LD_{50} values to mice intraperitoneally of Cuc A, 1.2; Cuc B, 1.1; and Cuc C, 6.8 mg/kg (David and Vallance, 1955); and orally of Cuc I, 5 and Cuc E glycoside, 40 mg/kg (Stroesand et al., 1985).

Mice fed freeze-dried *Cucurbita texana* fruit at 10–20% of the diet died in 3–6 days. When fed this fruit at 1%, 40% died within 10 weeks, and the survivors had severe diarrhea and anemia (Stroesand et al., 1985). Sheep and cattle that consumed bitter fruit of wild *Cucumis leptodermus*, *C. africanus* and *C. myricarpus*, under drought conditions in South Africa were severely poisoned (Watt and Breyer-Brandwyk, 1962). Twenty-two cases of human poisoning from eating *C. pepo* (zucchini) fruits that contained about 1.1 mg Cuc E glycoside per gram occurred in Queensland, Australia, in 1982 (Ferguson et al., 1983a; Herrington, 1983). Symptoms included severe cramps, persistent diarrhea, and collapse occurring within a few hours after eating the zucchini fruits which were apparently mutant reversions to the *Bi* heterozygote (Rhymal et al., 1984).

Allomonal effects of Cucs have also been demonstrated on invertebrate herbivores including red spider mites (Tetranychidae) and the stem borer *Margaronia hyalinate* (DaCosta and Jones, 1971a). The presence of Cucs E and I has been shown to provide feeding deterrents to numerous insect herbivores including the leaf beetles *Phyllotreta nemorum*, *P. undulata*, *P. tetrastigma*, *Phaeodon cochliariae*, and *P. cruciferae* (Nielson et al., 1977). Cuc B applied to bean leaves prevented the feeding of the bean leaf beetle *Ceratoma trifurcata* (Metcalf et al., 1980).

Chemical Identity of Cucurbitacins. Approximately 20 chemically different Cucs have been characterized from plants (Lavie and Glotter, 1971; Guha and Sen, 1975) (Figure 1). Cuc B is the predominant form found in about 91% of all species characterized, followed by Cuc D (69%), Cucs G and H (47%), Cuc E (42%), Cuc I (22%), Cucs J and H (9%), and Cuc A (7%). Cucs C, F, and L were each found in only a single species (2%) (Rehm et al., 1957). It appears that the two primary Cucs are Cuc B and Cuc E and that the other Cucs are formed by enzymatic processes occurring during plant development and matu-

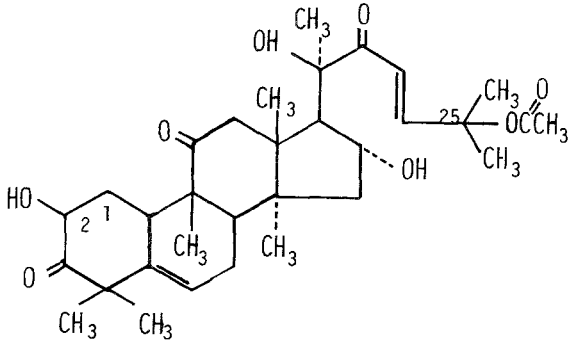

FIG. 1. Structure of cucurbitacin B. Cuc D is C_{25}—OH; Cuc E is C_1=C_2; Cuc I is C_1=C_2, C_{25}—OH; Cuc F is C_2—OH, C_3—OH, C_{25}—OH; Cuc G is C_{24}—OH, C_{25}—OH; Cuc L is C_1=C_2; C_{23}—C_{24}, C_{25}—OH.

ration. Cuc B can be metabolized to Cucs A, C, D, F, G, and H and is characteristic of *Coccinia*, *Cucumis*, *Lagenaria*, and *Trichomeria* (Rehm et al., 1957). Similarily Cuc E can be metabolized to Cucs I, J, K, and L and is characteristic of *Citrullus*. *Cucurbita* contains two groups of species characterized by either Cuc B or Cuc E (Metcalf et al., 1982). Cuc B is converted to Cuc E by Cuc Δ^1 reductase that produces the diosphenol grouping $C_1{=}C_2$, and Cuc Δ^{23} reductase converts the $C_{23}{=}C_{24}$ Cuc B and E series to the dihydro Cucs. Cucs B and E are converted to Cucs D and I by desacetylation by Cuc acetyl esterase (Schwartz et al. 1964, Schabort and Teijema 1968, Lavie and Glotter, 1971).

The Cucs in *Cucumis*, *Lagenaria*, and *Acanthosicyos* are present as free aglycones. However, in most species of *Citrullus* examined, the Cucs are present as glycosides (Rehm et al., 1957). In *Cucurbita* Cucs are present as aglycones in most species but glycosides are found in *C. cylindrata*, *C. foetidissima*, *C. palmata*, and *C. texana* (Metcalf et al., 1982). The presence of glycosides is related to the absence of β-glucosidase (elaterase) which may also be sequestered in intact plant tissues and released by crushing (Enslin et al., 1956).

Although the multiplicity of Cucs and their variable distribution in the Cucurbitaceae presents a complex situation for analysis, certain generalizations are valid: (1) Cucs B and E are the parent substances found most widely and the other Cucs, generally present in much lower quantities, are degradative products. (2) Cuc D is always associated with Cuc B and Cuc I is always associated with Cuc E. (3) Cucs G and H are always associated with Cucs B and D. (4) Cucs J and K are always associated with Cucs E and I. (5) Cuc A is always associated with Cuc B. (6) Cuc C is singular and occurs alone in *Cucumis sativus* (Rehm et al., 1957; Ferguson et al., 1983b). (7) Only Cucs B, C, and E sometimes occur alone.

Distribution of Cucurbitacins in Plants. Cucurbitacins, as the name suggests, are peculiarly associated with the family Cucurbitaceae where they have been characterized in at least 30 genera and more than 100 species (Rehm et al., 1957; Lavie and Glotter, 1971; Pohlmann, 1975). Cucs are also found in a few genera of the related plant families Begoniaceae, Brassicaceae (Cruciferae), and Datisceae, all of the superorder Violoflorae (Thorne, 1981). For example, Cucs have been identified in 16 species of *Iberis* (Brassicaceae) (Lavie and Glotter, 1971; Curtis and Meade, 1971). Cucs have also been identified in a few species of Euphorbiaceae, Scrophulariaceae and Rosaceae (Dryer and Trousdale, 1978).

Cuc synthesis in *Cucumis*, *Citrullus*, *Cucurbita*, and *Lagenaria* is initiated by a single dominant gene *Bi*. Nonbitter fruit may develop from bitter seedlings in the presence of a modifier gene *Su Bi* (Robinson et al., 1976). It is probable that a few major genes control the chemical nature of the Cuc formed and Cuc B is dominant over Cuc E (Ferguson et al., 1983b).

Cucs are found in all parts of the plant in the Cucurbitaceae: roots, stems, leaves, fruit, and occasionally in the seeds.

Roots. The concentration in the roots increases with age and can reach very high levels in perennial plants, e.g., 1.4% in *Citrullus naudinianus*, 1.1% in *Acanthosicyos horrida*, and 0.9% in *Colocynthis ecirrhosa* (Rehm, 1960). In 18 species of *Cucurbita*, Cucs B–D were detected in the roots of seven, up to 0.43% in *C. ecuadorensis*, and Cucs E–I were identified in six up to 0.38% in *C. palmata* (Metcalf et al., 1982).

Leaves. Although Rehm (1960) indicates that the leaves of Cucurbitaceae seldom contain Cucs even when the roots have very high concentrations, this generalization has many exceptions. Young rapidly growing leaves of *Colocynthis* (*Citrullus*) *vulgaris* and *C. ecirrhosa* have only about 0.01% Cucs, but this reaches 0.1–0.3% by the end of the vegetative season. Of 18 species of *Cucurbita* examined, Cucs B–D were found in seven species up to 0.059% in *C. lundelliana*, and Cucs E–I in six species up to 0.1% in *C. okeechobeensis* (Metcalf et al., 1982).

Fruits. The fruits of a variety of Cucurbitaceae contain high amounts of Cucs. Rehm et al., (1957) found Cuc concentrations of >0.1% in the fruits of *Citrullus colocynthis* and *C. ecirrhosis* (Cuc E), *Cucumis angolensis* and *C. longyies* (Cuc D), *C. myriocarpus* (Cuc A), and *C. sativus* (Cuc C). Of 18 species of *Cucurbita* examined, the fruits of seven contained Cucs B–D up to 0.31% in *C. andreana*, and the fruits of five contained Cucs E–I up to 0.23% in *C. foetidissima* (Metcalf et al., 1982).

Seeds. Cucs are not commonly found in the seeds of Cucurbitaceae. Rehm et al. (1957) reported that three of 45 species had bitter seeds, presumably from Cucs present in the surrounding tissue.

CUCURBITACINS AS KAIROMONES FOR ROOTWORM BEETLES

The ancestral association between the leaf beetles of the tribe Luperini (Coleoptera: Chrysomelidae: Galerucinae) and the plants of the family Cucurbitaceae seems to have been effected through the presence of the cucurbitacins acting secondarily as kairomone cues for host selection by the beetles. The scope of this association is presently worldwide. The Luperini is comprised of two very similar but geographically isolated subtribes, the Diabroticina of the New World, containing about 993 species all evidently radiating from tropical America, and the Aulacophorina of the Old World, containing about 535 species apparently radiating from tropical Australasia (Maulik, 1936; Wilcox, 1972). The Aulacophorina are presently all restricted to Asia except *Aulacophora foveicollis* whose range extends to Europe and *A. africanus* extending into Africa. Maulik (1936) cites the remarkable similarity between these two

large groups of phytophagous beetles: "In the Old World, Aulacophora represents Diabrotica . . . In larval, pupal and adult structures, in breeding habits and in food plants, there is a remarkable resemblance between the two genera." The present distribution of the two subtribes of the Luperini is shown in Metcalf (1985).

At least 50 species of Luperini, representing more than 80% of the available host records, have been recorded as feeding on Cucurbitaceae (Wilcox, 1972; Takizawa, 1978; Metcalf, 1985). The common names of notable pest species attest to the significance of this relationship:

Diabroticina (New World): *Diabrotica balteata*, the banded cucumber beetle; *D.* (*Paranapiacaba*) *connexa*, the saddled cucumber beetle; *D. picticornis*, the painted cucumber beetle; *D. speciosa*, the cucurbit beetle; *D. u. howardi*, the spotted cucumber beetle; and *A. vittatum*, the striped cucumber beetle.

Aulacophorina (Old World): *Aulacophora abdominalis*, plain pumpkin beetle; *A. femoralis*, cucurbit leaf beetle; *A. foveicollis*, red pumpkin beetle; and *A. hilaris*, pumpkin beetle.

The role of cucurbitacins in host selection by Luperini is exemplified by two recent studies relating Cuc content of *Cucurbita* cotyledons to damage by rootworm beetles, rated on a five-point scale. For *A. foveicollis* attacking *Cucurbita moschata* cultivars there was a substantial correlation between Cuc content and feeding damage, $r = 0.62$, $N = 32$, SD = 0.14[2], and it was concluded that "low cucurbitacin content appeared to impart resistance" (Pal et al., 1978). In a similar study of *D. u. howardi* and *A. vittatum* attacking *Cucurbita pepo* cultivars, the correlation between beetle damage and Cuc content was, $r = 0.77$, $N = 12$, SD = 0.20, and it was concluded that there was a "strong positive correlation between seedling cucurbitacin content and Diabroticina beetle attacks" (Ferguson et al., 1983b). Similar correlations were found between the average numbers of Diabroticina beetles feeding on crumpled leaves or sliced fruits of various *Cucurbita* spp. and their total Cuc content (Metcalf et al., 1982): with *D. u. howardi*, leaves $r = 0.74$, $N = 16$, SD = 0.20; fruits $r = 0.70$, $N = 11$, SD = 0.24. With *D. v. virgifera*, the correlations were leaves $r = 0.64$, $N = 16$, SD = 0.24; and fruits $r = 0.58$, $N = 11$, SD = 0.27.

The systematic relationships between Aulacophorina and Diabroticina and their common responses to Cucs indicate a common ancestral relationship with a Cucurbitaceae progenitor during a geologic period no later than the Miocene ca. 30 million years BP when continental land bridges were still present (Metcalf, 1979). This argument is supported by the presence of fossil *Diabrotica* spp. in Florisant shales of Colorado, ca. 25–30 million years BP (Wickham, 1914). Isozyme studies of the Diabroticina have produced evidence of the divergence of *Acalymma* from *Diabrotica* at about 2.7 genetic distances or approximately 45 million years BP (unpublished data).

[2] r = correlation coefficient, N = number of observations.

TABLE 1. LIMIT OF RESPONSE (LR) OF DIABROTICINA BEETLES TO PURE CUCURBITACINS ON SILICA GEL PLATES

Species	Cuc LR (μg)							
	B	D	E	F	G	I	L	E-gly
D. balteata	0.01			10	3	5		0.1
D. l. barberi	0.1		0.3					5
D. cristata	0.1	1.0	0.3	>10	3		>1.0	50
D. u. howardi	0.001	0.03	0.01	1.0	3.0	0.1	0.01	0.05
D. u. undecimpunctata	0.003		0.03					
D. v. virgifera	0.01	0.1	0.3	0.1	3	0.3	1.0	0.03
A. vittatum	0.3		10					50

Sensory Receptors of Diabroticina Beetles for Cucurbitacins. The high degree of sensitivity to the Cucs and the specificity of the behavioral responses of arrest and compulsive feeding induced by proximity to Cucs demonstrate that specific receptor organs are present in the beetles. Both sexes of *D. u. howardi*, *D. v. virgifera*, and *A. vittatum* responded to pure Cucs with identical arrest and feeding behavior. Amputation of the antennae of *D.u. howardi* beetles had little effect on the feeding response to Cucs but amputation of the maxillary palpi abolished arrest and feeding (Metcalf et al., 1980). Scanning electron microscopy of the maxillary palpi of the several species of Diabroticina studied (Table 1) has demonstrated *sensilla basiconica* arranged around the tip of the maxillary palpus that seem likely to contain the Cuc receptor. Detailed study of the comparative morphology in the several species is in progress (J.R. Larsen, in preparation).

Diabroticina Beetle Sensitivity to Cucurbitacins. Field observations of the mass attacks of various Diabroticina beetles on cotyledons, leaves, and fruits of Cucurbitaceae suggest that the Cucs present are effective at low concentrations in producing arrest and compulsive feeding of adult beetles.

Qualitatively, all the species of Diabroticina investigated (see Table 1) will feed to some degree on pure crystalline Cucs A, B, C, D, E, F, G, I, and L placed on filter paper or silica gel thin-layer chromatography (TLC) plates (Chambliss and Jones, 1966; Metcalf et al., 1980). The various beetle species produced identical spectra of feeding patterns on TLC plates developed from chloroform extracts of *Cucurbita andreana* (Cucs B–D), *C. okeechobeensis* (Cucs E–I), or *C. texana* (Cuc E glycoside) (Metcalf et al., 1982). These "beetle prints" (Figure 1) are valuable in characterizing the distribution of Cucs in the Cucurbitaceae. When the TLC plates developed from plant extracts were presented simultaneously, Cuc B was always fed upon before Cuc E and Cuc D before Cuc I, indicating the greater beetle sensitivity to the Cuc B–D compounds (Table 1). The beetle bioassay used in conjunction with TLC to separate

the individual Cucs is both qualitative and semiquantitative as the areas eaten from the TLC plates are proportional to the amounts of Cucs present.

Quantitatively, there are substantial differences in the threshold amount of the various chemically purified Cucs that produces a detectable feeding response and different species of beetles show different responses to the individual Cucs (Table 1). This quantity is the limit of response (LR) and is measured on silica gel TLC plates exposed to about 100 Diabroticina beetles for four days. The LR represents the relative degree of complementarity of the various Cucs to the maxillary palpi receptors of the Diabroticina (Metcalf et al., 1980). *D. u. howardi* and *D. u. undecimpunctata* beetles consistently responded to 1–3 ng of Cuc B under these standard conditions as shown in Table 1. This beetle bioassay is therefore about 250× more sensitive than HPLC (Ferguson et al., 1983b) and 1000× more sensitive than UV spectrophotometry (Metcalf et al., 1982).

Table 1 also shows that there are major differences in the LR values for the various Diabroticina beetles to the individual Cucs. These differences have evolutionary and behavioral significance. The conclusions that can be drawn from the data in Table 1 are: (1) Cuc B was consistently detected in the lowest amount and is probably the parent Cuc to which Diabroticina receptors are attuned, as it was found in 91% of the species characterized. (2) Cuc B was consistently detected at levels of about 0.1 that of Cuc E. (3) The acetoxy Cucs B and E were detected at levels about 0.1 those of the corresponding desacetoxy Cucs D and I, respectively. (4) Saturation of the desacetoxy Cucs at $C_{23}=C_{24}$ double bond (Cuc L) had little effect on level of detection. (5) Sensitivity to the 2-OH, 3-C=O Cuc D was greater than to the 2-OH, 3-OH Cuc F. (6) The *D. undecimpunctata* subgroup (*D. balteata*, *D.u.u.*, *D.u.h.*) is more sensitive to Cucs than the *D. virgifera* subgroup (*D.l. barberi*, *D. cristata*, and *D.v. virgifera*). (7) *A. vittatum* is substantially less sensitive to Cucs than the *Diabrotica* spp.

The range of variations in maxillary receptor sensitivity to the various Cucs (Table 1) is relatively consistent for all the species examined. It appears that Cuc B has maximum complementarity to the Cuc receptor. Receptor depolarization most probably results from allosteric changes in the conformation of the receptor protein resulting from interactions of the free paired electrons (dipoles) associated with the several oxygen atoms of the Cuc molecules (Metcalf et al., 1980). The structural change from Cuc B to Cuc E by introduction of a single C=C bond at C_1-C_2 in ring A (Figure 1) seems trivial, yet this change produces a 10-fold decrease in receptor affinity (Table 1). The introduction of the C=C bond into ring A changes the orientation of the three contiguous O atoms (C_3=O, C_2—OH, and C_{11}=O) from a staggered configuration on the cyclohexyl moiety of Cuc B to a planar configuration in Cuc E. From observations of molecular models, this change seems ample to decrease receptor affinity and depolarization. Cucs D and I exhibit about a 10-fold decrease in receptor affinity

as compared to the C_{25} acetoxy derivatives B and E (Figure 1). This suggests that the acetoxy C=O dipole must also be involved in complete binding to the receptor.

CUCURBITACINS AS ALLOMONES FOR DIABROTICINA BEETLES

The incredibly bitter taste of Cucs and their toxic effects on vertebrates after ingestion suggest a further role in the behavioral ecology of the Diabroticina beetles, i.e., as protective allomones against predators. Thus the ingestion of Cucs by Diabroticina species feeding on bitter Cucurbitaceae host plants may provide protection against birds and predaceous vertebrates, as first suggested by Howe et al. (1976).

Storage and Sequestration of Cucs. Diabroticina beetles can concentrate and sequester relatively large amounts of Cucs in free and derivatized form. A single elytron of *D. undecimpunctata* or *D. balteata* fed on bitter *Cucurbita andreana* × *C. maxima* cultivar (Rhodes et al., 1980), containing 1–3 mg Cuc B–D per gram fresh weight, had an extremely bitter taste and the numbing effect on lips and tongue was perceptible 2 hr later (personal observation).

Groups of *D. balteata* adults were fed on bitter *C. andreana* × *C. maxima* fruit for various periods of time and the Cuc content was measured in the hemolymph by UV spectrophotometry after isolation by TLC. The presence of a Cuc conjugate (R_f 0.4 in chloroform–methanol 1 : 1) was readily detected in 1 μl or less of insect hemolymph following TLC, after one and two days of feeding of the adult beetles on bitter squash (Ferguson et al., 1985). The concentration of the Cuc conjugate rapidly increased in the hemolymph of *D. balteata* and reached an apparent plateau level after 30–40 days when blood levels were about 30 μg/μl (3%). Similar blood measurements on *D. u. howardi* adults collected from bitter squash plants indicated hemolymph levels of Cucs of 20–26 μg/μl. This Cuc content is equivalent to about 15 mg Cucs per gram of body weight. No Cucs were detectable in *D. balteata* adults reared on pollen food (Ferguson et al., 1985).

Adverse Effect of Cucurbitacin Sequestration upon Predators. The Chinese mantis *Tenodera aridifolia sinensis* is an omnivorous insect predator and has been shown to acquire learned aversion to cardiac glycosides sequestered in the milkweed bug, *Oncopeltus fasciatus* (Berenbaum and Miliczky, 1984). Paired experiments where mantids were offered first Diabroticina beetles reared on pollen and then beetles reared on bitter squash fruit showed that 72% of *D. balteata*, 46% of *D. u. howardi*, and 24% of *D. v. virgifera* fed on bitter fruits were rejected. The results were highly significant ($P < 0.001$–$P < 0.05$) (Ferguson and Metcalf, 1985). No mantid ever rejected a beetle fed on pollen, but the rejection reaction of the Cuc-containing beetles typically consisted of the

mantid violently flinging away the beetle after a single bite on an elytron. Approximately 70% of the rejected beetles survived such encounters. The predator was obviously disturbed by the Cucs and underwent a period of excessive grooming, unsteadiness, and/or regurgitation. In some instances the mantis would fall from its perch and, after holding the bitter prey away from its body, would taste it one or two more times prior to discarding it.

Although the Chinese mantis consistently rejected *D. balteata*, *D. u. howardi*, and *D. virgifera* adult beetles fed on bitter squash in favor of those fed on pollen, the striped cucumber beetle, *A. vittatum*, was an exception, in that there was no significant difference in rejection rate between beetles fed pollen and those fed bitter squash (Ferguson and Metcalf, 1985). However, larvae of the *A. vittatum* beetles were reared on the roots of *Cucurbita maxima* (blue hubbard squash). The roots of this species contain relatively large amounts of Cucs, and taste and beetle bioassays showed the presence of large amounts of Cucs in both the pollen-fed and bitter-squash-fed adults. Thus, the larvae feeding on the bitter roots sequestered Cucs that were transferred to the adult beetles during metamorphosis. The larval sequestration of Cucs was effective and persistent, and *D. u. howardi* adult beetles fed as larvae on bitter *Cucurbita* and then placed as adults on a pollen diet for three months were still rejected by the Chinese mantid. Bioassay by TLC and beetle feeding demonstrated that the hemolymph of these beetles free from exposure to Cucs for six weeks still contained quantities of Cucs comparable to those fed continuously on bitter fruit (Ferguson and Metcalf, 1985).

The feeding deterrent effect of pure Cuc B was evaluated by topical application in acetone of 14 μg to the elytra of pollen fed beetles devoid of Cucs. Such treated beetles were consistently rejected by the Chinese mantid (Ferguson and Metcalf, 1985).

The protective effect of Cucs against predation of Diabroticina species appears to extend to larvae and to eggs. The larvae of several species reared on roots of bitter squash had hemolymph that was distinctly bitter. Females of *D. u. howardi*, *D. balteata*, and *A. vittatum* laid eggs that contained substantial quantities of Cucs as detected by beetle bioassay (Ferguson et al., 1985) (Figure 2). These "bitter" eggs could be effective in discouraging ant egg predators such as by *Solenopsis geminata* and *Pheidole* spp. (Risch, 1981).

Metabolism of Cucurbitacins by Diabroticina. The ability of Diabroticina beetles to grow, develop, and reproduce on host bitter *Cucurbita* containing as much as 0.32% fresh weight of Cucs (Metcalf et al., 1982) demonstrates the evolutionary development of effective metabolic mechanisms for disposition of these toxic triterpenoids. For example, groups of 25 *D. u. howardi* or *D. v. virgifera* adults completely consumed 1 mg of Cuc B in 72 hr without any perceptible ill effect. These insects weighed about 20 and 10 mg, respectively, so that the oral LD_{50} values for Cuc B are $>>2000$ mg/kg (Metcalf et al., 1980).

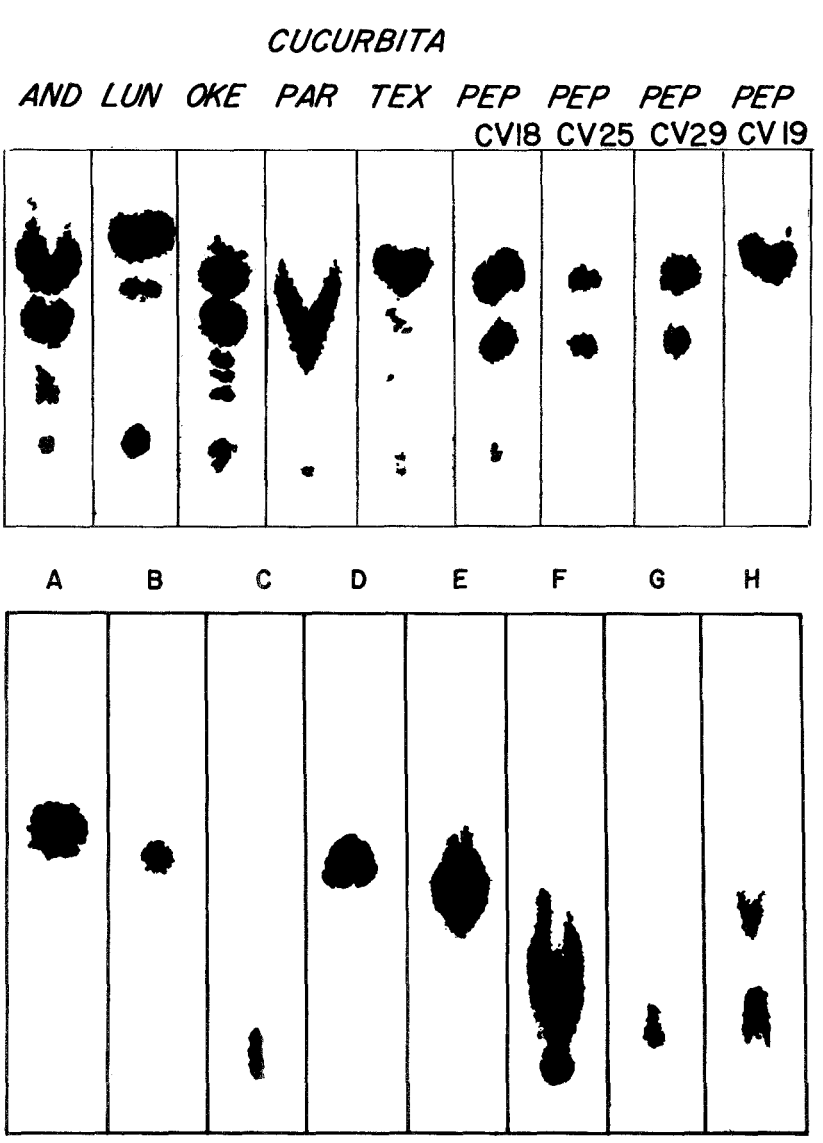

FIG. 2. Profiles of thin-layer chromatograms showing areas eaten from silica gel plates, "beetle prints," by feeding of adult *Diabrotica u. howardi*. Top: chloroform extracts of cotyledons of *Cucurbita* spp. and cultivars. AND = *andreana*, LUN = *lundelliana*, OKE = *okeechobeensis*, PAR = *palmeri*, TEX = *texana*, PEP = *pepo* cv. Ambassadore, Diplomat, Greenbay, and Black, respectively. (Ferguson et al., 1983b). Bottom: A, B = Cuc B and D standards, C = 0.75 μl of hemolymph from field collected *D. u. howardi*, D. = same hemolymph treated with pectinase, E = chloroform extract of excreta from *D. u. howardi*, F = chlorofrom extract of the body of *D. u. howardi* with no exposure to Cucs for six weeks, G = chloroform extract of 200 eggs from *D. u. howardi* adults fed on Cucs, and H = extract of 400 eggs of *A. vittatum* exposed to Cucs only as larvae (Ferguson et al., 1985).

[^{14}C]Cuc B was synthesized from DL-[2-^{14}C]mevalonate in seedlings of *C. maxima* and purified by preparative TLC to specific activity of 2.5 μCi/m mol. Approximately 1 mg of the [^{14}C]Cuc B was fed to groups of 20 one- to two-week-old Diabroticina beetles over a period of 48 hr. The disposition of ^{14}C-labeled products was examined in excreta, hemolympyh, gut, and body remainder after extraction and TLC. The total amounts of ^{14}C label excreted ranged from 67.2% for *A. vittatum* to 94.6% for *D. balteata*. Cuc B was identified in the excreta ranging from 1.7% in *D. u. howardi* to 30.0% in *D. v. virgifera*, but only traces of Cuc D were found, suggesting that deacetylation is not a major step in the formation of excretory products. Most of the ^{14}C was excreted in all five species of Diabroticina as three polar metabolites, R_f 0.0, 0.15, and 0.23 in TLC with chloroform–methanol 95:5. These polar metabolites comprised from 46.8% of the total excreted ^{14}C in *D. v. virgifera* to 91.0% in *D. balteata*. The major excretory metabolite in all five species, R_f 0.0, was a Cuc conjugate that comprised from 24.7% of the total excreted ^{14}C in *A. vittatum* to 43.8% in *D. cristata* (Ferguson et al., 1985). A small proportion of a Cuc-conjugate was permanently sequestered in the hemolymph, from 0.98 to 2.76% of the total ^{14}C. The remainder of the ^{14}C was retained, largely as conjugates, in the body and gut. In the five species examined, there were markedly different ratios of unmetabolized Cucs/polar metabolites, probably related to the present-day dietary habits of the beetles.

When *D. u. howardi* were fed ^{14}C Cucs B–D followed by two weeks on an artificial pollen diet, 93.9% of the total ^{14}C was excreted, but the hemolymph retained 1.12% of the total ^{14}C and 1058 eggs collected contained 0.21% (Ferguson et al., 1985). The characteristic polar metabolite of the hemolymph in all five species of beetle fed Cuc B had R_f 0.40 upon TLC in chloroform–methanol 1:1. This metabolite was further purified by HPLC (Ferguson et al., 1985), a fraction with retention time (RT) of 4.26 min eliciting strong beetle feeding on TLC plates (Figure 2). This Cuc conjugate was incubated overnight with pectinase to free the cucurbitacin which was extracted with chloroform. The cucurbitacin had an R_f 0.46 upon TLC with chloroform–methanol 95:5, and a RT on HPLC of 5.60 min, identical to that of Cuc D. Mass spectrometry by electron impact produced a fragmentation pattern identical to Cuc D (Audier and Das 1966). When beetles were fed *Cucurbita okeechobeensis* fruit containing Cuc E, or *C. texana* fruit containing Cuc E glycoside (Metcalf et al., 1982), hemolymph conjugates were formed that released Cuc I (R_f 0.52, blue-violet color with $FeCl_3$) after treatment with pectinase. Pure Cucs B and E, when incubated with pectinase, were not deacetylated to Cucs D and I as demonstrated by TLC. Thus, the deacetylation to produce the hemolymph conjugates was occurring in vivo in the beetles (Ferguson et al., 1985).

Consumption of Bitter Cucurbita and Diabroticina Longevity. Paired experiments were conducted where 50 newly emerged beetles were fed ad libitum on uniformly sized pieces of either bitter *C. andreana* × *C. maxima* F_1 hybrid

squash fruit containing 1–2 mg/g of Cuc B–D or on *Cucurbita* cultivars, *C. pepo* or *C. moschata*, devoid of Cucs (Ferguson et al., 1985). The total Cuc consumption was estimated over the lifetime of the beetles as 1–2 mg per beetle for *D. balteata*, 1.7–3.4 mg for *D. virgifera*, and 0.8–1.6 mg for *A. vittatum*. Beetle mortality was recorded daily, and the weekly mortality pattern and mean longevities for each species were determined (Ferguson et al., 1985). The lifespans of the adult beetles are remarkably long, up to 250 days. However, exclusive feeding on Cuc-containing fruit significantly decreased the mean longevity of *D. virgifera* from 126 days to 59 days ($P < 0.001$) and of *D. balteata* from 129 days to 70 days ($P < 0.001$). With *A. vittatum*, there was no significant difference in the longevity between the beetles fed on sweet fruits, mean 136 days, and those fed on bitter fruit, mean 122 days.

There were significant differences in the accumulated mortalities of male versus female beetles: the longevity of male *D. virgifera* was reduced 147% by feeding on bitter fruit as compared with only 39% for the female. With *A. vittatum*, feeding on bitter fruit decreased the male longevity by 27% ($P = 0.018$) but had no effect on the female longevity (Ferguson et al. 1985).

These effects on longevity can be interpreted in terms of the stress produced over the insects' lifetimes for energy requirements for metabolism, excretion, and tissue storage of the Cucs. These costs appear to be greatest in *D. virgifera*, whose normal life-style as a Poaceae feeder does not expose it to Cucs. The stress factor is less for *D. balteata* that commonly feeds on Cucurbitaceae and was barely perceptible for *A. vittatum* that is monophagous on Cucurbitaceae.

Incorporation of Cuc D into a pollen diet at 0.5 mg/g increased male food consumption substantially over that of females: *D. balteata* male 36%, female 3 %; *D. howardi* male 25%, female 1%; *D. virgifera* male 15%, female 9%; and *A. vittatum* male 24%, female 7% (Ferguson, 1985). This suggests that the male beetles in the longevity experiments may have consumed more Cucs, thus reducing their survival rate.

COEVOLUTIONARY IMPLICATIONS OF DIABROTICINA AND CUCURBITACEAE ASSOCIATION

The original coevolutionary strategy of the Cucurbitaceae to synthesize the intensely bitter and toxic Cucs as allomones to restrict herbivory has been very successful, as major herbivores are relatively few. This evolutionary strategy has produced three major variations in the structures of these Cuc allomones, (1) C_1-C_2 unsaturation, (2) acetylation at C_{23}, and (3) glucosylation.

A comparison of Cuc content of *Cucurbita* spp. with phyllogeny as constructed from genetic compatibilities and numerical taxonomy (Rhodes et al., 1968) suggests that the primitive form of Cucs was *B–D* and that Cucs *E–I* and the glycosides appeared as secondary modifications. These two modifications

each evolved at least twice independently in *Cucurbita*, i.e., $C_1 = C_2$ (E, I) in *C. martinezii* and *C. palmata* subgroups, and formation of glycosides in *C. texana*, *C. palmata*, and *C. cylindrata* (Metcalf et al., 1980, 1982).

All species of Diabroticina examined have specific Cuc receptors on the maxillary palpi that are structurally attuned to Cuc B. The presence of trace quantities of Cuc B elicits arrest and compulsive feeding in these Diabroticina species whether they are monophagous on Cucurbitaceae as *A. vittatum*, oligophagous on Cucurbitaceae as *D. balteata*, polyphagous as *D. u. howardi*, oligophagous on Poaceae as *D. l. barberi* and *D. v. virgifera*, or monophagous on *Andropogon* as *D. cristata*.

Stimulation of the Cuc receptors in Diabroticina beetles produces behavioral responses of arrest and compulsive feeding that are so fundamental as to override even the male response to the female sex pheromones, e.g., in *D. u. howardi* (Hummel and Andersen, 1982).

All species of Diabroticina examined have well-developed detoxification and storage mechanisms to partially metabolize, conjugate, and concentrate Cucs. Thus they appear to be immune to the general repellent and acutely toxic effects of Cucs. (Ferguson et al., 1985).

Major natural enemies of the Diabroticina appear to be few, and judged from the incredibly bitter taste of beetles fed on bitter *Cucurbita* and their repellent effect to a mantid predator (Ferguson et al., 1985), the coevolutionary strategy of the Diabroticina to conjugate Cucs and concentrate these bitter products in the hemolymph provides a successful survival mechanism.

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PHYTOCHEMICAL BASIS OF LEARNING IN *Rhagoletis pomonella* AND OTHER HERBIVOROUS INSECTS

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Abstract—Examples of phytochemically-based learning of host preference in herbivorous insects are reviewed in the context of traditionally important issues: the number and kinds of chemicals involved; which sensory modalities are affected; whether peripheral or central nervous processing is altered; and whether learning is associative or not. A fifth issue addressed here—whether experience enhances a feeding or ovipositing insect's propensity to accept familiar chemical stimuli or to reject novel chemical stimuli—has been ignored in previous studies. Following the review, evidence is presented indicating that female apple maggot flies (*Ragoletis pomonella*) learn to reject both novel physical and novel chemical stimuli.

Key Words—Learning, phytochemistry, host preference, herbivorous insects, *Rhagoletis pomonella*, Diptera, Tephritidae.

INTRODUCTION

The location and selection of host plants by herbivorous insects has been portrayed historically as a simple, invariant behavioral sequence (Thorsteinson, 1960). In particular, responses to the secondary and nutrient chemical constituents of plants have been viewed generally as consequences of innate releasing mechanisms (*sensu* Tinbergen, 1969), stereotyped and relatively resistant to environmental factors. The continuing accumulation of evidence for learning of host preference in herbivorous insects (Jermy et al., 1968; Dethier, 1980; Prokopy et al., 1982; Rausher, 1983; Papaj and Rausher, 1983; Stanton, 1984; Papaj, 1986a, b) and saprophagous insects (Jaenike, 1982, 1983, 1986), as well as recent theoretical discussions of learning as a factor in the evolution of host discrimination (Rausher, 1983; Papaj and Rausher, 1983; Stanton, 1984), de-

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mand a critical examination of the thesis that an insect's responses to phytochemicals are static and independent of prior feeding or egg-laying history. In this paper, we first review the evidence for learning involving plant chemicals and then introduce our own work on learning to chemical cues in fruit acceptance behavior by the apple maggot fly, *Rhagoletis pomonella.* We define "learning" as a reversible change in behavior with experience; we use "induction" synonymously with learning, but apply that term particularly to effects of diet on the feeding preference of lepidopterous larvae.

GENERAL REVIEW

Induction of Larval Feeding Preference. Yamamoto and Fraenkel (1960) and Jermy et al. (1968) can be credited with first showing convincingly that host preference depends on prior feeding history. Since then, feeding or oviposition preference has been altered by feeding or oviposition experience almost whenever the issue has been examined, predominantly in lepidopterous larvae (see reviews by Hanson, 1983; De Boer and Hanson, 1984), but also in adult Lepidoptera (Stanton 1984; Traynier 1984; Papaj 1986a–c), Diptera (Jaenike, 1982, 1983, 1985; Prokopy et al., 1982, 1986; Cooley et al., 1986) Orthoptera (Cassidy, 1978) and Coleoptera (Phillips, 1977; Rausher, 1983).

Most studies of so-called induction of feeding preference adopted the assay developed by Jermy et al. (1968) in which individuals reared on a particular host plant or artificial medium are presented simultaneously with an array of hosts, nonhosts, or artificial media. Preference was usually defined by the relative proportion of material of each type consumed after a certain time has elapsed or after a certain absolute amount of material is ingested. In the original work by Jermy et al. (1968), for example, larvae of the tobacco hornworm, *Manduca sexta,* were reared on tobacco (*Nicotianea* spp.), Jerusalem cherry (*Solanum* spp.), and tomato (*Lycopersicon* spp.); larvae were subsequently offered plant disks of all three species simultaneously. The assay was terminated when a larva had consumed approximately half of one disk. The results were unambiguous: larvae always consumed more leaf material of the plant species on which they were reared.

In other words, the order in which host species were preferred was altered by prior experience—in the sense of Papaj and Rausher (1983), a modification of the rank order of preference. In other cases the rank order of preference remained the same, but the degree to which different species were consumed still depended on feeding history—in the sense of Papaj and Rausher (1983), a modification of the specificity of preference. For example, when *Manduca* larvae were reared on certain nonhost species, those species were consumed more frequently than if the larvae were reared on host plants, but never more fre-

quently than the host plants themselves (De Boer and Hanson, 1984). In general, the outcome of induction, i.e., whether rank order or specificity is altered by experience, may depend upon the taxonomic relatedness of the test plant species (Wasserman, 1982; De Boer and Hanson, 1984).

The notion that host experience causes changes in an herbivorous insect's responses to host chemical stimuli was implicit in an important hypothesis about the processing of host-selection information advanced by Jermy et al. (1968). Presuming that induction of preference in lepidopterous larvae was mediated by changes in responses to phytochemicals, Jermy and colleagues argued that discrimination among plant species must involve, at least in part, assessment of qualitative differences in the profiles of phagostimulants and deterrents of different plant species. If quantitative differences only were important, e.g., if larvae discriminated among plants merely by measuring the level of a compound shared by those plants, then induction to one host should have enhanced the acceptability of all host species more or less equally. In fact, induction always resulted in increased consumption of the inducing plant relative to alternative plants. Qualitative differences among plants could reflect, for example, differences among plants in the kinds of compounds perceived by the insect or differences in the relative concentrations of more than one compound shared by those plants.

Evidence for Phytochemical Basis of Larval Induction. The assumption of a phytochemical basis of larval induction of feeding preference has been supported by a number of studies. In a study by Schoonhoven (1969), for example, the gustatory organs of *Manduca* larvae reared on tomato plants exhibited electrophysiological responses to saps from an array of host and nonhost species that differed from those of larvae reared on an artificial medium. Impulse rates from a gustatory organ, the medial maxillary sensilla styloconica, were generally higher for larvae fed on artificial diet than for those fed on tomato leaves. Similarly, the impulse rates generated by contact with a variety of pure chemicals (sodium chloride, salicin, solanin, and inositol) were greater after feeding on artificial diet lacking these compounds than after feeding on tomato.

The responses of larvae to single compounds can also be modified by rearing on media containing those compounds. Electrophysiological responses to the secondary compound, salicin, by *Manduca* larvae were altered by prior feeding on diets containing salicin (Schoonhoven, 1969). When salicin was incorporated into artificial medium or tomato leaves, the medial maxillary sensilla styloconica of larvae exposed to the salicin-laced diet fired less rapidly than the sensilla of larvae exposed to diet alone.

Induction of preference for diets containing particular compounds is not confined to secondary plant compounds only. The medial maxillary sensilla styloconica of *Manduca* larvae fed on artificial diet spiked with 0.01 M inositol (a weakly stimulatory sugar) fired at a lower rate upon contact with inositol than

did those of larvae fed on artificial diet alone (Schoonhoven, 1969). Similarly, Städler and Hanson (1978) induced successfully *Manduca* larvae to an artificial diet containing the nutrient linoleic acid. Larvae fed on a standard wheat germ diet and larvae fed on a similar diet lacking lineoleic acid and other nutrient compounds consumed proportionately more of their respective diets in choice tests. Städler and Hanson used choice tests featuring a variety of extracts of the diets as well as tests with pure lipid components to confirm that experience altered feeding responses to linoleic acid.

Components of Behavior Altered by Induction. Like most induction studies using natural foliage for testing, preference for a host chemical or host test extract has often been deduced from the relative amounts of chemically treated and untreated material consumed by larvae in choice assays. These so-called "cafeteria tests" facilitate a large number of replicates and experiments (e.g., Städler and Hanson, 1978; De Boer and Hanson, 1984), but compromise efforts to define which components of host-selection behavior are altered by prior experience. Saxena and Schoonhoven (1978, 1982), in sharp contrast, used behavioral assays in which individual insects were observed directly to elucidate the behavioral mechanisms underlying induction of larval preference.

Saxena and Schoonhoven (1978) reared a group of *Manduca* larvae on a wheat germ diet containing the terpene, citral, and another group on a plain wheat germ diet. Results of a tightrope choice test in which larvae walked along a wire or Plexiglas edge between two experimental disks revealed that citral-exposed larvae turned more frequently towards a citral-medium disk than a plain-medium disk. Moreover, induction to diet was symmetrical. Larvae reared on plain medium turned more frequently towards a plain-medium disk than a citral-medium disk. Larvae reared on citral medium consumed more citral medium than plain medium in both choice and no-choice presentations; larvae fed plain medium consumed more plain medium in both choice and no-choice presentations. Although differences in consumption between citral-experienced and citral-naive larvae presumably represent some unknown composite of differences in orientation, biting and feeding responses, and/or arrestment, Saxena and Schoonhoven contended that both orientational and contact feeding responses were altered by prior experience with citral medium or plain medium.

Saxena and Schoonhoven (1982) extended these assays using *Manduca* larvae reared and tested on natural foliage. As commonly found in such studies, prior experience with a plant species tended to increase the extent to which that species was consumed relative to other plants. In addition, larvae tended to turn more frequently towards the plant on which they were fed, in both choice and no-choice tests. Interestingly, the rank order of orientational preferences of larvae reared on different diets did not always correspond to the rank order of feeding preferences. This result suggests that some other behavior besides orientation is also altered by prior feeding history. Furthermore, the alteration in orientational preference was apparently olfactorily-based, at least in part. Lar-

vae reared on tomato, for example, chose tomato leaves over blank controls in the turning assay much more often than did larvae reared on an artificial diet. This effect of experience on orientation behavior disappeared entirely when test tomato leaves were presented behind a glass barrier.

Sensory Modalities and Multicomponent Chemical Basis of Induction. The contention that both orientation and contact behavior are affected by prior feeding history suggests that more than one sensory modality is involved in induction of larval preference. More direct and detailed information about the sensory modalities affected by induction was supplied by the ablation experiments of Hanson and Dethier (1973). After raising *Manduca* larvae on either tomato or Jerusalem cherry, they extirpated systematically the olfactory and gustatory organs of fourth- and fifth-instar larvae. Using standard leaf-disk choice tests, the relative consumption of tomato and Jerusalem cherry was measured for normal and operated larvae. Loss of either the gustatory organ (maxillary sensilla styloconica) or both olfactory organs (antennae and maxillary palps) reduced markedly the strength of induction, relative to unoperated larvae. A lesser but significant degradation of induction followed the removal of the antennae alone or the palps alone. The residual effect of prior experience on relative consumption of tomato and Jerusalem cherry after ablation of either the olfactory or gustatory systems suggested strongly that both systems were involved independently in induction of host preference.

This systemic redundancy in induction of preference was paralleled in Hanson and Dethier's studies by a redundancy in the number of discriminatory organs within each system: both discrimination and induction of discrimination persisted after the unilateral ablation of the maxillary sensilla styloconica, antennae, and palps. The unilateral extirpation procedure not only indicated that more organs of each type were present than were required to discriminate or to be induced to discriminate among food plants, but confirmed that the effects of the bilateral gustatory and olfactory ablations on induction were not merely the result of surgical trauma.

The activity of both olfactory and gustatory systems in induction of preference in lepidopterous larvae implies that responses to more than one class of chemical compound are modified by prior feeding history. This inference was supported by Städler and Hanson (1976, 1978), who recorded the behavioral and electrophysiological responses of larvae reared on tomato and Jerusalem cherry to host extracts prepared with a variety of solvents. Using a modification of the standard cafeteria test, larval preference was measured as the proportion of glass-fiber disks impregnated with a particular solvent extract that was consumed by a larva. While larvae discriminated among a number of extracts, only the nonpolar hexane and polar water extract carried the inducing activity. Thus, experience with a host plant apparently altered responses to more than one but less than all of the phytochemicals used in discrimination. The fact that responses to only a subset of the phytochemicals used to select hosts were appar-

ently altered by prior experience may help to explain the common observation (De Boer and Hanson, 1984) that the extent to which preference among particular plant species is induced in late-instar larvae is not necessarily coincident with the extent to which early-instar larvae discriminate among those species.

Adult as well as immature insects may be conditioned to more than one chemical in plant or plant-derived resources. A multicomponent chemical basis of learning of adult oviposition preference in *Drosophila* spp. was postulated by Jaenike (1983). After exposing larval and adult *D. melanogaster, D. pseudoobscura,* and *D. immigrans* to artificial media containing one of various substances (sodium chloride, ethanol, ethyl acetate, lactic acid, piperidine, and peppermint oil), Jaenike (1982) presented adults simultaneously with media with and without these substances. Rearing larvae on these media had no effect on the number of eggs laid by adults in various substrates, adding to the tremendous body of evidence that adult preference is rarely susceptible to larval conditioning (Papaj and Rausher, 1983). Exposure of adult flies to peppermint-containing food, however, significantly reduced the aversion of adults of all three species towards laying eggs in this medium. While this result may be a bonafide example of habituation to a deterrent substance (Manning, 1967; McGuire, 1984), Jaenike (1983) nevertheless questioned the extent to which learning of acceptance of individual deterrent compounds (particularly ones that were not present in natural food resources) reflected the kind of learning possible in natural breeding substrates.

Jaenike (1983) therefore exposed larval and adult *Drosophila melanogaster* to more natural breeding substrates derived from apple, tomato, squash, and banana. As before, adult preference did not depend on the type of food on which larvae developed. In two-choice tests, however, individuals laid a greater proportion of eggs in the particular substrate on which they fed as adults than did individuals that fed on the other substrate. In a second experiment, adult flies were exposed to either apple, tomato, orange, grape, or onion food and tested simultaneously on all five media. Adult exposure to a certain medium sometimes, but not always, induced a preference for ovipositing on that medium. Adults that fed on grape or tomato, for example, laid proportionately more eggs in grape or tomato, respectively, than did adults in the other treatments. Feeding on apple, orange and onion, however, did not increase the proportion of eggs laid on those substrates, relative to feeding on alternative food sources. In addition, exposure to one food source sometimes increased the relative acceptability of other foods. Flies kept on apple, for instance, laid proportionately more eggs on grape than flies kept on tomato, orange, or onion. This so-called "cross-induction" phenomenon was not reciprocal: flies maintained on grape medium did not show an enhanced propensity to accept apple medium.

A mechanism proposed by Jaenike (1983) to explain cross-induction assumed that prior experience with a given food resource influences responses to an array of repellent and attractive compounds. While details of this scheme

were entirely speculative, cross-induction itself is probably genuine. Enhancement of relative acceptibility of one host species upon experience with another is evident in a great number of studies on induction of preference (even though it was not usually acknowledged; e.g., Jermy et al., 1968; Yamamoto, 1974; Saxena and Schoonhoven, 1982; De Boer and Hanson, 1984). Indeed, its generality may be very much understated simply because induction experiments typically assay discrimination between only two plant species, conditions under which cross-induction cannot be deduced.

Effects of Experience on Peripheral and Central Processing. Presuming a multicomponent chemical basis of induction of lepidopterous larvae, Schoonhoven (1977) proposed alternative schemes in which experience with a food resource was accompanied by changes in either the central or the peripheral nervous system. Both models embrace the notion that discrimination is the outcome of comparison of the across-fiber firing pattern of sensory receptors with a centrally located template. The first model supposes that prior experience affects feeding behavior by changing the internal template of the phytochemical spectrum against which the across-fiber firing pattern of the sensory receptors is matched. The second model supposes that prior experience alters the across-fiber firing pattern of the sensory receptors itself.

Both Schoonhoven (1969) and Städler and Hanson (1976) reported evidence that changes in the sensitivity of the gustatory maxillary sensilla styloconica to particular chemicals accompany induction of host preference. While these studies clearly corroborate a hypothesis of peripheral control of induction, they do not exclude the possibility of correlative changes in the central nervous system.

A central nervous system component in conditioning of feeding behavior was emphasized in Szentesi and Bernays' (1984) study of the physiological basis of habituation to nicotine hydrogen tartrate (NHT) by grasshopper nymphs (*Schistocerca gregaria*). Habituation, defined as the waning of response to a stimulus with repeated exposure to that stimulus, is widely presumed to involve central nervous processes. While a number of investigators have defined effects of host experience on feeding (e.g. Schoonhoven, 1969; Gill, 1972, cited in Szentesi and Bernays, 1984) or oviposition (Jaenike, 1982, 1983) preference that could potentially be attributed to habituation (McGuire, 1984), few studies controlled for effects of resource deprivation on preference. In most procedures, food with and without deterrent was offered to groups of insects ad libitum for a specified period of time, after which responses to the stimulus were measured. No attempt was made to control for the effect of differences in consumption rate of the alternative diets on subsequent preference. Thus, a difference in preference among treatments could be due to the specific effect of repeated exposure to the deterrent or to the general effect of resource deprivation or to both. By ensuring that all locusts consumed food at the same average rate, regardless of the presence or absence of a deterrent in their diet, Jermy et al.

(1982) and Szentesi and Bernays (1984) verified that a depression of deterrency was due to repeated exposure to the deterrent and not to food deprivation.

Periods of sensory stimulation even without ingestion of the deterrent substance was sufficient to cause habituation to NHT by locust nymphs. Grasshoppers whose maxillary palp sensilla had been encapsulated with nicotine hydrogen tartrate (NHT) solutions ate more NHT-treated diet than control grasshoppers whose palps were encapsulated with water, even though both groups were reared on an NHT-free diet of fresh wheat (Szentesi and Bernays, 1984). An additional result demonstrated that habituation via sensory stimulation was associated with changes in the central nervous system. Before testing, the maxillary palps of groups of NHT-exposed and NHT-naive individuals were covered with a nylon sleeve and rendered inaccessible to sensory stimulation. Despite concealment of the palps, individuals whose palps were previously immersed in NHT solutions ate more NHT-impregnated food in the test period than did individuals whose palps were previously immersed in water.

Chemical Cues Involved in Associative Learning by Herbivorous Insects. Associative learning, defined as the association of a neutral stimulus with a nonneutral stimulus through the temporal pairing of those stimuli, is usually considered to entail changes in the central nervous system. Two instances in which a nonneutral host-selection chemical cue is associated with a neutral visual cue are known, both in adult papilionoid Lepidoptera.

Searching behavior of ovipositing cabbage butterflies, *Pieris rapae*, was altered by prior experience with particular varieties of their cabbage hosts (Traynier, 1984). Exposure of adult females to either Earlyball or Wongbok cabbage varieties for as short as 30 min predisposed individuals to land more frequently on those types in the first 5 min of a two-choice test. Efforts to reverse the effects of training in this experiment were unsuccessful, suggesting that short training periods induced relatively long-term changes in oviposition preference.

Taking advantage of the fact that paper disks wetted with sinigrin (a common component of cabbage and other crucifers) and water solutions elicited oviposition, Traynier (1984) next attempted to train female butterflies to disks of two shades of green, Shoalhaven Green and Great Green. Although females tended to land first on Shoalhaven Green disks regardless of prior experience, this preference in two-choice tests was greater for females preexposed to sinigrin-treated Shoalhaven Green disks than for females preexposed to sinigrin-treated Great Green disks. A single contact with a sinigrin-treated Shoalhaven Green disk—even without oviposition—altered significantly a butterfly's choice of colored disks. Thus, the visual cue (color or monochromatic hue) was apparently associated with the perception of sinigrin solution directly and not indirectly via the events corresponding to egg-laying.

Association of a visual cue with chemical releasers of oviposition behavior also occurs in pipevine swallowtail butterflies (Papaj, 1986a, b). In a large

outdoor screened enclosure, adult female *Battus philenor* butterflies were trained to search selectively in an array of host and nonhost plants for leaves of a shape (defined by maximum leaf length–maximum width ratio) similar to that of the *Aristolochia* host species to which they were exposed. Unlike cabbage butterflies, the effects of learning were reversed readily upon exposure to an alternative host species with a significantly different leaf shape. As in cabbage butterflies, contact with a host plant without oviposition was sufficient to induce a change in alighting responses.

Methanolic extracts of *Aristolochia macrophylla* applied to artificial substrates or to nonhost plants elicited oviposition by gravid females. When placed on nonhost plants sprayed with these extracts, butterflies alighted predominantly on leaves of a shape similar to that of the treated nonhost species. Contact with treated nonhost plants even without oviposition was sufficient to increase significantly a female's propensity to alight on leaves of a shape similar to that of the treated nonhost plant.

Many studies of associative learning (e.g., Traynier, 1984) neglect to control for the nonassociative effects of sensitization and pseudoconditioning. Sensitization is defined as a change in response coincident with repeated presentations of a neutral, conditioned stimulus whether or not that stimulus is paired with a nonneutral, unconditioned stimulus (McGuire, 1984; Tully, 1984). Cabbage butterflies, for example, may have come to prefer to land on green disks even if green disks without sinigrin treatment had been presented repeatedly. The fact that experienced *Pieris* butterflies often laid eggs on untreated disks in subsequent tests may be evidence of this nonassociative process.

The searching behavior of pipevine swallowtail butterflies, by contrast, provided an internal control for sensitization. *Battus* butterflies searching for a host plant on which to deposit eggs land periodically on leaves. Upon alighting, they drum their tarsi and presumably stimulate tarsal chemoreceptors (Feeny et al., 1983). If the leaf belongs to a nonhost species, the female immediately resumes search. If the leaf belongs to a host, the female often lays a small egg cluster and resumes search. The great majority of leaves on which a female alights are nonhost plants and, in fact, the standard assay for leaf-shape response (Rausher, 1978) records only the kinds of nonhost leaves on which an individual lands.

Whereas a single contact with a treated nonhost plant induced a search mode for leaves of a shape similar to that of that plant, repeated contacts with untreated nonhost plants in the enclosure array did not. Moreover, both in the enclosure (Papaj, 1986a) and in the field (Papaj, 1986b), a single contact with a host plant whose leaf shape differed from that for which an individual was searching originally often induced a new search mode for the leaf shape of the newly discovered host plant. Such abrupt switches in nonhost alighting behavior never occurred after "mistaken" contacts with a nonhost plant with the "wrong" leaf shape. The most parsimonious explanation for these host-re-

leased switches is that female butterflies associated the neutral shape stimuli of the host leaves on which they landed with positive host phytochemical stimuli.

Pseudoconditioning is defined as a change in response coincident with repeated presentations of the nonneutral, unconditioned stimulus whether or not that stimulus is paired with the neutral, conditioned stimulus (McGuire, 1984; Tully, 1984). Pseudoconditioning of leaf-shape response could have been possible if repeated contact with host extract (i.e., the unconditioned stimulus) resulted in search modes for particular leaf-shapes. Specifically, repeated contact with extracts prepared from the broad-leaved host species, *A. macrophylla*, should have induced preferences for broad leaves. In fact, search modes depended only on the leaf shape of the nonhost species (i.e., the conditioned stimulus) to which the extract was applied.

LEARNING IN APPLE MAGGOT FLY

Repeated oviposition in a particular fruit species alters the degree to which female apple maggot flies, *Rhagoletis pomonella* (Walsh), attempt to oviposit into various fruit species in future encounters (Prokopy et al., 1982, 1986). Females ovipositing successively in hawthorn fruits, for example, accepted subsequent hawthorn fruits more frequently than did females ovipositing successively in apples. The training effect was reciprocal: females exposed to apples accepted apples more frequently than females exposed to hawthorn fruits. The effect of experience on responses to alternative fruit species could be reversed by repeated exposure to a different fruit species. Although the effect of experience on apple maggot fly oviposition-site selection was previously described as associative learning (Prokopy et al., 1982), this assignation was premature. In fact, almost nothing was known at the time about the stimuli involved in learning. Hence, it was not possible to show that one cue was associated with another through prior experience.

Prokopy et al. (1986) established an alternative dimension along which to classify effects of experience on host preference. They pointed out that experience with a host type could alter the difference in acceptance of two host types in either of two ways. First, prior experience with a particular host type might act to enhance the likelihood of acceptance of that host type in subsequent encounters. Alternatively, experience with one host type might depress the tendency to accept alternative host types in subsequent encounters. Although previous studies of learning of preference failed to distinguish between "learning to accept" and "learning to refuse" a host type, most investigators have assumed that learning enhances the propensity to accept the familiar resource type. In fact, Prokopy et al. (1986) and Cooley et al. (1986) discovered that

both the apple maggot fly and another tephritid fly, the Mediterranean fruit fly (*Ceratitis capitata* Weidemann), learn to reject the novel fruit type.

This conclusion was reached by comparing the fruit acceptance of flies exposed to a single fruit species with that of flies that had never been exposed to fruit (i.e., naive flies) or with that of flies exposed alternately to more than one fruit species (i.e., untrained flies). A female apple maggot fly ovipositing successively into hawthorn fruit, for example, had no greater propensity to accept a test hawthorn fruit than did a female ovipositing alternately into hawthorn and apple. Instead, the female trained on hawthorn was simply less prone than the untrained female to accept an apple. Similarly, females exposed to apples were less prone than the naive or untrained females to accept a test hawthorn fruit. In short, females exposed to a particular fruit species were always more likely to reject the alternative fruit species than were naive or untrained females.

Whether learning to reject the novel fruit type is mediated by responses to chemical stimuli was not known previously. In the following study, we investigated two cues—fruit surface chemistry and fruit size—that could be involved in learning of fruit acceptance by ovipositing female *R. pomonella.* These stimuli were chosen because both are known to be important in discrimination of oviposition sites by naive females (Diehl and Prokopy, 1986). We asked the following questions: (1) Do females exposed to particular fruit species discriminate among models differing in size or in chemistry? (2) If females learn based on fruit size or chemistry, do they learn to accept familiar fruit stimuli or to reject the novel fruit stimuli? (3) Is an association between fruit size and chemistry formed through prior experience with host fruit?

METHODS AND MATERIALS

Adult female *R. pomonella* flies originated from puparia collected from infested apple fruit on Orchard Hill at the University of Massachusetts at Amherst. Adult flies were kept at 25°C, 16:8 light–dark photoperiod, and about 60% relative humidity. On day 1, 25 females and five males were placed in each of three 30 × 30 × 30-cm Plexiglas and aluminum screen cages. On day 7, two Red Delicious apples (ca. 60 mm diam) were hung with wire from the ceiling of cage 1. On the same day, 10 *Crataegus mollis* fruits (ca. 15 mm diam) were hung with wire from the ceiling of cage 2. Cage 3 received no fruit. Every two days, fruits were replaced with fresh fruit. This training regime was replicated three times. The number of eggs deposited in a single sample of used apples and hawthorn fruits was estimated by counting the number of oviposition punctures into the fruit surface.

On day 14, we began testing the propensity of experienced and naive flies to attempt to oviposit in a variety of fruit models. The models were prepared

by coating plastic and clay spheres of apple (65 mm in diameter) and hawthorn (15 mm in diameter) sizes, respectively, with ceresin wax dyed red. Ceresin wax in the form of hollow hemispheres is known to elicit relatively high rates of oviposition by apple maggot flies (Prokopy and Bush, 1973).

Models of each size were treated with surface fruit chemicals of either apple or hawthorn fruits, using the following procedure. Parafilm (American Can Co.) was stretched and wrapped around individual apples or groups of between six and eight hawthorn fruits. The parafilm was left on the fruits for between three and seven days at room temperature. The parafilm wrap was then removed and applied to wax models of each size category. The surface originally in contact with the surface of the fruit was always placed in contact with the wax surface of the model. The parafilm wrap was removed from the models after 2 hr, and the models were used immediately in testing. Each day, freshly treated models were used in testing. A preliminary study (Papaj, unpublished) revealed that ceresin wax domes pretreated with parafilm wrapped in host fruit received significantly more oviposition punctures and eggs than domes pretreated with blank parafilm.

Assay flies were selected by introducing, in alternating fashion, a large or small red ceresin wax sphere into each treatment cage. When a female flew or walked onto the model, she was placed in an empty test cage and allowed to rest for 5 min. Virtually all flies were eventually tested using these criteria. The fly was then allowed to walk onto the first model. An acceptance of the model was recorded if the female attempted to oviposit (i.e., bore into) in the model. A rejection was recorded if the female flew or walked from the model or if the female remained on the fruit 5 min without boring. Following acceptance (which was interrupted before egg deposition could occur) or rejection, the fly was permitted to rest 5 min before presentation of the next model.

The order of presentation of the models was randomized, changing each time a series of flies from each treatment was tested. Approximately 85 flies from each training regime were tested on each of the following four models: (1) apple size treated with apple-exposed parafilm, (2) apple size treated with hawthorn-exposed parafilm, (3) hawthorn size treated with apple-exposed parafilm, and (4) hawthorn size treated with hawthorn-exposed parafilm.

About 25 flies were tested on two additional models: one apple-sized and one hawthorn-sized model, each exposed to blank parafilm only.

RESULTS

Although flies in each fruit-exposure group were given equal opportunity to lay eggs in fresh fruit, apple-exposed flies laid many fewer eggs than hawthorn-exposed flies. In one sample three-day period, flies laid 234 eggs into one

set of 10 hawthorn fruit. Flies laid only 34 eggs into two apples in the same period (this difference is highly significant; $G = 167.35$, $P < 0.0001$). Casual observations suggested that flies had difficulty penetrating the apple skin with their ovipositor.

For all groups of females tested on models treated with blank parafilm, large, apple-sized models were accepted less frequently than small, hawthorn-sized models; this difference was significant for hawthorn-exposed and naive flies (Table 1). In addition, females exposed previously to hawthorn fruit exhibited an even greater difference in their propensity to bore into large vs. small models (0.12 vs. 0.36, respectively) than did naive females (0.27 vs. 0.54, respectively), but this trend was not significant. Previous experience with apple, by comparison, had no detectable effect on a female's propensity to attempt to oviposit in apple- vs. hawthorn-sized models.

Likewise, there was no effect of apple exposure on acceptance of models pretreated with parafilm wrapped previously around fruit. Females exposed to apples attempted to oviposit into apple- and hawthorn-treated spheres of either size as frequently as naive females (Table 2). Both apple-exposed and naive flies attempted to oviposit less frequently in large models compared to small models. Neither group of flies, however, exhibited a difference in propensity to bore into an apple-treated model or a hawthorn-treated model of a given size.

Females exposed to hawthorn fruits, by contrast, differed markedly from naive females in their acceptance of particular models pretreated with parafilm wrapped previously around fruit (Table 2). First, although each group attempted to bore more often into small models than large models of a given parafilm treatment, hawthorn-exposed females attempted to oviposit proportionately less frequently into apple-sized models of a given parafilm treatment than did naive females. Second, the training reponse to fruit model size was paralleled by a

TABLE 1. PROPORTION OF ACCEPTANCES BY FEMALES EXPOSED TO APPLE OR HAWTHORN FRUITS AND BY NAIVE FEMALES ON MODELS DIFFERING IN SIZE AND PRETREATED WITH BLANK PARAFILM

		Model type	
Exposure	N	Apple-sized[a]	Hawthorn-sized[a]
Apple	26	0.19 a1	0.38 a1
Hawthorn	25	0.12 a1	0.36 a2
Naive	26	0.27 a1	0.54 a2

[a] Proportions within a column that are followed by the same letter are not significantly different by a two-way G-test at $P = 0.05$. Proportions within a row that are followed by the same number are not significantly different by a two-way G-test at $P = 0.05$.

TABLE 2. PROPORTION OF ACCEPTANCES BY FEMALES EXPOSED TO APPLE OR HAWTHORN FRUITS AND BY NAIVE FEMALES ON MODELS DIFFERING IN SIZE AND PARAFILM PRETREATMENT

Exposure	*N*	Apple-sized[a]	Hawthorn-sized[a]
A. Apple-treated model			
Apple	81	0.35 a1	0.63 a2
Hawthorn	84	0.14 b1	0.48 b2
Naive	86	0.43 a1	0.72 a2
B. Hawthorn-treated model			
Apple	81	0.39 a1	0.62 a2
Hawthorn	84	0.28 b1	0.69 a2
Naive	86	0.49 a1	0.69 a2

[a] Proportions within a column that are followed by the same letter are not significantly different by a two-way *G*-test at $P = 0.05$. Proportions within a row that are followed by the same number are not significantly different by a two-way *G*-test at $P = 0.05$.

training response to fruit model parafilm treatment. Naive females exhibited no difference in acceptance of hawthorn-treated and apple-treated models within a size category. Hawthorn-exposed females, by contrast, attempted to bore considerably less often into an apple-treated model than into a hawthorn-treated model of a given size category.

In summary, responses to both size and surface chemistry were altered by prior experience with hawthorn fruits. The hawthorn-exposed flies accepted least often an apple-treated and apple-sized model and most often a hawthorn-treated and hawthorn-sized model. Training was associated with the rejection of novel fruit stimuli: the propensity of hawthorn-exposed females to attempt to oviposit into the hawthorn-treated and hawthorn-sized models was no greater than that of naive females. Rather, the propensity of hawthorn-exposed females to attempt to oviposit into apple-sized and/or apple-treated models was consistently less than that of naive females.

Finally, a log-linear chi-square model (Bishop et al., 1975) was used to assess the interaction of learned responses to model size and chemistry. The analysis tested the independence of the following factors: response (accept vs. reject), exposure (hawthorn-experienced vs. naive), size (apple-sized vs. hawthorn-sized), and parafilm treatment (apple-treated vs. hawthorn-treated). The significant response × exposure × size effect ($G = 4.32$, $P < 0.05$) confirms statistically that flies exposed to hawthorn fruits differed from naive flies in propensity to bore into large and small fruits. The significant response × exposure × parafilm treatment effect ($G = 6.39$, $P < 0.01$) confirms that flies

exposed to hawthorn fruits differed from naive flies in propensity to bore into apple-treated and hawthorn-treated fruit. Most importantly, the lack of a four-way interaction (response × exposure × size × parafilm treatment effect, $G = 0.44$, $P > 0.05$) indicates that the effect of training on size discrimination did not depend on the chemical features of the fruit model. Conversely, the effect of training on discrimination of fruit chemistry did not depend on the size of the fruit model.

DISCUSSION

A fundamental dichotomy in learned responses to novel vs. familiar chemical stimuli has been ignored previously. In many investigations of induction of feeding preference, the proper controls by which to identify this kind of conditioning were not executed. "Naive" insects were typically first-instar larvae that had not yet fed on plants or older larvae that had fed on a "neutral" artificial medium. When naive insects are much younger than experienced insects, however, level of experience is necessarily confounded with developmental stage. Moreover, insects feeding on artificial diets are not "naive" because such diets are not "neutral": lepidopterous larvae can be induced to prefer particular artificial diets over others and even over natural foliage (Städler and Hanson, 1978; Saxena and Schoonhoven, 1982). Using "untrained" individuals that have been exposed to a mixture of host species or a mixture of artificial media is a viable, but rarely considered, alternative. Of course, feeding insects can be "untrained" only to the extent that they will consume alternative foods.

An additional methodological complication has afflicted most studies of induction of larval feeding preference. Although cafeteria tests have assayed adequately the relative consumption of alternative foods, the *absolute* consumption rate of alternative food items across control and experimental groups has rarely been measured. Without measuring absolute consumption rates, however, it is almost impossible to deduce whether experience with a particular host species causes an absolute increase in the tendency to consume that host or an absolute decrease in the tendency to consume novel hosts, or both.

Despite these difficulties, many investigators have assumed that prior host experience enhances the acceptability of familiar food. Schoonhoven (1969), for example, presumed that induction in *Manduca* larvae was associated with decreased deterrence of the familiar diet. Similarly, Jaenike (1982, 1983) and McGuire (1984) contended that exposing adult *Drosophila* flies to peppermint-oil medium reduced the flies' aversion to peppermint oil. Städler and Hanson (1978) stated that induction of *Manduca* larvae to particular artificial diets both increased consumption of the rearing media and decreased consumption of alternative media.

Our present results indicate that alteration of a female apple maggot fly's responses to both physical (i.e., model size) and chemical (i.e., parafilm treatment) cues took the form of learning to reject the novel model type, paralleling the results of previous experiments (Prokopy et al., 1986; Cooley et al., 1986) with tephritid flies tested on natural fruit.

Interestingly, the propensity of hawthorn-exposed flies to bore into the hawthorn-treated, hawthorn-sized model was identical to that of naive flies. This result could be interpreted in one of two ways. First, fruit size and the fruit chemical constituents extracted and transferred with the parafilm treatment may be the only characters which females learned. Hawthorn-experienced flies might have accepted the hawthorn-sized, hawthorn-treated model as frequently as did naive flies because all of the relevant stimuli with which they were familiar were present. If so, experienced flies may be attending either to the absence of familiar stimuli or to the presence of novel stimuli when they reject novel fruit models. Such a fortuitous result seems unlikely. Fruit size and parafilm-extracted chemicals probably represent only a subset of the stimuli.

Alternatively, experienced flies may not be attending to the absence of familiar stimuli at all when they reject novel fruit or fruit models. Rather, they may reject novel fruit or novel fruit models because of the presence of novel stimuli. Thus, even though the hawthorn-sized, hawthorn-treated model does not bear all of the stimuli learned by flies, it would still be accepted by the experienced flies as vigorously as by the naive flies because of the absence of any novel stimuli.

In this study, apple maggot flies clearly learned to reject models with novel chemical stimuli, but only when exposed to hawthorn fruit. Exposure to apples failed to alter the females' fruit acceptance pattern, relative to that of naive females. In fact, apples always receive many fewer oviposition attempts than do hawthorn fruit in laboratory assays (Prokopy et al., 1985) and have consistently yielded a weaker training effct than hawthorn with real test fruit in previous experiments (Prokopy et al., 1986). Nevertheless, we do not yet know if females trained successfully to apple learn to accept familiar chemical stimuli or to reject novel chemical stimuli.

We also do not know if learning in apple maggot flies constitutes associative learning. The lack of an interaction in training to models of different sizes and parafilm treatments implies that size and chemistry cues were not associated during learning. If size and chemistry became associated through experience, the hawthorn-exposed flies should have been proportionately less inclined to bore into an apple-sized, apple-treated model than was expected based on their responses to the models with mixed size and chemistry characteristics (i.e., the apple-sized, hawthorn-treated and hawthorn-sized, apple-treated models). This negative result does not, however, exclude an association between size and/or chemistry and some other unknown stimuli.

By using fruit models to train as well as to test female apple maggot flies, we hope to address these questions. We hope also to shed light on the principal issues outlined in our review: whether learning based on host chemistry involves one or more than one chemical constituent; which sensory modalities are involved in learning of preference; and whether changes in the central or peripheral nervous system accompany experience with particular host species.

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BIOCHEMICAL INSIGHT INTO INSECTICIDAL PROPERTIES OF L-CANAVANINE, A HIGHER PLANT PROTECTIVE ALLELOCHEMICAL

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Abstract—L-Canavanine manifests potent insecticidal properties in a canavanine-sensitive insect such as the tobacco hornworm, *Manduca sexta* (L.) (Sphingidae). Investigations of the biochemical basis for the antimetabolic properties of this arginine analog reveal that it is activated and aminoacylated by arginyl tRNA synthetase and incorporated into the nascent polypeptide chain. This creates structurally aberrant, canavanine-containing proteins that can possess altered physicochemical properties. Evidence is presented in studies with the tobacco hornworm; the canavanine-adapted bruchid beetle, *Caryedes brasiliensis* (Bruchidae) and the weevil, *Sternechus tuberculatus* (Curculionidae); as well as the canavanine-resistant larvae of *Heliothis virescens* [Noctuidae] to support the contention that formation of aberrant, canavanyl proteins produce deleterious biological effects and is a significant basis for canavanine's antimetabolic properties.

Key Words—L-Canavanine, *Manduca sexta*, Lepidoptera, Sphingidae, *Caryedes brasiliensis*, *Dioclea megacarpa*, Leguminosae, *Sternechus tuberculatus*, Coleoptera, Bruchidae, Curculionidae, *Heliothis virescens*, Noctuidae, allelochemicals, toxicology.

INTRODUCTION

L-Canavanine, L-2-amino-4-(guanidinooxy)butyric acid, is a potentially toxic allelochemical synthesized by hundreds of leguminous plants (Bell et al., 1978). This arginine analog (Figure 1) elicits potent antimetabolic properties in developing larvae of canavanine-sensitive insects such as the tobacco hornworm, *Manduca sexta* (L.) (Sphingidae). Of the various mechanisms whereby canavanine manifests its toxicity in insects, one of the most important is through

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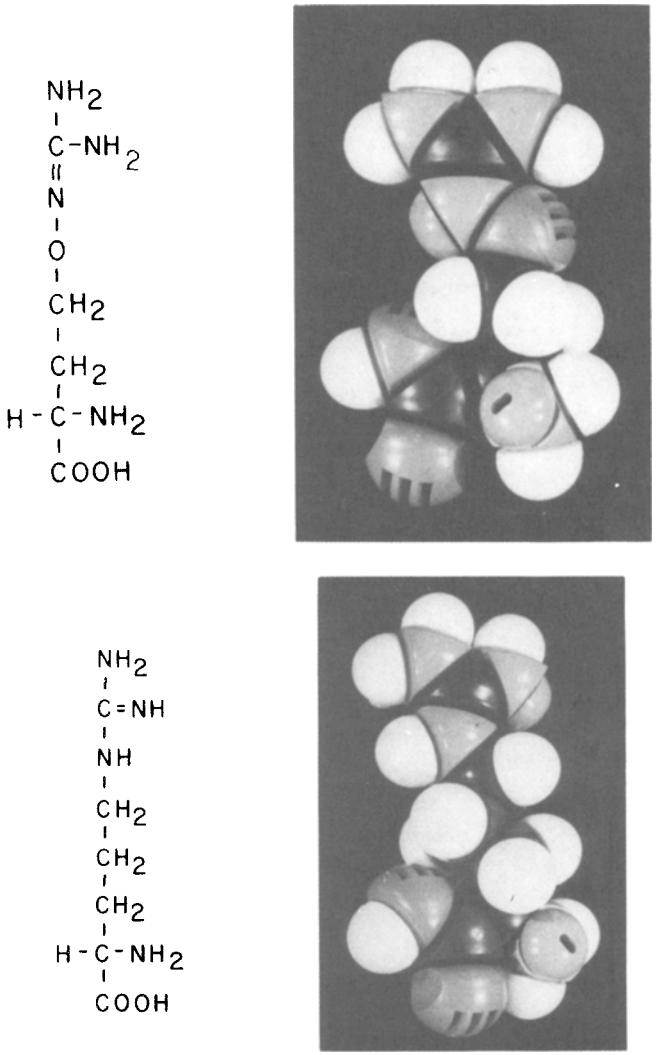

FIG. 1. CPK space-filling model of L-canavanine bottom and L-arginine top.

the formation of canavanine-containing, aberrant proteins. Due to the marked structural analogy between L-canavanine and L-arginine, arginyl-tRNA synthetase activates and aminoacylates L-canavanine, eventually linking it to the cognate tRNA for arginine (Allende and Allende, 1964). These reactions obligate the erroneous incorporation of canavanine into the nascent polypeptide chain.

CANAVANINE AND ABERRANT PROTEIN PRODUCTION

Administration of 1 g canavanine per kilogram fresh body weight (the LD_{50} larval dose) via parenteral injection to newly ecdysed fifth-instar larvae causes canavanine incorporation into the de novo-synthesized proteins of various larval tissues. Canavanine incorporation into hemolymph proteins is ascertained by precipitating the hemolymph proteins obtained from L-[*guanidinooxy*-^{14}C]canavanine-treated larvae with trichloroacetic acid (TCA). After exhaustively washing the TCA-precipitated materials, the hemolymph proteins are hydrolyzed with 6 N HCl and the hydrolysate canavanine separated from arginine by ion-exchange chromatography (Rosenthal and Thomas, 1985). The canavanine-containing fraction is treated simultaneously with arginase (EC 3.5.3.1) and urease (EC 3.5.1.5) to convert L-[*guanidinooxy*-^{14}C]canavanine to [^{14}C]urea and L-canaline prior to conversion to NH_3 and $^{14}CO_2$. The latter compound is trapped in hydroxide of hyamine and quantitated by liquid scintillation spectroscopy (Rosenthal and Janzen, 1983a).

About 0.4% of the parenterally injected L-[*guanidinooxy*-^{14}C]canavanine is recovered in the hemolymph proteins of *M. sexta* after 24 hr (Figure 2). Thus, canavanine-sensitive insects such as *M. sexta* can incorporate significant amounts of canavanine into newly synthesized proteins. Evidence for replacement of a residue other than arginine by canavanine has not been found.

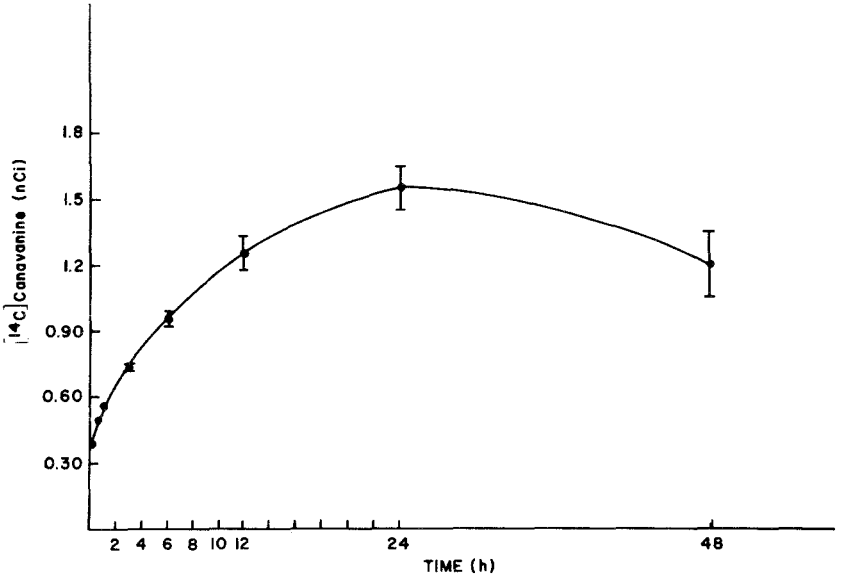

FIG. 2. Incorporation of L-[*guanidinooxy*-^{14}C]canavanine into de novo-synthesized hemolymph proteins of *M. sexta*. Each value represents the mean and SE of three samples. See original (Rosenthal and Dahlman, 1985) for additional experimental details.

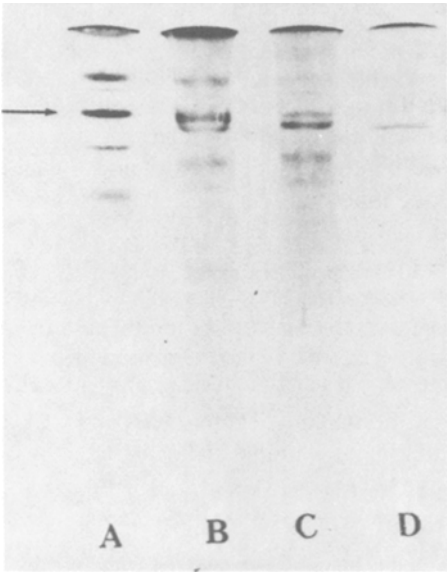

FIG. 3. Polyacrylamide gel electrophoresis of secreted fat body proteins of the migratory locust, *Locusta migratoria migratorioides*. Lane A, protein pattern from fat bodies maintained on arginine-free incubation medium for 3 hr at 30°C; lanes B, C, and D, from comparable materials maintained on 10 mM canavanine for 3, 6, and 9 hr, respectively (lane C, transfer to fresh incubation medium after 3 hr, lane D, transfer after 3 and 6 hr). In each instance, the incubation media from five fat body samples were pooled (2.5 ml) and 0.1 ml was applied to the gels. Arrow, position of vitellogenin; canavanyl vitellogenin is 1.5 mm below vitellogenin. See Pines et al. (1981) for additional experimental details; reproduced with permission.

A much more meaningful question, however, is that of the biochemical and biological ramifications of canavanyl protein production. This question has been addressed in studies of vitellogenin synthesis and secretion by the fat body of the adult migratory locust, *Locusta migratoria migratorioides*. Vitellogenin is the principal extraovarial protein; it is transported to the oocyte where it functions in vitellin formation, a key protein of the egg yolk. Isolated fat body of *Locusta* can be maintained in culture for 9 hr; during this time, the fat body produces vitellogenin and secretes it into the external medium. *Locusta* fat body maintained in complete medium produces appreciable vitellogenin (Figure 3, lane A). If the arginine of the culture medium is replaced by canavanine, both native vitellogenin and a novel canavanyl vitellogenin are produced over the first 3 hr (lane B). During the next hour, the proportion of canavanyl vitellogenin to the native molecule increases further (lane C). Lane D reveals the limited biosynthetic capacity of the fat body during the final 3-hr incubation

period. Of the detectable protein, only canavanyl vitellogenin can be discerned (Pines et al., 1981).

The shift in the electrophoretic mobility of canavanyl vitellogenin attests to the alteration that results from canavanine incorporation into this protein. It reveals that sufficient canavanine can be incorporated into vitellogenin to alter significantly its physicochemical properties. While these studies are of interest biochemically, they still leave unresolved the important biological question of whether canavanine incorporation into protein alters functional parameters of the macromolecule.

Work is in progress by Douglas Dahlman and the author to isolate enzymes from *M. sexta* and to compare these macromolecules with their canavanine-containing counterparts. These efforts are intended to provide the first direct experimental evidence on how canavanine replacement for arginine affects the functionality of a structurally aberrant insectan enzyme. The above biological question is also being addressed in a collaborative effort with Peter Dunn at Purdue University. Dunn is investigating the protein lysozyme and a group of peptides, known collectively as bacteriacidins, that are produced by the fat body of *M. sexta.* These hemolymph components function to protect the larvae against pathogenic organisms.

Incorporation of canavanine into these molecules diminishes significantly their biological activity and protective efficacy. Antibodies raised against lysozyme are immunoreactive with canavanyl lysozyme. This reactivity provides the means to determine the amount of lysozyme formed by canavanine-treated, cultured fat body. Under conditions in which canavanine inhibits about 50% of lysozyme's biological activity, no appreciable reduction in the amount of lysozyme produced relative to control fat body cultures occurs. Thus, canavanyl protein formation affects adversely the functionality of the molecule, not its synthesis. This point is most important, for addition of sufficiently high canavanine to the culture medium can terminate lysozyme formation.

Such direct experimental evidence addressing the question of canvanine incorporation and impaired functionality is still limited; however, convincing indirect evidence continues to accumulate. If, for example, one obtains an insect either able to use or resist canavanine, the capacity of such an insect to avoid canavanyl protein production can be evaluated. Development through evolutionary time of a significant preventive or avoidance capacity suggests that benefit is accrued from preventing aberrant protein production and therefore that such production carries deleterious biological consequences.

The bruchid beetle, *Caryedes brasiliensis* (Bruchidae) represents a canavanine adapted insect. *Caryedes brasiliensis* is the sole insect predator of *Dioclea megacarpa* (Leguminosae), a plant inhabiting the Neotropical forests of Costa Rica (Janzen, 1980). The seed of this legume is a veritable canavanine storehouse; as much as 13% of its dry weight is contributed by canavanine (average seed canavanine content may be closer to 9%). Canavanine can ac-

count for 55% of every nitrogen atom and more than 95% of the nitrogen allocated to free amino acids of the seed (Rosenthal, 1977). This stored canavanine undoubtedly creates a very formidable chemical defensive barrier that contributes to the significant freedom from insect predation that occurs in the seed.

Caryedes brasiliensis incorporates little [^{14}C]canavanine into total larval protein (Rosenthal et al., 1977). Comparable experiments with *M. sexta* result in the incorporation of 3 $\pm$ 0.5% of the administered canavanine into de novo synthesized proteins that constitute the musculature and body wall of the larvae (Rosenthal et al., 1976).

The appreciable capacity of this seed predator to avoid canavanyl protein production is revealed by direct comparison of the incorporation of radioactive arginine and canavanine, when the specific activity of each amino acid is equal. Under these conditions, the bruchid beetle incorporates into protein one canavanine molecule for each 365 molecules of arginine. This compares to one canavanine in 5.6 arginine molecules for *M. sexta* (Rosenthal and Janzen, 1983b). Comparable analyses with an array of arginine analogs indicates that *M. sexta* can incorporate all of the tested compounds into proteins. On the other hand, *C. brasiliensis* exhibits little ability to link these amino acids to arginyl tRNA (Table 1). These experiments provide evidence that *C. brasiliensis* may have evolved a discriminatory arginyl-tRNA synthetase that not only limits canavanine placement into protein, but also imparts a general resistance to the inclusion of such nonprotein amino acids into newly synthesized proteins.

A very important dimension is added to this research by the studies of John Bleiler, a graduate student in my laboratory. As part of his field work in Costa Rica, Bleiler discovered a weevil, *Sternechus tuberculatus* (Curculionidae) that is a seed predator of the legume, *Canavalia brasiliensis* (Figure 4). The female

TABLE 1. INCORPORATION OF ARGININE AND CERTAIN OF ITS STRUCTURAL ANALOGS INTO SOLUBLE INSECT PROTEIN[a]

Substrate	^{14}C incorporation (pCi/mg soluble protein)	
	Manduca sexta	*Caryedes brasiliensis*
Arginine	20,278	12,393
Canavanine	3,611	34
Homoarginine	264	<5
5-Hydroxyhomoarginine	480	<5
2-Amino-4-guanidinobutyric acid	262	<5
2-Amino-3-guanidinopropionic acid	112	<5

[a] See original for experimental details (Rosenthal and Janzen, 1983b).

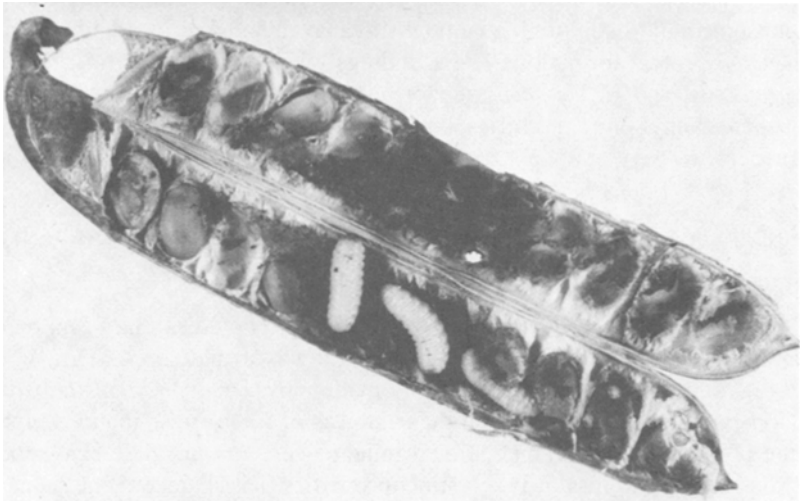

FIG. 4. Terminal instar larvae of the curculionid weevil, *Sternechus turberculatus*. The larvae are seen feeding on the canavanine-containing seeds of the legume, *Canavalia brasiliensis*.

weevil bores through the pericarp and deposits her eggs on the inside wall of the fruit; the larvae consume the seeds. Thus, in contrast to *Caryedes brasiliensis*, *Sternechus tuberculatus* develops within the fruit, not the seed.

Canavalia Brasiliensis stores about 8% of its dry matter as L-canavanine. Comparable analysis of the discriminatory capacity of *S. tuberculatus* reveals that so little canavanine is incorporated into larval proteins of this canavanine-utilizer that the arginine-to-canavanine ratio is in excess of 1:500. Thus, both of these canavanine-utilizing seed predators exhibit a pronounced ability to avoid the accumulation of canavanyl proteins.

The tobacco budworm, *Heliothis virescens* (Noctuidae), a generalist herbivore, is remarkably resistant to canavanine. Milan Berge, in my laboratory, determined the LC_{50} value for terminal instar larvae at 300 mM. High dietary levels of canavanine attenuate larval growth and the resulting pupae are depauperate, but the massive developmental aberrations noted with canavanine-fed *Manduca sexta* larvae are not observed. Since *Heliothis virescens* larvae are so canavanine resistant, it is relevant to ask if the larvae incorporate significant canavanine into their newly formed proteins. Such determinations indicate an amino acid incorporation ratio of canavanine to arginine of about 1 to 100, under the same conditions that this ratio is 1 to 5.6 for *Manduca sexta*. Moreover, comparison of canavanine incorporation into de novo-synthesized proteins reveals that while *Manduca sexta* places about 3.5% of the injected L-[*guanidinooxy*-^{14}C]canavanine into larval proteins of the body wall, the value for *He-*

liothis virescens is about 0.04%. Larvae of *Heliothis virescens* which are able to consume abnormally high quantities of canavanine, do so without significant canavanyl protein formation. These findings with *M. sexta*, *C. brasiliensis*, *S. tuberculatus*, and *H. virescens* support my contention that formation of anomalous, canavanyl proteins carries a marked potential for adverse biological consequences.

INDEPENDENT EVIDENCE FOR DYSFUNCTION IN INSECTAN CANAVANYL PROTEINS

Independent experimental evidence from insect studies that bears on canavanine incorporation and protein functionality is available. As part of their investigation of heat-shock protein formation by *Drosophila melanogaster*, DiDomenico et al. (1982) forced the synthesis of these stress-induced proteins in the presence of canavanine. Canavanine placement into these heat-shocked proteins causes the loss of two distinct properties that characterized them. The canavanyl heat-shocked proteins fail to concentrate in the nuclei of treated salivary gland cells (Figure 5A and B). These canavanyl proteins also lose the ability to bind RNA. This finding was established by treating normal heat-shock proteins with RNA-degrading enzymes. Removal of the RNA component results in a distinctive pattern that displays only the heat-shocked protein (Figure 5D) rather than the broad streaking characteristic of the RNA–heat-shocked protein complex (Figure 5C). Canavanyl heat-shocked proteins electrophorese as the normal, RNA-treated heat-shock proteins do (Figue 5D).

An equally dramatic effect of canavanine incorporation into heat-shocked proteins is indicated by electrophoretic analysis of *Drosophila* proteins from heat-shocked cells returned to 25°C (Figure 6). Heat-shock proteins gradually disappear and synthesis of normal proteins ensues concomitantly.

Canavanyl heat-shock proteins are not only not degraded, they actually increase. These canavanine-treated cells fail to resume production of the usual protein complement. Clearly, canavanine incorporation into these stress-induced proteins has a profound effect on normal cellular function. DiDomenico et al. (1982) concluded that their canavanyl heat-shock proteins are " . . . nonfunctional or, at the very least, to have profoundly disturbed function."

CANAVANINE AND INSECT PROTEIN SYNTHESIS

Another aspect of canavanine and insect protein synthesis and turnover emanates from investigation of canavanine and *M. sexta* conducted by the author and Douglas L. Dahlman. Our studies of canavanine and hemolymph protein synthesis in fifth-instar *M. sextra* larvae reveal that a parenterally injected, LC_{50} dose stimulates de novo protein synthesis. Yet, positive canavanine-me-

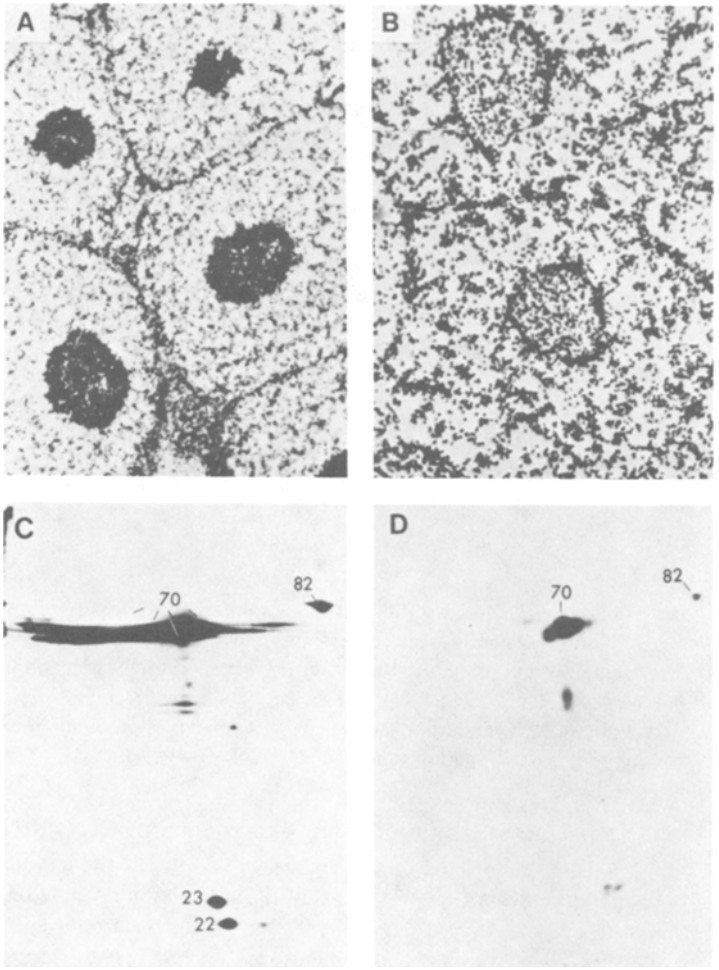

FIG. 5. The effect of L-canavanine on the heat-shock proteins of *Drosophila melanogaster*. (A and B) Salivary glands from a third stadium *D. melanogaster* larva were split and incubated under conditions that generated heat-shock proteins either in the presence of arginine or canavanine. (C and D) Analysis of heat-shock proteins by two-dimensional electrophoresis. See original (DiDomenico et al., 1982) for additional experimental details. Photo kindly supplied by S. Lindquist; reproduced with permission.

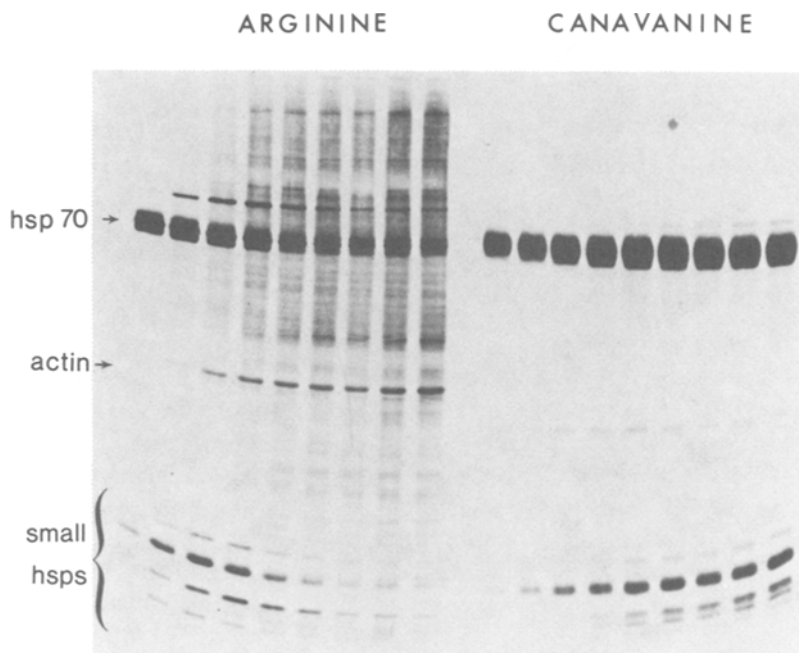

FIG. 6. Loss of heat-shock protein synthesis and resumption of normal protein production. Heat-shock proteins were synthesized in the presence of arginine or canavanine and placed under conditions where normal protein synthesis resumes. The proteins were pulse-labeled with tritiated leucine at consecutive 60-min intervals. See original (DiDomenico et al., 1982) for additional experimental details. Photo kindly provided by S. Lindquist; reproduced with permission.

diated stimulation of protein production is of short duration; it exists for between 1.5 and 3 hr (Table 2). While the biochemical basis for this enhancement is still under study, an explanation for the short duration of this effect may be known.

Manduca sexta possesses a very active system for the preferential degradation of aberrant, canavanyl proteins. Terminal instar larvae degrade erroneous hemolymph proteins several times faster than their normal counterparts, and there is evidence that the level of anomalous protein governs the rate of aberrant protein catabolism. The short period of canavanine-mediated stimulation of protein synthesis may represent the time required for these reactions to attain an equilibrium with those of preferential degradation (Rosenthal and Dahlman, 1985).

In a corollary experiment, *M. sexta* larvae were treated initially with canavanine for 24 hr, under conditions in which appreciable canavanyl hemolymph proteins are formed (as in Figure 2). These insects and control larvae were then

TABLE 2. INCORPORATION OF L-[^{3}H]LEUCINE INTO HEMOLYMPH PROTEINS

Time (hr)	[^{3}H]Leucine incorporation (nCi)[a]	
	Without canavanine	With canavanine
0.5	1.05 ± 0.04	1.74 ± 0.07
1.5	1.17 ± 0.03	1.89 ± 0.05
3.0	1.19 ± 0.09	1.11 ± 0.04
6.0	1.09 ± 0.06	0.99 ± 0.05
12.0	1.21 ± 0.03	1.21 ± 0.03
24.0	1.88 ± 0.13	1.91 ± 0.05

[a] At the specified time after producing the canavanine-treated or control larvae, each larva received 2.0 μCi L-[^{3}H]leucine/g fresh body weight. Thirty minutes later, the hemolymph was collected. Each value is the mean ± SE of nine larvae. See original for further experimental details (Rosenthal and Dahlman, 1985).

injected with [^{3}H]arginine-containing hemolymph proteins. Careful monitoring of these normal, tritiated proteins indicated that they are degraded at the same rate by canavanine-treated and control larvae. This finding indicates that *M. sexta* larvae can distinguish effectively between normal and aberrant proteins. Even in a large excess of anomalous canavanyl protein, when very active protein degradation is occurring, normal proteins are not swept out in purging abnormal macromolecules.

ECOLOGICAL CONSIDERATIONS

The experiments conducted to date with representative canavanine-utilizing, canavanine-resistant, and canavanine-sensitive insects reveal the importance to canavanine-utilizing insects of avoiding the adverse biological consequences manifested by aberrant, canavanyl protein formation. If anomalous proteins are formed by an insect such as *M. sexta*, the larvae can respond by preferential degradation of their aberrant macromolecules without inadvertent destruction of functional proteins. This capacity of insects to respond to canavanyl protein formation is particularly critical in insect ability to cope with this potentially toxic allelochemical, because canavanine not only mediates the production of pernicious aberrant proteins, but stimulates the rate at which these deleterious macromolecules are built.

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CARDENOLIDE CONNECTION BETWEEN OVERWINTERING MONARCH BUTTERFLIES FROM MEXICO AND THEIR LARVAL FOOD PLANT, *Asclepias syriaca*[1]

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Abstract—The majority (85%) of 394 monarch butterflies sampled from overwintering sites in Mexico contain the same epoxy cardenolide glycosides, including most conspicuously a novel polar glycoside with a single genin-sugar bridge (aspecioside), as occur in the milkweeds *Asclepias speciosa* and *A. syriaca*. This cardenolide commonality was established by isolating aspecioside and syriobioside from the wings of overwintering monarchs and the two plant species, and comparing chromatographic and NMR spectrometric characteristics of the isolates. When combined with the migratory pattern of monarchs and the distribution of these two milkweed species, this chemical evidence lends strong support to the hypothesis that *A. syriaca* is the major late summer food plant of monarchs in eastern North America. This finding may be of ecological importance, for *A. syriaca* contributes less cardenolide and cardenolides of lower emetic potency to monarchs than most milkweeds studied to date.

Key Words—*Danaus plexippus*, monarch butterflies, Lepidoptera, Danaidae, Asclepiadaceae, milkweeds, *Asclepias syriaca*, *Asclepias speciosa*, ecological chemistry, chemical defense, cardenolides, aspecioside, desglucosyrioside, syriobioside, overwintering.

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INTRODUCTION

Monarch butterfly (*Danaus plexippus* L.) larvae sequester cardiac glycosides (cardenolides) from their milkweed (*Asclepias* spp., Asclepiadaceae) host plants, and there is much evidence that these emetic heart poisons defend the monarch from potential vertebrate predators in the larval, pupal, and adult stages (references in Brower, 1984; Seiber et al., 1983). Presumably this protection extends to adults during migration and in their overwintering aggregations in Mexico and along the California coast (Brower and Fink, 1985). The overwintering sites in the neovolcanic plateau of central Mexico are of particular interest, for these well-defined areas apparently serve as resting colonies for the bulk of the monarch population migrating from east of the Continental Divide—the majority of monarchs on the North American continent (Urquhart and Urquhart, 1976; Brower, 1985). Monarch specimens collected recently from the sites in Mexico were found to contain a broad quantitative spectrum of cardenolides, but it is unclear exactly how and to what extent these chemicals provide protection in the colonies from certain opportunistic avian and rodent predators (Brower and Fink, 1985; Fink and Brower, 1981; Fink et al., 1983; Brower et al., 1985; Brower and Calvert, 1985).

The sequestration and storage of milkweed-derived cardenolides by monarchs is a selective process. Monarchs reared as larvae on milkweeds low in cardenolide concentrate the chemicals, whereas those reared on cardenolide-enriched plants exclude more cardenolide than they store (Brower et al., 1982, 1984a, b). This suggests regulation to achieve a cardenolide capacity, at least for cardenolides in specific structural classes (Reichstein et al., 1968; Roeske et al., 1976; Seiber et al., 1980). Monarchs also selectively store just some of the array of cardenolides typically present in individual *Asclepias* plants. This qualitative regulation involves, at least in part, rapid larval metabolism of some cardenolides (generally the less polar ones) to other cardenolides (generally of intermediate polarity) which are stored (Reichstein et al., 1968; Roeske et al., 1976; Brower et al., 1982; Seiber et al., 1980; Marty and Krieger, 1984). The variation in cardenolide structures from one *Asclepias* species to another, along with the selective storage of just some of the cardenolides by each monarch reared on an individual plant, have led us to investigate the possibility of "fingerprinting" wild captured adults to their larval *Asclepias* food plant as reflected in their specific cardenolide thin-layer chromatography (TLC) profiles (Brower et al., 1982, 1984a, b).

Application of the TLC fingerprinting technique to adult monarchs collected from overwintering sites thus provides an opportunity for delineating which of the 108 known species of *Asclepias* are the major larval food plants in North America. With this goal in mind, we analyzed the cardenolide content of monarchs collected from various overwintering sites in Mexico (Brower et al., 1977). The majority of these individuals had the same major cardenolides,

thereby indicating a potential commonality of larval food plant species. This was in sharp contrast with our prior survey of overwintering monarchs collected in California coastal colonies, which showed several distinct cardenolide TLC patterns spanning a broad R_f range (Roeske et al., 1976). We here present evidence that the major cardenolides in the majority of individuals from Mexico are identical to two of the major cardenolides present in *Asclepias speciosa* and *A. syriaca*. At least one other, less prevalent, cardenolide in monarchs from Mexico also coincides with a third cardenolide constituent of these same two plant species. This chemical evidence, coupled with the known geographical and temporal occurrence of *Asclepias* species (Woodson, 1954), allow us to establish that *A. syriaca* is the most probable major food plant of fall migrant monarchs in eastern North America—a subject of copious speculation in the prior literature (Urquhart, 1960; Urquhart and Urquhart, 1978, 1979, 1980; Fink and Brower, 1981; Brower et al., 1984b).

METHODS AND MATERIALS

Butterfly and Plant

Whole live monarch butterflies of both sexes were collected from clusters on branches of the oyamel fir (*Abies religiosa*) in January and March, 1978, 1979, and 1980 from several overwintering areas in the states of Michoacan and Mexico (Calvert et al., 1979). They were individually packed in glassine envelopes and kept frozen until oven dried and prepared for analysis. For chemical isolation, wings were excised from an additional sample of approximately 8000 live monarchs collected in January, 1983, from Site Alpha. They were dried at 60°C for 16 hr, milled to a fine powder (166.7 g dry weight), and kept frozen until extracted.

Whole, above-ground *A. speciosa* plants were collected near state route 89 just south of Sierraville (Sierra County, California) on July 12, 1982. They were dried in a forced draft oven at 50–60°C for 48 hr, ground in a Wiley mill to pass a 2.0-mm screen, and then stored frozen until extracted. Leaves of *A. syriaca* plants collected in Hampshire County, Massachusetts, in August 1967, were dried at 60°C for 16 hr, similarly screened, and stored at room temperature. The validity of this *A. syriaca* plant sample was verified by comparing cardenolide TLC profiles with those from more recent collections of *A. syriaca* from the same general area.

Analytical Procedures

Gross Cardenolide Content. Plant and butterfly specimens were analyzed for gross cardenolide content by a spectrophotometric assay based upon the base-catalyzed reaction of cardenolide with 2,2′,4,4′-tetranitrodiphenyl (TNDP)

as described in Brower et al. (1972) and modified by Brower et al (1975). This assay provides results in micrograms of cardenolide (equivalent to digitoxin) per 0.1 g dry weight.

Thin-Layer Chromatography. The lead acetate precipitation method described in Brower et al. (1982) was used, with the modification described in Brower et al. (1984b), to clean up samples prior to TLC.

The conditions for analytical TLC development in the chloroform–methanol–formamide (CMF) primary solvent system or the ethyl acetate–methanol (EAM) solvent systems, visualization with TNDP, and photography of developed chromatograms were the same as described previously in Brower et al. (1982). Preparative TLC conditions were as noted in subsequent sections of this paper.

High-Performance Liquid Chromatography (HPLC). The HPLC consisted of two Altex (Berkeley, California) model 110a pumps, a model 410 solvent programmer, an LDC (Riveria Beach, Florida) Spectromonitor I variable-wavelength UV detector (set at 220 nm), with range = 0.08–0.64 Absorbance Units Full Scale (AUFS), a Rheodyne injector (model 7125, Cotati, California) with a 50-μl loop, and a Linear (model 155, Irvine, California) recorder with the chart speed set at 20 cm/hr. The column was a Partisil 10 column (Whatman, 10 μm, 25 cm $\times$ 4.6 mm ID) and the flow rate was 1.2 ml/min.

The sample was injected with 100% methyl *tert*-butyl ether as the eluting solvent, and immediately a gradient to 100% tetrahydrofuran (THF) was begun extending over 10 min. The THF was then held for 15 min. Fractions (2 ml) containing the major cardenolide components were collected. The usual elution time was ca. 15–20 min after injection. After the solvent program was completed, solvent was recycled to 100% methyl *tert*-butyl ether and held there for 30 min to allow for reequilibration.

Nuclear Magnetic Resonance (NMR) Spectrometry. NMR was run in $CDCl_3$–CD_3OD solvent on a Bruker WM 400 NMR at the University of Sydney.

Isolation of Cardenolides

Butterfly Wings. Butterfly wing powder (10 g) was soaked overnight in 150 ml 50% aq EtOH. The mixture was blended twice in a Polytron homogenizer for 2–5 min followed by standing 2 hr. The process was repeated with a second volume of solvent and the filter cake resulting from filtration of the first batch. The combined filtrates were concentrated to 100 ml and methanol (20 ml) added. The solution was partitioned with petroleum ether (3 $\times$ 50 ml), sodium chloride added to the aqueous solution to saturation, and then partitioning was carried out with 10% isopropyl alcohol in chloroform (3 $\times$ 40 ml). The isopropanol–chloroform extract was filtered through sodium sulfate and evaporated to dryness to give 85.8 mg of viscous liquid. TLC showed at least four cardenolide

spots, with one at R_{Dig} = 0.60 in CMF being the most prominent. The above residue was combined with another 56 mg from a second batch of wings similarly processed and the mixture fractionated by preparative TLC on two 2-mm silica gel G plates developed twice each with ethyl acetate–methanol (97:3). Six fractions were obtained: fraction 2 (8.8 mg) was enriched in the major component (R_{Dig} = 0.60 on an analytical TLC plate); fraction 4 (5.0 mg) had primarily syriobioside, by matching R_{Dig} (=0.67) with a syriobioside standard; fraction 6 (6.1 mg) had primarily desglucosyrioside, by matching R_{Dig} (=1.3) with a desglucosyrioside standard.

Preparative TLC fractions 2 and 4 were separately subjected to preparative HPLC yielding 1.8 mg of the major cardenolide from TLC fraction 2 (subsequently identified as aspecioside) and 0.75 mg of syriobioside from TLC fraction 4. Both collections were single components, by analytical TLC.

A. syriaca. Plant material (100 g) was processed similarly to monarch wings with the following exception: Prior to preparative TLC, the isopropanol–chloroform extract was chromatographed on 30 g of Biosil A silica gel eluted with chloroform (200 ml) followed by 1% increments of methanol in chloroform (100 or 200 ml/increment). The 6 and 7% methanol in chloroform increments contained the major cardenolide (R_{Dig} = 0.60), subsequently identified as aspecioside, and the 3 and 4% contained syriobioside. Completion of the isolation scheme yielded 1.9 mg aspecioside and 1.0 mg of syriobioside. Desglucosyrioside also appeared in TLC analysis of early Biosil A chromatographic fractions.

A. speciosa. Plant material (1800 g) yielded 21.5 mg of aspecioside, 9.7 mg of syriobioside, and amounts of desglucosyrioside and uzarigenin which were not isolated. The procedure, reported in detail in Cheung et al. (1986), was similar to that described for *A. syriaca*.

RESULTS

In order to demonstrate cardenolide commonality among *A. speciosa*, *A. syriaca*, and overwintering monarchs from Mexico, extracts of composite samples of each (dried, ground aerial plant parts or dried, ground wings) were carried through a multistep isolation scheme (Table 1). The stages varied with the sample, requiring more steps for the plants due to a higher level of coextractives than for the butterfly wings. Each sample type contained desglucosyrioside, based upon coincidence of TLC R_f in two solvent systems; this was a major cardenolide component in the two plant species but a minor one in the butterfly wing extract. Each sample type also contained two polar cardenolides by TLC analysis in two solvent systems; one had R_{Dig} = 0.37 (EAM) and 0.60 (CMF) while a second had R_{Dig} = 0.90 (EAM) and 0.67 (CMF).

Initial spectrometric measurements were made on the two polar compounds

TABLE 1. CARDENOLIDE ISOLATION FROM *Asclepias speciosa*, *A. syriaca*, AND MONARCH BUTTERFLY WINGS.

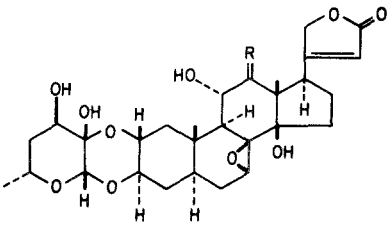
	A. speciosa	*A. syriaca*	Monarch wings
Location of collection	Sierra Co., California	Hampshire Co., Massachusetts	Michoacan, Mexico (Site Alpha)[a]
Quantity extracted (g, dry weight)	1800	100	20
Gross cardenolide concentration (μg/0.1g)	104	114	> 20
Cleanup steps	Liq/liq partition Column chromatography (3×) Prep TLC Prep HPLC	Liq/liq partition Column chromatography Prep TLC Prep HPLC	Liq/liq partition Prep TLC Prep HPLC
Cardenolide yield			
Desglucosyrioside	+++[b]	+++[b]	+[b]
Aspecioside (mg)	21.5	1.9	1.8
Syriobioside (mg)	9.7	1.0	0.75

[a]Brower et al., 1977; collected during January 1983.
[b]Relative proportions by TLC analysis (+++ > ++ > +); desglucosyrioside was not isolated.

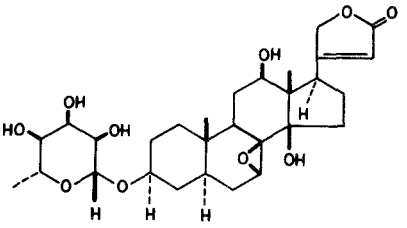
from *A. speciosa*. One (R_{Dig} = 0.67 in CMF) had a [^{1}H]NMR spectrum identical in its major features with those reported for syriobioside (Brown et al., 1979) and, in fact, coincided in TLC R_f (two solvent systems) with a syriobioside standard.

The second (R_{dig} = 0.60 in CMF) had a [^{1}H]NMR spectrum showing H-1′ as a doublet, indicating the presence of H at carbon 2′ in contrast to syriobioside and desglucosyrioside; the carbohydrate of this compound is thus linked to the aglycone through only one oxygen atom, as distinct from the doubly linked system of syriobioside and desglucosyrioside. The carbohydrate NMR signals identified it as 6-deoxy-D-allose. The stereochemistry of the protons on each carbohydrate carbon was determined with the aid of decoupling experiments. The mass spectrum of this compound (desorption chemical ionization and cesium FAB) had MH^+ = 551 amu, indicating a molecular weight of 550 (vs. 564 for syriobioside and 562 for desglucosyrioside). The molecular formula of $C_{29}H_{42}O_{10}$ takes into account the structure of the 6-deoxy-D-allose, requiring the aglycone to be $C_{23}H_{32}O_6$. The NMR spectrum had a doublet at 3.24 ppm (J = 6 Hz) which was assigned to the 7α-H of a β-7,8-epoxide. The position of the remaining oxygen functional group is 12β, based upon NMR decoupling experiments given in detail elsewhere (Cheung et al., 1986). The structure for this compound, for which we proposed the name "Aspecioside" (Cheung et al., 1986), along with the structures of desglucosyrioside and syriobioside, are

shown below:



R = O, Desglucosyrioside
R = α-OH, β-H, Syriobioside



Aspecioside

[^{1}H]NMR was employed to confirm the presence of aspecioside in *A. syriaca* plants and the monarch wing sample from Mexico. The spectra for corresponding isolates from the latter two sources were identical, except for extraneous impurity peaks, with that of aspecioside from *A. speciosa* (Figure 1).

Desglucosyrioside and syriobioside were originally isolated from *A. syriaca* (Bauer et al., 1961; Masler et al., 1962) along with syrioside, and β-D-glucose conjugate (at C-3′) of desglucosyrioside, the relatively simple cardenolide genins syriogenin and uzarigenin, and the uzarigenin conjugate desglucouzarin. The structures of desglucosyrioside, syriobioside, and syrioside were recently determined (Brown et al., 1979; Cheung and Watson, 1980).

We previously showed that *A. speciosa* also contains desglucosyrioside, syriogenin, and uzarigenin, along with TLC spots in the R_f range of syriobioside, syrioside, and aspecioside (Brower et al., 1984b). The only other *Asclepias* species known to contain cardenolide epoxides are *A. eriocarpa* and *A. erosa*, both of which are found only in California, and *A. labriformis*, which is found in a relatively restricted habitat in Utah (Seiber et al., 1978; Cheung et al., 1980).

In our earlier study of the sequestration of cardenolides by monarchs reared on *A. speciosa*, we showed that desglucosyrioside (TLC spot 19 in CMF) was present in freshly emerged adult monarchs along with several other cardenolide TLC spots, including two now identified as syriobioside (TLC spot 11) and aspecioside (TLC spot 10) (Brower et al., 1984b). The latter compound, which was particularly enriched (factor of ca. 2.5) in the adult butterflies relative to its concentration in *A. speciosa* leaves, was followed in order of spot intensity by syriobioside and desglucosyrioside. This order of intensity occurred also in the monarch wings sampled from the overwintering sites in Mexico. There was a total of 21 resolvable TLC spots in whole adult monarchs freshly emerged from *A. speciosa*-reared larvae (Brower et al., 1984b). Nine of these, including spots 10, 11, and 19, had probabilities of occurrence greater than 0.50 (i.e., half or more of the 111 butterflies examined from six geographical areas in California had these spots). For 10, 11, and 19, which were among the five most intense spots, the intensity values (most intense = 5.00) were 4.28 (spot

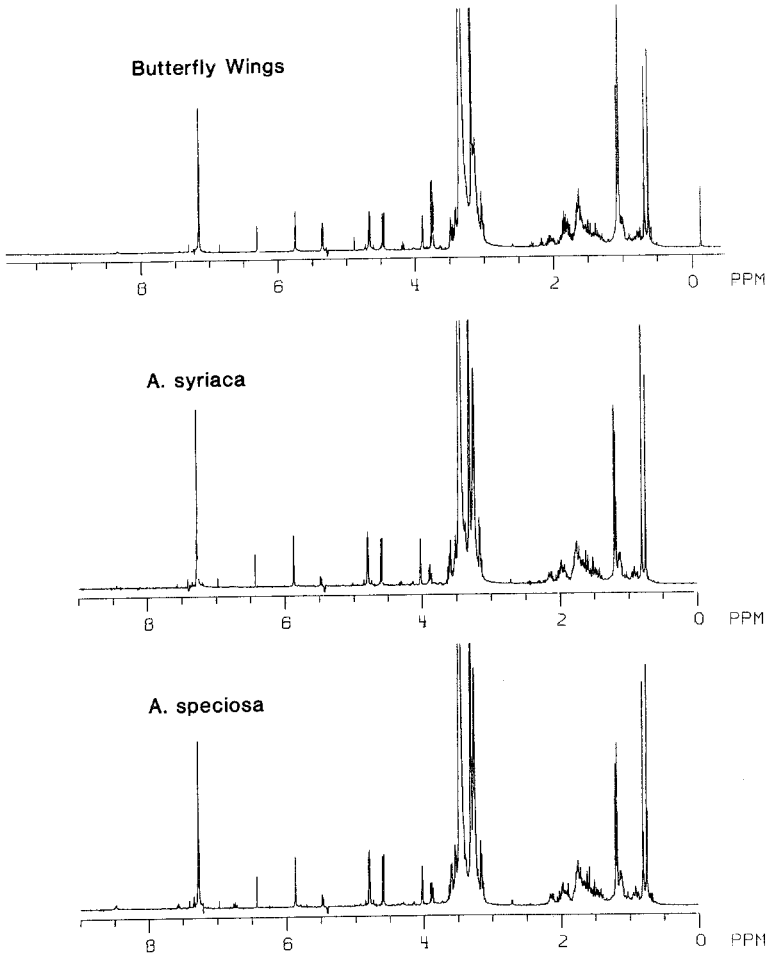


FIG. 1. [^{1}H]NMR spectra (400 MH_z) of aspecioside isolated from monarch wings collected from overwintering Site Alpha in Mexico, *Asclepias syriaca,* and *A. speciosa.* The NMR spectrum of the wing isolate is offset by approximately 0.15 ppm relative to the two other spectra.

10), 2.04 (spot 11), and 1.97 (spot 19), which is roughly in agreement with observations made on the monarch wing samples from Mexico.

A detailed analysis of the *A. syriaca* plant–butterfly cardenolide fingerprint is underway but not yet completed (Malcolm and Brower, in preparation). Enough *A. syriaca* plant–butterfly pairs have been analyzed by TLC to compare with the *A. speciosa* profile and that from overwintering butterflies (both whole specimens and wings only) from Mexico. This comparison is in the redrawn TLC channels in Figure 2. The four sample types are qualitatively similar, particularly in the predominance of aspecioside and, to a lesser extent, syrioside and desglucosyrioside in all four. Several other unidentified TLC spots

are common to the four sample types as well. From these preliminary data with *A. syriaca*-reared monarchs, we conclude that they provide a cardenolide profile very similar to the more extensively studied *A. speciosa* monarchs (Brower et al., 1984b). This is in keeping with the common cardenolide chemistry in the two plant species (Cheung and Watson, 1980; Cheung et al., 1986) as well as other similarities between the two species discussed further below.

The extent to which the *A. speciosa/A. syriaca* cardenolide TLC pattern was represented in the overwintering monarchs collected in Mexico was gauged in the following manner: Individual, whole monarchs (394) collected over a three-year period from Site Alpha were analyzed, and the cardenolide TLC patterns subjectively categorized in four groups (Figure 3): (1) Those having the greatest cardenolide spot intensity at the R_{Dig} of ca. 0.60 (aspecioside) and/

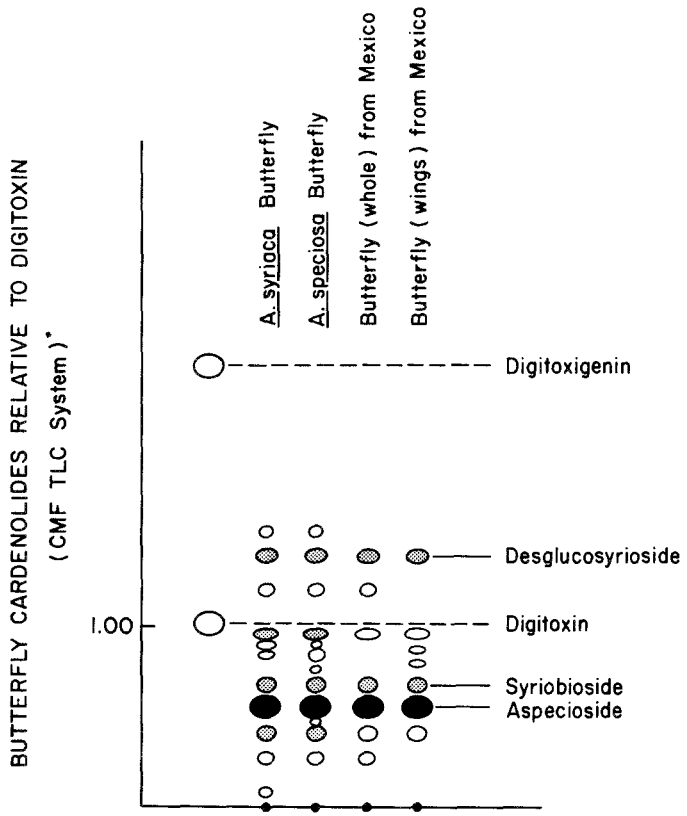


FIG. 2. Redrawn thin-layer chromatographic channels showing typical cardenolide profiles from *A. speciosa*- and *A. syriaca*-reared monarchs, Site Alpha monarch whole bodies and Site Alpha monarch wings. These channels are from plates developed four times in chloroform–methanol–formamide (90:6:1). The most heavily shaded spots were the most intense in the original chromatograms.

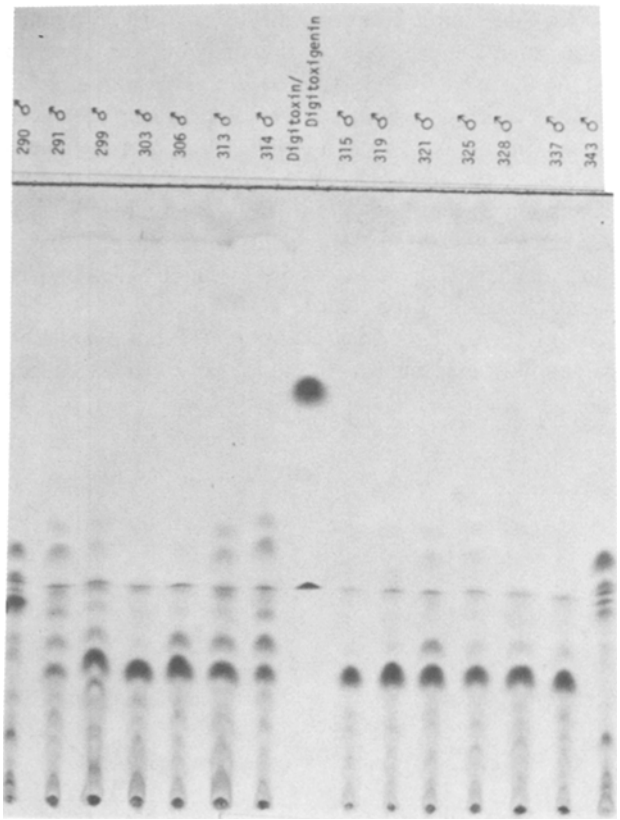


FIG. 3. Photograph of a thin-layer chromatography plate spotted with cleaned extracts of 14 individual whole monarch males collected on January 23, 1979, at Site Alpha N-15 in Mexico. Butterflies 290 and 343 show a pattern with more high R_f ($R_{Dig} > 1.0$) than low R_f cardenolides ($R_{Dig} < 1.0$). Butterfly 291 has approximately equal amounts of high and low R_f cardenolides. Butterflies 299, 303, 306, 313, 314, 315, 319, 321, 325, 328, and 337 show predominately aspecioside ($R_{Dig} = 0.6$) with lesser amounts of syriobioside ($R_{Dig} = 0.7$) and, in some cases, desglucosyrioside ($R_{Dig} = 1.3$); these represent the *A. syriaca* profile discussed in the text. Altogether 394 monarchs from Site Alpha were similarly analyzed. Chromatograms were developed as in Figure 2, with 75 μg digitoxin equivalents spotted for each butterfly.

or ca. 0.67 (syriobioside)—the most recognizable features of the *A. speciosa/A. syriaca* fingerprint pattern—included 337 individuals. (2) Those having their greatest cardenolide spot intensity at the $R_{Dig} > 1$, which probably had not been reared as larvae on *A. speciosa* and/or *A. syriaca*, included nine individuals. (3) Those having cardenolides of approximately equal intensity low ($R_{Dig} < 1$) and high ($R_{Dig} > 1$) included 40 individuals. This latter group may have included some individuals fed as larvae on either *A. speciosa* or *A. syriaca*, but the evidence was not clearcut. (4) Those lacking any observable cardenolide

spots at concentrations applied to the TLC plates included 10 individuals. In sum, 85% of the specimens examined conformed clearly to the *A. speciosa/A. syriaca* fingerprint.

DISCUSSION

The range of *A. syriaca* is reported to extend from Maine to Virginia, west to North Dakota and Kansas, and south to, roughly, 35° latitude (Figure 4) (Woodson, 1954). It is an abundant species—"the preeminent weedy species of the northeastern U.S." (Woodson, 1954)—whose frequency and range have been increased by agricultural and transportation activities (Marks, 1983; Brower, unpublished observations). Its range coincides closely with that of the major population of summer monarchs in North America (Urquhart, 1960), and it is readily available as a food plant into late summer when the last generation of monarchs which eventually migrates south is produced. *A. speciosa* shares many of these characteristics with the important exception that its range extends from Minnesota to Texas in the east westward to the Pacific coast, that is, approximately up to but only marginally overlapping with the range occupied by *A. syriaca* (Figure 4). In fact, the similarity of the two species is demonstrated by spontaneous hybridization in their overlapping range in Minnesota,

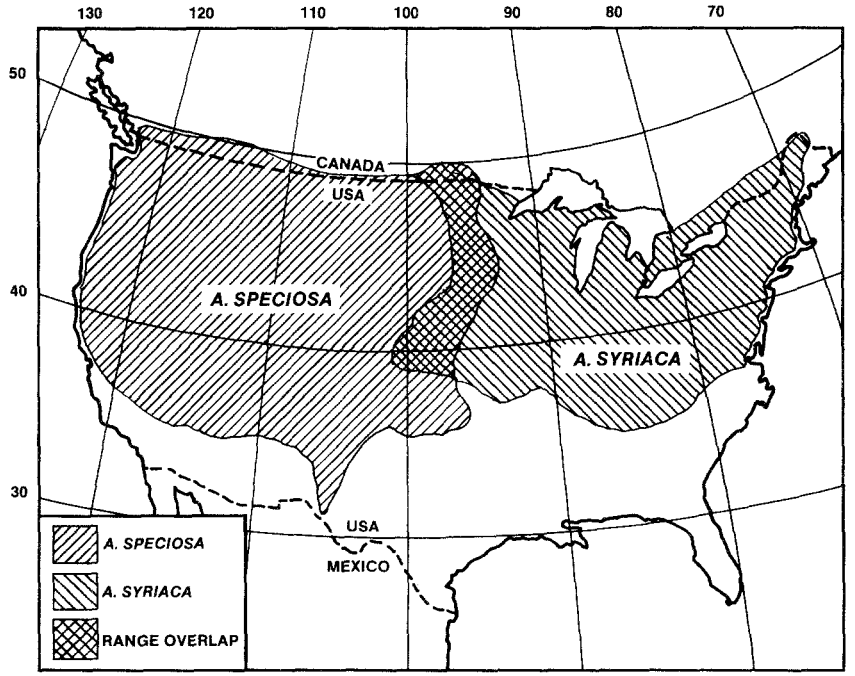


FIG. 4. Map showing approximate ranges of *Asclepias speciosa* and *A. syriaca* in North America (from collection data in Woodson, 1954).

Iowa, and the Dakotas (Woodson, 1954) as well as their very similar cardenolide chemistry. Because *A. speciosa* does not grow in the midwest–northeast U.S., it is clear that *A. syriaca* is the more likely late season host plant for monarchs which migrate to the overwintering sites in Mexico—that is, the major population in North America.

Several other *Asclepias* species which have a range similar to *A. syriaca* cannot be definitively excluded as potential contributors to the dominant TLC fingerprint observed in the sample from Mexico because their plant–butterfly fingerprints have been studied less extensively than those of *A. speciosa* and *A. syriaca* or have not been examined at all. Some speculation, however, may be offered on the potential contribution of these other species. Among those species which are relatively abundant and widespread within the *A. syriaca* range, *A. amplexicaulis, A. incarnata, A. tuberosa,* and *A. verticillata* have nil or very low cardenolide concentrations when compared with *A. syriaca* (Seiber et al., 1983) and are therefore unlikely candidates. *A. humistrata, A. quadrifolia, A. variegata, A. viridifolia,* and *A. viridis* are early season species (Woodson, 1954) which are not abundant late in the season when the fall migratory population is formed. Several other less-abundant species in similar geographical ranges cannot be excluded as minor contributors based upon available evidence.

If *A. syriaca* is, in fact, the principal late-season host plant for monarchs in North America, as others have suggested (Urquhart, 1960; Urquhart and Urquhart, 1978, 1979, 1980; Fink and Brower, 1981) and our cardenolide analyses strongly support, the finding may prove to be of much ecological importance. Historical changes in the eastern North American flora have apparently favored the adaptable and hearty *A. syriaca,* increasing its abundance relative to other milkweeds and thus increasing its availability as a monarch food plant (Brower et al., 1984b; Marks, 1983). We have previously shown that its botanical relative, *A. speciosa,* is a poor cardenolide source for monarchs relative to other *Asclepias* species we have examined (Brower et al., 1984b) and that the cardenolide concentration range of *A. syriaca* is similar to that of *A. speciosa* (Malcolm and Brower, in preparation). Moreover, as noted above, *A. syriaca* contains virtually the same cardenolides as *A. speciosa*. In addition, we have shown (Brower et al., 1984b) that the polar epoxy cardenolides now identified as syriobioside and aspecioside, which predominate in monarchs reared on *A. speciosa* and *A. syriaca,* have a markedly lower emetic potency than those of intermediate polarity which are sequestered from the California milkweed species, *A. eriocarpa* (Brower et al., 1982). This may account for the lower emetic potency of migrating monarchs collected in Massachusetts (Brower and Moffitt, 1974) and also in Mexico (Brower and Fink, 1985) than was observed in monarchs sampled from the overwintering sites in California, most of which probably fed on *A. eriocarpa* and other milkweeds contributing cardenolides of intermediate polarity (Brower et al., 1982). The consequence of an increase in abundance and distribution of *A. syriaca* plants may have been to lower the monarch's cardenolide content, as well as to diminish its emetic potency (Brower

and Fink, 1985) throughout the entire eastern range. This may account for the more extensive avian and rodent predation in the overwintering colonies in Mexico than occurs in the overwintering sites in California (Calvert et al., 1979; Fink and Brower, 1981; Fink et al., 1983; Brower et al., 1984b; Brower and Calvert, 1985).

The potential connection between an increasing abundance of a milkweed host plant, *A. syriaca,* which is a relatively poor source of cardenolide defensive chemicals, and the increased vulnerability of the major monarch butterfly population in eastern North America warrants further study.

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SEQUESTRATION OF CARDENOLIDES IN *Oncopeltus fasciatus*[1]: MORPHOLOGICAL AND PHYSIOLOGICAL ADAPTATIONS

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Abstract—The morphological and physiological adaptations associated with sequestration of cardenolides by the lygaeid *Oncopeltus fasciatus* are summarized and discussed. Cardenolides are efficiently accumulated in *O. fasciatus*; however, the insect does not appear to suffer any physiological cost as a result of handling large amounts of these plant toxins. Morphological adaptations of the insect include a modified integument composed of a double layered epidermis with an inner layer (the dorsolateral space) specialized for cardenolide storage. Special weak areas of the cuticle are found on both the thorax and abdomen, which rupture when the insect is squeezed, resulting in the cardenolide-rich contents of the inner epidermal layer being released onto the body surface in the form of discrete spherical droplets. Physiological adaptations include selective sequestration of food plant cardenolides, concentration of cardenolides in the dorsolateral space, passive uptake of cardenolides at the gut and dorsolateral space requiring little energy output, reabsorption of secreted cardenolides by the Malpighian tubules, high in vivo tolerance to cardenolides, and the presence of cardenolide-resistant Na,K-ATPases.

Key Words—Hemiptera Heteroptera, Lygaeidae, *Oncopeltus fasciatus*, Asclepiadaceae, cardenolides, sequestration, morphological adaptations, physiological adaptations.

INTRODUCTION

The Heteroptera, including the Lygaeidae, have adult metathoracic and larval abdominal scent glands (Staddon, 1979), that produce a diversity of chemicals

[1]Hemiptera-Heteroptera: Lygaeidae.

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(Baggini et al., 1966; Calam and Youdeowei, 1968; Tsuyuki et al., 1965; Weatherston and Percy, 1978). These chemicals often function for defense (Remold, 1962, 1963; Calam and Youdeowei, 1968; Dazzini and Pavan, 1978).

Lygaeidae are generally brown, cryptically colored, and ground dwelling seed feeders. In contrast, members of the subfamily Lygaeinae are typically brightly colored arboreal plant feeders, with red and black aposemetic coloration (Scudder and Duffey, 1972). The coloration and off-ground habit in the Lygaeinae correlates with a reduced adult metathoracic scent apparatus (Schaefer, 1972), although scent glands persist and function in both larvae and adults (Games and Staddon, 1973a,b; Henrici, 1938, 1939; Johansson, 1957; Remold, 1962, 1963; Staddon, 1979).

Most of the Lygaeinae feed on Asclepiadaceae and Apocynaceae (Scudder and Duffey, 1972), and hence, the warning coloration in this taxon also appears to be correlated with the use of cardenolides for defensive purposes. This has been clearly documented in other insect species (Brower, 1969; Brower and Brower, 1964; Rothschild, 1966, 1972; Rothschild and Kellett, 1972; Rothschild et al. 1970, 1973).

Oncopeltus fasciatus (Dallas) is a typical member of the Lygaeinae, and a common research animal (Feir, 1974). It normally lives on species of Asclepiadaceae (Eggermann and Bongers, 1971, 1972), although it can also live on *Nerium oleander* (Klausner et al., 1980) and can be reared on peanuts (Frings and Little, 1955), sunflower seeds (Bongers, 1969), and on a mixture of cashew and sunflower seeds or pumpkin and sunflower seeds (Gordon and Loher, 1968).

While *O. fasciatus* clearly utilizes endogenously produced chemicals for its defense (Games and Staddon, 1973a, b), it is also able to sequester large quantities of cardenolides from its food plants thoughout its life cycle (Duffey and Scudder, 1974). It is assumed that these exogenous chemicals also have an antipredator defensive function (Duffey and Scudder 1974; Scudder and Meredith, 1982a).

This paper reviews the role of cardenolides in the complex defensive system of *O. fasciatus*. In it is summarized the morphological and physiological adaptations associated with cardenolide uptake and accumulation. These adaptations are discussed with particular reference to the high efficiency of cardenolide accumulation seen in *O. fasciatus* and the lack of any apparent physiological cost of cardenolide storage to the insect. Contrasts are made between cardenolide sequestration and deployment in *O. fasciatus* and that in the monarch butterfly (*Danaus plexippus* L.). Finally, comments are offered on predation experiments with this insect and other Lygaeinae.

MORPHOLOGICAL ADAPTATIONS

The Integument. The integument of arthropods characteristically has a single-layered epidermis bound by a basal lamina (Richards, 1951; Neville, 1975).

In *O. fasciatus* a double-layered epidermis is present in both males and females, regardless of diet, from late embryo through to the adults (Scudder and Meredith, 1982a). This unusual epidermal structure is a special morphological feature of *O. fasciatus* adapted for cardenolide storage and release.

The outer layer of the epidermis (Figure 1) is a typical epidermal monolayer (Ep), concerned with cuticle production and support. It is pigmented and nonvacuolated. In contrast, the inner epidermal layer (Ev) is extensively vacuolated and unpigmented. The irregularly shaped, membrane-bound vacuoles (v) are probably the site of cardenolide storage in *O. fasciatus*. The vacuolation of the inner cell layer becomes more and more extensive during development, until in the adult the cells are essentially fluid-filled.

In the adult integument, the inner cell layer is very fragile, and cells may lack lateral cell membranes. When the cardenolide compartments are expanded, the cytoplasm of the cells is drastically reduced, and persists only in the areas where the inner cells are attached to the basal lamina by hemidesmosomes. However, study of the nuclei of these cells shows that they are not necrotic, suggesting that these cells must be cardenolide resistant. Since the cells can be experimentally loaded with ouabain (Scudder and Meredith, 1982a), they would appear to be as resistant as mammalian cell lines that have been established in culture (Baker et al., 1974; Landolph et al., 1980; Mankovitz et al., 1974; Robbins and Baker, 1977).

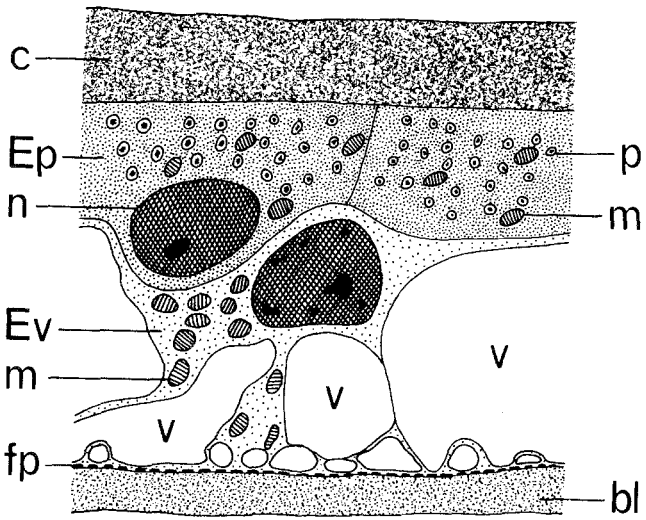

FIG. 1. Diagrammatic section through the integument of adult *O. fasciatus* showing the double layered epidermis. Abbreviations: Ep, pigmented epidermal cell layer; Ev, vacuolated epidermal cell layer; bl, basal lamina; c, cuticle; fp, foot processes; m, mitochondria; n, nucleus; p, pigment granule; v, vacuole.

The basal lamina (bl) underlying the inner epidermal cell layer in *O. fasciatus* thickens throughout development, and in the adult is 1.02 μm thick. This is at least twice the usual thickness (Ashurst, 1968) reported for the insect basal lamina. It is suggested (Scudder and Meredith, 1982a) that this thick basal lamina functions structurally to prevent the inner cell layer from rupturing internally, and releasing the contents into the hemocoel.

Thus, the integument of *O. fasciatus* is especially adapted in that (1) it has a double-layered epidermis with an inner layer specialized for cardenolide storage; (2) the inner layer is cardenolide and ouabain resistant; and (3) it has an especially thick basal lamina to provide structural reinforcement preventing the inner cell layer from rupturing internally.

Although a similar double-layered epidermis has been found in *Lygaeus kalmii* (Scudder and Meredith, 1982a), it is not known if it also occurs in other Lygaeinae. It is also not yet clear whether the cardenolides stored in the inner cell layer of immature stages are passed from one instar to the next.

Since the inner epidermal layer cells lack extensive membrane elaboration and abundant mitochondria, such as is seen in transporting tissues, it is suggested that the cardenolide uptake process into the cells is not an active process. (See section on physiological adaptations for further discussion).

In the adult *O. fasciatus*, injection experiments with India ink show that the cardenolide-containing areas of the integument appear to be continuous between the thorax and abdomen. The area which we have called the dorsolateral space is especially expanded in the epimeral lobes of the metathorax and the dorsolateral margins of the mesothorax and abdomen. The cardenolide-containing space occurs throughout the integument of the thorax and abdomen and is interrupted only in regions of muscle attachment and where the cuticle is modified as invaginations.

Thoracic Release Sites. In both the thorax and abdomen of *O. fasciatus*, cardenolide-rich fluids are released onto the surface of the body by integument rupture: there are no special pores or glands. Only when the insect is forcibly squeezed will discrete spherical droplets appear at precise points of weakness. Mere disturbance will not elicit release.

In the meso- and metathorax, these areas of weakness occur along the dorsolateral margins of the segment. In the mesothorax, droplets of cardenolide-rich fluid may form anywhere along the margin, where the cuticle is somewhat thinner (Scudder and Meredith, 1982a).

In the metathorax, there is a curved slit at the posterior, dorsolateral margin of the epimeral lobe. Here cuticular flanges abut and are underlayed by the epidermis. When pressure is applied, the flanges are forced apart, and the epidermal layer seal is broken to release the cardenolide-rich contents of the inner epidermal layer. Normally, the cardenolides will be released on the thorax before they will appear on the abdomen.

Abdominal Release Sites. On the abdomen, special weak areas of cuticle

occur in the lateral black pigment spots at the anterolateral corners of sterna II–VII. On the surface, this area of the cuticle is smooth and is surrounded by dense microtrichia. This smooth area of cuticle is about one fourth the normal thickness of the surrounding cuticle. This weak area and underlying epidermis is ruptured under pressure to release the cardenolide-rich contents of the inner epidermal layer. On release, these droplets are emphasized by their location on aposematic areas and are positioned such as to be immediately encountered by a predator.

These structural specializations seen in the abdomen of *O. fasciatus* are also present in other Lygaeinae, but are not a universal characteristic of the subfamily (Scudder and Duffey, 1972). In those taxa without structural weak points on the sterna, the mode of deployment of the cardenolides is not known.

PHYSIOLOGICAL ADAPTATIONS

Oncopeltus fasciatus sequesters and tolerates large amounts of cardenolides in its body tissues. A number of physiological adaptations are associated with this ability. We have studied sequestration of cardenolides from its natural food plants (*Asclepias* spp.), the distribution of cardenolides in body tissues, the uptake of cardenolides at the gut and into the dorsolateral space, the metabolism and excretion of cardenolides, and the tolerance of the insect and its Na,K-ATPases to the toxic effects of cardenolides. Since commercial preparations of host plant cardenolides are not available, two nonasclepiad cardenolides, ouabain and digitoxin, have been used as indicator cardenolides in many of the experiments we have undertaken. Ouabain and digitoxin are not found in the food plants of *O. fasciatus* and have a 5β *cis* A/B steroid ring juncture rather than the 5α *trans* A/B steroid ring juncture characteristic of asclepiad cardenolides. Ouabain and digitoxin were chosen owing to their contrasting polarities and to their availability in pure and radiolabeled forms. In addition, a number of the metabolites of these compounds have been identified and are also commercially available.

Uptake of Cardenolides from Natural Food Plants (Asclepias, *spp.). Oncopeltus fasciatus* sequesters cardenolides from the seeds and vegetative parts of its food plants (Duffey and Scudder, 1974; Feir and Suen, 1971; Moore and Scudder, 1985) with no apparent physiological costs (Isman, 1977; Chaplin and Chaplin, 1981; Jones et al., 1983). Several studies suggest that milkweed cardenolides are sequestered in proportion to the cardenolide content of the food source (Isman, 1977; Duffey et al., 1978; Vaughan, 1979). The wide range in the cardenolide contents detected in *O. fasciatus* (0–900 μg cardenolide per insect) reflects the large intra- and interspecific differences in the cardenolide contents of the insect's food plants (Isman et al., 1977a; Duffey et al., 1978; Vaughan, 1979; Isman, 1977, 1979, unpublished data). In contrast, a study by

Vaughan (1979) found quantitative regulation of the cardenolide content in *O. fasciatus*. A greater percentage of the seed cardenolide content was sequestered in insects reared on *A. syriaca* seeds of low cardenolide content than in insects reared on *A. syriaca* seeds of high cardenolide content. In general, females contain more cardenolide per insect than males (usually >50% more; Duffey and Scudder, 1974; Isman, 1977), possibly as a result of cardenolide accumulation in developing eggs, although in one study no sexual differences in total cardenolide content per insect was found (Moore and Scudder, 1985).

These results suggest that uptake of cardenolides in *O. fasciatus* is similar to that in the monarch in the following aspects: (1) neither insect suffers any apparent physiological cost because of the sequestration and storage of cardenolides, (2) both species usually exhibit sexual dimorphism in cardenolide contents, and (3) both species exhibit differences in cardenolide content which reflect interspecific differences in their food plants (Brower and Moffitt, 1974; Seiber et al., 1980; Brower et al., 1984).

Distribution and Selective Sequestration of Milkweed Cardenolides. The distribution of cardenolides in *O. fasciatus* has been determined in insects reared on two natural food plants, *Asclepias syriaca* and *A. speciosa* (Table 1 and Figure 2). The results of these experiments (Duffey and Scudder, 1974; Moore and Scudder, 1985) indicate that cardenolides are accumulated primarily in the dorsolateral space while low cardenolide levels are maintained elsewhere in the insect (Table 1).

Comparison of the cardenolide profiles of insects and their food plants shown in Figure 2 indicate that individual cardenolides are selectively sequestered and stored in *O. fasciatus*. Most, but not all, of the cardenolides detected

TABLE 1. DISTRIBUTION OF CARDENOLIDES IN *Oncopeltus fasciatus* EXPRESSED AS PERCENT OF ADULT TOTAL

	Adult total (%)	
Sample	*A. syriaca*-reared insects (Duffey and Scudder, 1974)	*A. speciosa*-reared insects (Moore and Scudder, 1985).
Dorsolateral space	60–95[a]	46–89[a]
Hemolymph	trace	trace
Urine plus feces	trace	trace
Metathoracic gland	trace	—
Fat body (male)	—	trace
Fat body (female)	—	4–5
Gut with contents	—	trace
Wings	—	trace

[a] Estimate based on assumption of 2–3 μl volume for dorsolateral space, but this may be an underestimate (see Moore and Scudder 1985)

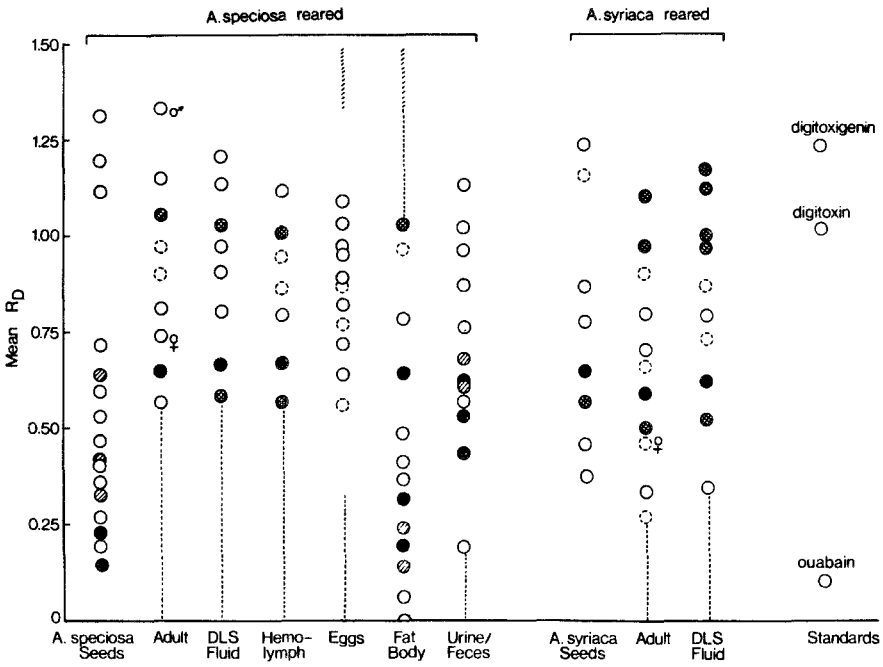

FIG. 2. Cardenolide profiles of *A. speciosa* and *A. syriaca* seeds and extracts of *O. fasciatus* adults reared on each seed. Each spot represents an individual cardenolide. The relative concentrations of the cardenolides of the different samples are not comparable. Symbols: $R_D = R_f$ spot/$\bar{X}$ R_f of digitoxin; ⊘, noncardenolide; ○, low concentration; ○, medium concentration; ⊗, high concentration; ●, highest concentration; ◐, high or highest concentration depending on the extract; ♂, seen in male only; ♀, seen in female only; ⁞, light tailing of cardenolides; ≡, light tailing of noncardenolides.

in the seeds were present in the insect, and many of the cardenolides that were highly concentrated in the insect did not correspond to highly concentrated cardenolides in these seeds (Figure 2; see also Moore and Scudder, 1985). Selective sequestration of food plant cardenolides has also been noted in *Danaus plexippus*, *Aphis nerii*, *Caenocoris nerii*, *Spilostethus pandurus*, and *Poekilocerus bufonius* (von Euw et al., 1967, 1971; Rothschild et al., 1970; Brower et al., 1982). Many of the cardenolides detected in *O. fasciatus* which do not correspond to seed cardenolides may represent products of metabolism.

The cardenolide profiles shown in Figure 2 also indicate that cardenolides of a wide polarity range were sequestered, as well as concentrated, in insects reared on either species of milkweed. These results indicate a further similarity between *O. fasciatus* and the monarch since the monarch also sequesters cardenolides covering a wide polarity range (Brower et al. 1982). This was unexpected since earlier studies indicated that polar cardenolides are preferentially

sequestered in *O. fasciatus* (Duffey and Scudder, 1974; Duffey et al., 1978; Scudder and Meredith, 1982b). The role of physical-chemical characteristics other than polarity in cardenolide sequestration in *O. fasciatus* is also suggested by the sequestration and concentration of intermediate and more polar cardenolides in the fat body of insects reared on *A. speciosa* (Figure 2). This result was also unexpected since the fat body often accumulates nonpolar compounds and toxins owing to their lipophilic nature (Kilby, 1963), and earlier observations indicated preferential sequestration of the nonpolar cardenolide digitoxin in the fat body of adult *O. fasciatus* (Duffey et al., 1978).

Uptake of Cardenolides by the Gut. Uptake of the polar cardenolide, ouabain, and the nonpolar cardenolide, digitoxin, by the gut of *O. fasciatus* has been investigated by Yoder et al. (1976) and Scudder and Meredith (1982b). The selective permeability of the gut to digitoxin seen in both experiments suggests that the gut may be more permeable to nonpolar cardenolides than to polar cardenolides. Uptake of ouabain and digitoxin was linearly related to concentration, was nonsaturable, and was not observed to occur against a concentration gradient, which suggests that gut uptake of cardenolides is probably passive and occurs by diffusion. The results of Yoder et al. (1976) also provide evidence that uptake of cardenolides by the gut may be sensitive to Na^+ levels and that uptake of individual cardenolides may not be affected by the presence of other cardenolides in the gut lumen.

Scudder and Meredith (1982b) demonstrated that the gut of two insects which do not normally encounter cardenolides in their diet, namely the locust *Schistocerca gregaria* and the cockroach *Periplaneta americana*, was not permeable to either ouabain or digitoxin. These results suggest that gut permeability to cardenolides may be one of the specializations that has allowed certain insects, such as *O. fasciatus*, to accumulate these plant toxins in their body tissues and defensive secretions.

Uptake of Cardenolides into Dorsolateral Space. The uptake of ouabain and digitoxin into the dorsolateral space has been investigated by Duffey et al. (1978) and Scudder and Meredith (1982b). The selective permeability of the dorsolateral space seen in these experiments (ouabain $>>$ digitoxin) is the reverse of that seen in the gut. This indicates that the accumulation of some cardenolides, i.e., ouabain, in *O. fasciatus* may be limited by their rate of uptake across the gut, whereas for other cardenolides, i.e., digitoxin, accumulation may be limited by their rate of uptake into the dorsolateral space (Scudder and Meredith, 1982b).

Uptake of ouabain and digitoxin by the dorsolateral space, is in proportion to the cardenolide content in the food source and the hemolymph, is nonsaturable, and displays first-order kinetics which suggests that the uptake of cardenolides into this epithelial layer is passive and occurs by diffusion (Duffey et al., 1978; Scudder and Meredith, 1982b). We have already noted ultrastructural characteristics of the inner epidermal layer that suggest uptake of cardenolides

into the dorsolateral space is passive (Scudder and Meredith 1982a). Estimated ratios of cardenolide concentration in the dorsolateral space to cardenolide concentration in the hemolymph (DLS/H) were as high as 73 in insects injected with ouabain (Duffey et al., 1978). In insects reared on natural host plants, DLS/H ratios up to 13,000 were found (Duffey and Scudder, 1974; Duffey et al., 1978; Moore and Scudder, 1985). Since diffusion of a compound can only occur along its electrochemical gradient, the cardenolides in the dorsolateral space must have a lower chemical activity than the cardenolides in the hemolymph. The chemical activity of the cardenolides in the dorsolateral space could be lowered by processes such as phase separation (i.e., an emulsion phase as suggested by Duffey et al., 1978), binding to proteins or binding to other cellular constituents; however, at this time the actual process involved is not known.

Some of the characteristics of ouabain uptake into the dorsolateral space suggest that this epithelial layer may function in the tolerance of *O. fasciatus* to cardenolides by maintaining low levels of these toxins in the hemolymph (less than 6.5×10^{-6} M, Moore and Scudder, 1985a). Uptake of ouabain can occur when cardenolide levels in the hemolymph are as low as 3.5×10^{-7} M (Duffey et al. 1978). In addition, rates of ouabain uptake increase as hemolymph concentrations of ouabain increase, aiding in rapid clearance of ouabain from the hemolymph (Table 2).

Metabolism of Cardenolides. Several studies have indicated that ouabain and digitoxin, as well as milkweed cardenolides, are metabolized in *O. fasciatus* adults (Duffey and Scudder, 1974; Scudder and Meredith, 1982b; Meredith et al., 1984; Moore and Scudder, 1985), and metabolism of digitoxin has been

TABLE 2. UPTAKE OF OUABAIN INTO DORSOLATERAL SPACE 3 HOURS AFTER HEMOLYMPH INJECTION[a]

Initial ouabain concentration in hemolymph after injection (M)[b]	Concentration in dorsolateral space fluid 3 hr after injection (pmol/μl)	Injected ouabain in dorsolateral space after 3 hr (%)[c]
4×10^{-8}	3×10^{-2}	17–26
4×10^{-7}	2×10^{-1}	8–13
4×10^{-6}	5	25–38
4×10^{-5}	100	50–75
4×10^{-4}	1000	50–75
4×10^{-3}	10000	50–75

[a] Calculated from data in Duffey et al., (1978).
[b] Calculated using hemolymph volume estimate of 10 μl (Duffey et al., 1978).
[c] Calculated using dorsolateral space volume estimate of 2–3 μl (Duffey and Scudder, 1974).

demonstrated in larval instars (Duffey and Scudder, 1974). Possible sites of cardenolide metabolism in adult insects were investigated using ouabain and digitoxin. To date, metabolism of ouabain has only been found in the Malpighian tubules of *O. fasciatus* (Meredith et al., 1984), whereas metabolism of digitoxin has been found in both the Malpighian tubules and the midgut (Scudder and Meredith 1982b). Digitoxin was not metabolized in isolated hemolymph or dorsolateral space fluid (Scudder and Meredith, 1982b).

Metabolism of cardenolides has also been indicated in a number of other insects and may serve to rid the insects of particularly toxic cardenolides, as well as provide the insects with cardenolides that are readily accumulated and especially effective for defense (von Euw et al., 1967; Scudder and Meredith, 1982b; Brower et al., 1982; Levey, 1983).

Excretion of Cardenolides by Malpighian Tubules. Meredith et al. (1984) investigated the excretion of ouabain by the Malpighian tubules under a variety of in vitro conditions. Their results demonstrated that ouabain was passively secreted and metabolized by the Malpighian tubules which suggests that the tubules are a potential site of cardenolide loss in *O. fasciatus*. The discovery, however, of a specialized tubule segment that actively reabsorbed ouabain indicates that the excretory loss could be minimized and that reabsorption of secreted cardenolides may be an important factor in the sequestration of cardenolides in *O. fasciatus*. Indeed, in situations of high ouabain secretion by the tubules, 84–93% of the ouabain that otherwise would be excreted was reabsorbed. Further investigation is necessary to establish the role in vivo of the Malpighian tubules in the overall process of sequestration of cardenolides in *O. fasciatus*.

Ouabain-Resistant Na,K-ATPases and Tolerance to Cardenolides. Two additional factors may function in the ability of *O. fasciatus* insect to tolerate and store cardenolides from its food plants: in vivo tolerance of the insect to large amounts of cardenolides injected into the hemolymph, and the presence of cardenolide-resistant Na,K-ATPases in the insects' nervous tissue (Moore and Scudder, 1986). The in vivo tolerance of *O. fasciatus* to ouabain was compared to that of *Schistocerca gregaria* and *Periplaneta americana*, two orthopteroid insects which do not encounter cardenolides in their diet. The results clearly show that *O. fasciatus* was highly resistant to the toxic effects of ouabain (Table 3); correcting for interspecific weight and hemolymph volume differences, *O. fasciatus* tolerated 1954× and 7288× the LD_{50} for *S. gregaria* and *P. americana*, respectively.

Cardenolides exert their toxic effects by specifically inhibiting the enzyme $Na^+ + K^+ - ATPase$ (Na, K-ATPase; E.C. 3.6.1.3), and among vertebrates, species differences in sensitivity to cardenolides are known to parallel differences in the sensitivity of their Na,K-ATPases (Akera, 1977; Bodeman, 1981; Schwalb et al., 1982). Our studies (Moore and Scudder, 1986) indicate that such differences may also account for interspecific sensitivities to ouabain in *O.*

TABLE 3. OUABAIN TOLERANCE OF *O. fasciatus*, *S. gregaria*, AND *P. americana*[a]

Insect	Ouabain injected (nmol/insect)	Ouabain injected (nmol/mg wet weight insect)	Initial ouabain concentration in hemolymph after injection (M)[b]
O. fasciatus, 100% survival	200.0	4.3	12.5×10^{-3}
S. gregaria, LD_{50}	6.1	2.2×10^{-3}	3.0×10^{-5}
P. americana, LD_{50}	0.9	5.9×10^{-4}	5.4×10^{-6}

[a] Adult females—*O. fasciatus* and *P. americana* random ages, *S. gregaria* 16–18 days after ecdysis. Survival after 24 hr (48 hr survival is the same).

[b] Calculated using the following hemolymph volume estimates: *O. fasciatus* and *S. gregaria* 11 and 200 μl, respectively (Meredith, unpublished results), *P. americana* 165 μl (Guthrie and Tindall, 1968), and assuming complete mixing of injected ouabain with hemolymph.

fasciatus and *S. gregaria* (Table 4); the ouabain concentration resulting in 50% inhibition of the Na,K-ATPase activity in nervous tissue lyophilates of *O. fasciatus* was 100× greater than that for *S. gregaria* brain and 200× greater than that for *S. gregaria* rectum. Thus, the presence of cardenolide-resistant Na,K-ATPases in *O. fasciatus* appears to be one more factor in the ability of this insect to tolerate and store cardenolides from its food plants.

Characteristics of Sequestration and Tolerance of Cardenolides in O. fasciatus: *Comparison to Monarch Butterfly.* Throughout this review, we have noted the similarities and differences in the sequestration and tolerance of cardenolides in *O. fasciatus* and the monarch. Of particular interest is the fact that neither insect appears to suffer ill effects from the sequestration of large amounts of cardenolides.

Our investigations suggest that the low physiological cost of sequestering cardenolides in *O. fasciatus* is the result, at least in part, of the following specializations: (1) high in vivo tolerance to cardenolide intoxication (Moore and Scudder, 1986); (2) passive uptake of cardenolides at the gut and dorsolateral space, requiring little energy output (Duffey et al., 1978; Scudder and Mere-

TABLE 4. INHIBITION OF NA, K-ATPASE ACTIVITY BY OUABAIN IN NERVOUS TISSUE OF *O. fasciatus* AND BRAIN AND RECTUM OF *S. gregaria*

Species	Tissue	I_{50} (M)[a]
O. fasciatus	nervous tissue	2.0×10^{-4}
S. gregaria	brain	2.0×10^{-6}
S. gregaria	rectum	1.0×10^{-6}

[a] I_{50} = the molar concentration resulting in 50% inhibition.

dith, 1982a,b); and (3) the presence of cardenolide-resistant NA,K-ATPases in the nervous tissue (Moore and Scudder, 1986).

It is of obvious interest to determine if these specializations are also found in the monarch and other cardenolide-accumulating insects. The work of Vaughan and Jungreis (1977) suggests that cardenolide-resistant Na,K-ATPases are a factor in the low physiological cost of cardenolide sequestration in the monarch. Further work is needed to determine the importance of cardenolide-resistant Na,K-ATPases, high in vivo tolerance, and the mode of cardenolide uptake in the storage and tolerance of host plant cardenolides in other insects.

Despite the similarities seen in the sequestration and tolerance of cardenolides in *O. fasciatus* and *D. plexippus*, there are some interesting differences between these two insects, particularly in the greater efficiency and apparent nonsaturability of cardenolide uptake in *O. fasciatus* and the different distribution of cardenolides in the body tissues of these insects (Tables 5 and 6). Table 5 demonstrates that *O. fasciatus* sequesters more cardenolides per g dry weight of insect than has been recorded for any other nonlygaeid insect species. This high efficiency in cardenolide uptake seems to be directly related to the presence of the dorsolateral space in the integument of *O. fasciatus* and the apparently nonsaturable accumulation of large amounts of cardenolides in the

TABLE 5. GROSS CARDENOLIDE CONTENT OF INSECTS FEEDING ON SPECIES OF *Asclepias*

Insect species	Host plant species	Cardenolide content (mg/g dry wt)[a]	Ref.[b]
Lepidoptera			
Cycnia tenera (Arctiidae)	*A. humistrata*	2.99	1
C. inopinatus	*A. humistrata*	4.02	2
Danaus plexippus (Danaidae)	*A. humistrata*	4.45	3
	A. curassavica	3.19	4
	A. curassavica	3.42	5
	A. eriocarpa[c]	3.17	6
Coleoptera			
Tetraopes basilis (Cerambycidae)	*A. eriocarpa*[c]	0.76	7
T. melanurus	*A. humistrata*[c]	8.86	8
Hemiptera			
Oncopeltus fasciatus (Lygaeidae)	*A. curassavica*	23.78	9
O. cingulifer	*A. curassavica*[c]	20.30	10
Lygaeus kalmii kalmii	*A. vestita vestita*	31.88	11

[a] Digitoxin equivalents (mol wt = 728); values are the average for means of males and females in cases where the sexes were separated for analysis.

[b] References: 1, Cohen and Brower, 1983; 2, Nishio, 1980; 3, Cohen, 1985; 4, Roeske et al., 1976; 5, Brower and Glazier, 1975; 6, Brower et al., 1982; 7, Isman et al., 1977a; 8, Nishio et al., 1983; 9, Isman, 1977; 10, Isman, 1979; 11, Isman et al., 1977b.

[c] Field-collected insects; all others were reared in the laboratory on field-collected or greenhouse-grown plant material.

TABLE 6. DISTRIBUTION OF CARDENOLIDES AMONG TISSUES OF THREE INSECTS FEEDING ON MILKWEED[a]

Insect	Cardenolide content			
	Hemolymph	Gut	Fat body	Wings
O. fasciatus (adult)	Low	Low	Low	Low
Danaus plexippus (adult)	High	Moderate	Low	High
Danaus plexippus (larvae)	High	High	?	—
Cycnia inopinatus (larvae)	High	Low	?	—
Cycnia inopinatus (adult)	?	?	?	High

[a] Data compiled from Brower and Glazier, 1975; Duffey and Scudder, 1974; Moore and Scudder, 1985; Nishio et al., 1983; Nishio, 1980.

highly specialized inner epidermal layer (Duffey and Scudder, 1974; Duffey et al., 1978; Scudder and Meredith, 1982b; Moore and Scudder, 1985).

Table 6 demonstrates that the tissue distribution of cardenolides in *O. fasciatus* differs from that of *Danaus plexippus* as well as the arctiid moth *Cycnia inopinatus*. This may reflect interspecific differences in maximizing the defensive use and minimizing the toxic effects of food plant cardenolides (see Blum 1981, 1983). High cardenolide concentrations in the wings of *D. plexippus* and *C. inopinatus* and the gut of *D. plexippus* larvae appear to be effective in defense against predators (Brower and Glazier, 1975; Nishio, 1980; Blum, 1983). In contrast, the low cardenolide content in the wings, gut, and other tissues of *O. fasciatus* probably reflects the greater effectiveness of concentrating cardenolides in the dorsolateral space for predator defense in this insect (Duffey and Scudder, 1974; Scudder and Meredith, 1982b; Moore and Scudder, 1985).

PREDATION EXPERIMENTS WITH LYGAEINAE

To date, few experiments have been carried out to investigate the effectiveness of cardenolides in *O. fasciatus* predator defense. Gelperin (1968) noted that *O. fasciatus* was rejected by mantids, and Feir and Suen (1971) mention that *O. fasciatus* is not acceptable to the common fence lizard, but these authors did not distinguish the effectiveness of cardenolides from the effect of the scent gland exudates. Similarly, the experiments of Abushama and Ahmed (1976) with *Spilostethus pandurus* failed to make this distinction. Sillén-Tullberg et al. (1982) reported that the great tit (*Parus major*) found adults of *Lygaeus equestris* more distasteful than larvae, and attributed the differences to the concentration of cardenolides, but noted the occurrence of scent glands in both stages. Sillén-Tullberg et al. (1982) found that many larvae and adults of *L. equestris* survived being seized and immediately dropped by the predator.

Endogenous defensive chemicals are certainly effective against many terrestrial predators (Staddon, 1979), and since these are released first by *O. fasciatus* when disturbed, this factor must be taken into account in any assessment of the function of cardenolides. There is some evidence to suggest that the cardenolides in *O. fasciatus* may be effective in some instances, but there is little experimental evidence for universal predator defense to vertebrates (unpublished data). However, Berenbaum and Miliczky (1984) indicate that cardenolides can be effective against mantids, although they are not effective against all insect predators (Sauer and Feir, 1972). Levey (1983) suggested that the cardenolides in *Spilostethus* may protect these insects from reduviids, but not from mantids. Jones (1932, 1934) found that *Oncopeltus sexmaculatus* and *Lygaeus kalmii* were left uneaten by birds and that *L. kalmii* (fresh and dried for up to 60 days) was refused by ants, although he drew attention to the fact (Jones, 1932) that the relative unacceptability of many insects to predators may depend on many factors, not only on the plant-derived deterrants.

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ECONOMICS OF CHEMICAL DEFENSE IN CHRYSOMELINAE

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Abstract—Chemical defense in chrysomelid larvae (subtribe Chrysomelina and Phyllodectina) is reviewed. Most species secrete autogenous monoterpenes. The diversity of their secretion is interpreted as a mechanism to reduce adaptation by predacious arthropods. The consequences of a host plant shift to the Salicacae are explored. Salicin from these host plants is used as a precursor for the salicylaldehyde secreted by the larvae of many species. This offers several advantages. It provides the larvae with an inexpensive and efficient defense. The recovery of the glucose moiety of the salicin contributes significantly to the larval energy budget. Adults sequester salicin in the eggs at concentrations which are toxic to ants. Owing to this maternal provisioning, neonate larvae produce salicylaldehyde from hatching onwards, whereas other species secreting monoterpenes are not protected at hatching. The secretion of salicylaldehyde by different species is considered to be chemical mimicry reinforcing visual aposematic signals.

Key Words—Defensive secretion, egg defense, allomones, *Salix*, salicin, Coleoptera, Chrysomelidae, monoterpenes, salicylaldehyde.

INTRODUCTION

Chrysomelidae, or leaf beetles, are a family of phytophagous insects, often forming large aggregations on their food plants. This makes them particularly apparent to predators and parasitoids, and spectacular chemical, mechanical, and behavioral defensive mechanisms have evolved in this family (Deroe and Pasteels, 1982). Defensive glandular secretions of both larvae and adults are particularly prominent in the brightly colored Chrysomelini, a tribe of the subfamily Chrysomelinae.

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In temperate regions, Chrysomelinae are often found in damp, open, relatively undisturbed habitats such as riversides, marshes, and meadows. There, the favored food plants include both herbs [docks (Polygonaceae), buttercups (Ranunculaceae) or mints (Labiatae)] and also some trees and shrubs [willows and poplars (Salicaceae) or alders (Betulaceae)]. The Chrysomelinae are monophagous or oligophagous on these plants.

Within the Chrysomelinae there is a fairly good parallel between systematic position and the distribution and chemical nature of chemical defense. This is true both in the larval and adult stages. At least in the larvae, the apparent exceptions can be explained by the influence of particular host plants. In the present paper, we will give an overview of larval chemical defense in this group and discuss in more detail the influence of host plant secondary chemistry.

DISTRIBUTION OF CHEMICAL DEFENSE IN CHRYSOMELINAE LARVAE

The larvae of the subtribe Chrysomelina and of the genus *Phratora* in the subtribe Phyllodectina all possess nine pairs of glands distributed over the meso- and metathorax and the first seven abdominal segments. The glands are eversible, and when the larvae are disturbed, a drop of secretion appears at the tip of the everted reservoirs. Most of this secretion can be withdrawn back into the reservoirs during the retraction of the latter into the body (Hollande, 1909; Garb, 1915). According to the descriptions of Cuénot (1896), Hollande (1909), Garb (1915), Berti (1968), and Renner (1970), there seems to be little morphological difference between the glands of the different genera.

The principal classes of compounds found so far in the larval secretions of Chrysomelini are listed in Table 1, together with the host plant of each species. Six different methylcyclopentanoid monoterpenes have been identified (Figure 1) (review in Pasteels et al., 1984). Many species secrete a mixture of these compounds which are most likely autogenously synthesized. The larvae producing them feed on a range of plants, and no direct host plant influence can be detected. The secretions of different species within a genus are, in some cases, more different than the secretions from species belonging to different genera. In addition quantitative and qualitative differences exist between geographically isolated populations of the same species.

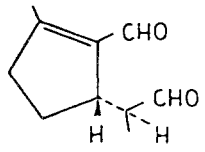
In Table 2, the data for different populations of *Plagiodera versicolora* are summarized. The European samples are rather homogenous, characterized by the presence of plagiodial and plagiolactone. They differ strongly from both the Japanese and the previous North American samples. The Japanese *Plagiodera* is a different subspecies but *Pl. versicolora* was introduced into North America from Europe. The reader must be cautioned about possible artifacts; these monoterpenes are unstable, and, for example, plagiodial can be easily transformed

TABLE 1. OCCURRENCE OF PRINCIPAL CLASSES OF COMPOUNDS FOUND IN LARVAL SECRETIONS OF CHRYSOMELINI SPECIES[a]

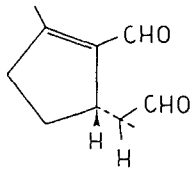
	Compound[b]					
	M	S	B	P	J	Host Plant
Subtribe Chrysomelina:						
8 *Chrysomela* sp.		7	(3)	1		Salicaceae, and *Alnus*
1 *Gastrolina* sp.					1	*Juglans*
3 *Gastrophysa* sp.	3					*Rumex*
1 *Hydrothassa* sp.	1					*Ranunculus*
1 *Linaeidea* sp.	1					*Alnus*
2 *Phaedon* sp.	2					*Brassica* and *Nasturtium*
1 *Prasocuris* sp.	1					*Ranunculus*
1 *Plagiodera* sp.	1	(1)				*Salix*
Subtribe Phyllodectina:						
4 *Phratora* sp.	3	1				Salicaceae

[a] Abbreviated from Pasteels et al., 1984.
[b] Abbreviations: M, methylcyclopentanoid monoterpenes; S, salicylaldehyde; B, benzaldehyde; P, phenylesters; J, juglone; For each genus, the number of species secreting one or the other classes of compounds are given. ( ), minor component or only present in some populations.

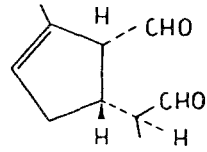
to chrysomelidial (reported from the American population) when stored at room temperature in the light. Even when stored for long periods in sealed capillaries at −20°C, degradation occurs. We recently analyzed freshly collected secretions from the United States (Wisconsin). This sample contained plagiodial and



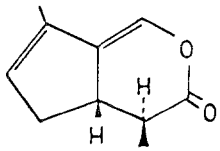
Chrysomelidial



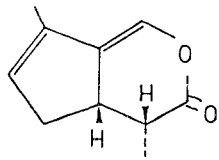
Epichrysomelidial



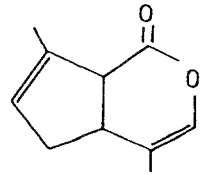
Plagiodial



Plagiolactone



Epiplagiolactone



Gastrolactone

FIG. 1. Methylcyclopentanoid monoterpenes identified in the larval secretions of Chrysomelini.

TABLE 2. PROPORTIONS OF DIFFERENT METHYLCYCLOPENTANOID MONOTERPENES IN SEVERAL POPULATIONS OF *Plagiodera versicolora*

Country	Methylcyclopentanoid monoterpenes[a]					Reference
	1	2	3	4	5	
Japan		3	90	3	3	Sugawara et al. (1979)
U.S.A.	66*			33		Meinwald et al. (1977)
U.S.A.			81	19		This paper
England			73	27		This paper
Belgium			70	30		Pasteels et al. (1984)
Switzerland			33	67		This paper
South of France			47	53		This paper

[a] 1: chrysomelidial, 2: epichrysomelidial, 3: plagiodial, 4: plagiolactone, 5: epiplagiolactone.
*66% of compound 1 and 2.

plagiolactone but no chrysomelidial. It has been reported that some populations of European *Plagiodera* larvae also produce salicylaldehyde (Hollande, 1909; Pasteels et al., 1984). In view of the relative homogeneity of the secretions from larvae originating from England to the south of France, this observation needs to be confirmed.

The significance of the diversity of chemical defenses between sympatric species or between allopatric populations of the same species is not clear. In most cases, no pheromonal function is known which could explain the specificity of the blends. The larvae are often gregarious, but defensive secretions do not have an aggregating function. Groups of mixed species are easy to obtain in the laboratory. Larvae experimentally made devoid of secretion remain grouped. It has been suggested that the secretion of the larvae of *Gastrophysa viridula* may lower intraspecific competition. Indeed, the larvae evert their glands at the approach of adults of the same species which are repelled (Renner, 1970). This epideictic function does not necessarily imply highly specific pheromones. Indeed, Raupp et al. (1984) suggested that the secretion might also lower interspecific competition by repelling other herbivores.

The diversity of defensive secretions could be due to genetic drift in the absence of selective pressure for uniformity. Tschinkel (1975), in his study of the defensive secretions of tenebrionids, suggested that their precise composition was not critical as long as the secretion met certain physical requirements. Variation on the theme would then be possible without any particular ecological significance. On the other hand, it is also possible that intraspecific variation between geographically isolated populations reflects adaptation to the different communities in which the beetles are living. If this is the case, secretions from

different species inhabiting the same food plant should be more similar to each other than those of species living in different habitats. This does not seem to be true. In nature, larvae of *Hydrothassa marginella* and *Prasocuris phellandrii*, for example, are often found together on *Ranunculus* spp.. They are probably exposed to the same set of potential predators and parasitoids, but they produce different mixtures of compounds.

Selection for diversity would be another possible explanation of the specificity of the defensive secretions in sympatric species. So far, the activity of the defensive compounds has only been demonstrated with "standard laboratory predators" such as ants, for which at least chrysomelidial and salicylaldehyde are deterrent (Blum et al., 1978; Sugawara et al., 1979; Pasteels et al., 1983). Using adult sawflies (*Tenthredo olivacea*), predator observed to feed on chrysomeline larvae in nature, Pasteels and Grégorie (1984) demonstrated that the sawflies can be conditioned and show a preference for the larvae producing the secretion they have already experienced. such behavior of predators favors the maintenance or evolution of diversity of defensive secretions in sympatric species. Especially for species feeding next to each other on a plant, a high diversity of defensive blends may be a good strategy to prevent adaptation by the predator. Similarly, interspecific and intraspecific diversity of plant secondary metabolites is thought to limit the adaptation of herbivores to these plants (Dolinger et al., 1973; Atsatt and O'Dowd, 1976).

The wide distribution of methylcyclopentanoid monoterpenes in the secretions of the larvae of Chrysomelina dnd *Phratora* suggests that these compounds represent their primitive defensive secretion. As already stated, these products are most likely synthesized de novo, since they have no obvious precursors in the food plants; for example, chrysomelidial is secreted by the larvae of different species feeding on *Alnus*, *Rumex*, *Ranunculus*, *Brassica*, *Nasturtium* and *Salix*, respectively.

The larvae of some species of Chrysomelina and *Phratora*, however, secrete not monoterpenes but aromatic compounds such as salicylaldehyde or benzaldehyde. These species feed on trees belonging to the Salicaceae or Betulaceae. In contrast to the situation described for the species producing monoterpenes, in at least some species producing aromatic compounds, the defensive secretion is derived from a host plant precursor. In these species there is no longer a chemical diversity in the defensive blends.

Pasteels et al. (1982) suggested that the secretion of aromatic compounds by chrysomeline larvae can be best explained as a consequence of a shift of food plant, associated with speciation, from herbs to Salicaceae or Betulaceae. The secretion of salicylaldehyde would then be an adaptation to the secondary chemistry of the Salicaceae.

In the remainder of this paper we will examine in more detail the mechanism of such adaptation and its consequence for the willow-feeding leaf beetles.

FOLIAR SECONDARY CHEMISTRY OF THE SALICACEAE

Among north temperate woody plants, the European Salicaceae are remarkable for the presence of phenolglycosides in their bark and in the leaves of some species. The leaves of the species containing phenolglycosides do not contain proanthocyanidins, a form of condensed tannins found in the leaves of the remaining salicaceous species (Hegnauer, 1973).

Salicaceous bushes and trees might have been particularly likely objectes of host plant switches of primitively herb feeding Chrysomelinae for various reasons. First, they are one of the most abundant woody plants in the biotopes favored by the beetles. Secondly, the leaves of these *Salix* species without proanthyocyanidins might be a less formidable evolutionary barrier for an herbivore adapted to digesting herbs (Rowell-Rahier and Pasteels, 1982) than those of most woody plants, which typically contain tannis.

Using published food plant lists for the weevils, the sawflies, and the caterpillers of the British moths, we searched for a correlation between the presence or absence of phenolglycosides in the leaves and the degree of dietary specialization of the insects feeding on these leaves.

The results showed that the *Salix* spp. with phenolglycosides (*S. babylonica*, *S. fragilis*, *S. incana*, *S. nigricans*, *S. pentandra*, *S. purpurea*, *S. repens* and *S. triandra*) tend to be the hosts of specialized herbivores and are avoided by generalists. Conversely, *Salix* spp. without phenolglycosides in their leaves (*S. alba*, *S. aurita*, *S. caprea*, *S. cinerea*, and *S. viminalis*) tend to be eaten by more generalist insects and are avoided by the more specialized ones. Moreover, the faunas of the different *Salix* species with phenolglycosides in their leaves are more similar to each other than to the faunas of the *Salix* species having no phenolglycosides in their leaves (Rowell-Rahier, 1984).

Salicin is one of the most frequently occurring of these glycosides. It is known to be toxic (Marks et al., 1961) and, together with the other phenolglycosides present in the leaves, may act as a wide-range feeding deterrent against nonadapted herbivores (Edwards, 1978; Tahvanainen, 1985). Salicin also proved to be deterrent and highly toxic for ants. In a toxicity test, 50% mortality was reached after two days of experimental regime, during which the LD_{50} may be estimated as 5 μg of salicin per ant in two days (Pasteels et al., in preparation).

INFLUENCE OF HOST PLANT ON DEFENSIVE SECRETION OF CHRYSOMELINE LARVAE

The best studied example within this group of beetles, whose defensive secretions are dependent on the host plant, is *Ph. vitellinae*, one of the species whose larvae secrete salicylaldehyde. It was suggested long ago that salicylal-

dehyde is derived from host plant phenolglycosides (Wain, 1943) such as salicin and populin. We tried to verify this hypothesis experimentally for *Ph. vitellinae* and *C. tremulae*, using two different approaches (Pasteels et al., 1983)

First, to confirm the role of salicin as a precursor of the salicylaldehyde secreted by the larvae of *Ph. vitellinae*, the consequences of a salicin-free diet on the defensive secretion of the larvae were examined. In nature, adult *Ph. vitellinae* are never seen on *S. caprea*, and the larvae do not normally accept these hairy leaves as food. After being denuded of trichomes, however, the leaves of *S. caprea* were readily accepted by the larvae of *Ph. vitellinae*. Larvae developed normally on "shaved" *S. caprea* leaves but did not secrete salicylaldehyde. Addition of salicin to *S. caprea* restored the secretion of salicylaldehyde. Salicin is thus a precursor for the larval defensive secretion, but it is not obligatory for food acceptance by the larvae. The hairiness of the leaves of *Salix* species containing no salicin is responsible for the mortality due to starvation of the young larvae reared on them.

Secondly, by feeding larvae of *C. tremulae* with labeled salicin, we demonstrated that also in this species salicylaldehyde is derived from salicin. This is most likely true for all species of the genus feeding on salicaceous plants.

After confirming the influence of the host plant secondary metabolite, we tried to establish the relationship between the quantity of precursor ingested and the quantity of defensive secretion produced and to determine where in the body this transformation occurred. The fate of the glucose moiety split from the original glycoside molecule was also examined.

The results show that the concentration of salicylaldehyde in the secretion is positively correlated with the amount of salicin in the food of the larvae. The transformation of salicin into salicylaldehyde probably occurs mostly in the defense glands, since the β-glucosidase activity is four times higher in the glands than in the gut (Pasteels et al., 1983).

The concentrations of glucose and of salicylaldehyde in the secretion are far from being equimolar, indicating that the glucose formed by salicin hydrolysis was in great part recovered by the larvae and transferred back into the blood (Pasteels et al., 1983).

WHEN A STRONG DEFENSE IS GOOD FOR THE ECONOMY

The energetic costs of defense are frequently referred to in discussions of the ecology and evolution of chemically defended organisms; it is generally assumed that any loss is fitness caused by these costs must be outweighted by the gain in fitness secured by an effective defense. However, there has been virtually no attempt to demonstrate these energetic costs or to measure them quantitatively, especially in animals (Blum, 1981).

Although no direct experimental data are available, it is very likely that

the methylcyclopentanoid monoterpenes secreted by the larvae of chrysomeline species (see above) arise from geranylpyrophosphate via mevalonic acid, as do other terpenes (Blum, 1981). This must involve finite, although perhaps small, costs. The salicylaldehyde secreted by other species is formed by the enzymatic hydroloysis of the glucosidic bond of salicin. A preliminary calculation shows that when the glands are emptied and refilled daily, this glucose could supply up to one third of the caloric requirements of the larva (see Table 3).

Both types of defense occur in closely comparable species of the same genera feeding on Salicaceae. This assemblage is thus very suitable for an investigation of the cost of defense, which would be expected to be maximal in those species synthesizing monoterpenes de novo and minimal in those secreting salicylaldehyde, which profit from the use of a plant precursor and also by gaining glucose.

Larvae of *Phratora tibialis*, *Ph. vitellinae*, *Plagiodera versicolora*, and *Chrysomela 20-punctata* were reared in the laboratory from egg to adult on appropriate *Salix* spp.; these, together with the chemical nature of the secretions of the beetles, are listed in the first three columns of Table 4. Each species sample was divided into control and experimental groups, which did not differ in population density, quantity, and quality of food provided, sex ratio, mortality during the experiment, or in time elapsed before eclosion as adults. Both groups were disturbed daily and induced to evert their glands: the exposed secretion was collected only from the experimental group, which then refilled their glands by the following day. The assumption is made that the control

TABLE 3. DERIVATION OF BENEFIT OF GLUCOSE RECOVERY IN *Ph. vitellinae* LARVAE[a]

A. Energy requirements
   Oxygen consumption: 0.5 ml O_2/g FW/hr
   1 ml O_2 consumed = 5.05 cal
   3.68 cal = 1 mg glucose, incorporating correction for energy loss during digestion.
   Mean weight of field-collected third-instar larvae: 8.54 mg; thus, one larva requires 140.65 μg glucose/day
B. Energy gain from salicin metabolism
   Daily salicin ingestion: 0.46 μM
   Daily glucose ingestion in form of salicin: 0.46 × 180 = 82.8 μg of which (0.46 − 0.20*) × 180 reach the glands = 46.8 μg
   [*: the glands only start to produce secretion after ingestion of 0.20 μM of salicin].
   99.9% of this glucose is recovered by the larvae; thus the larva recovers 46.7 μg glucose/day, covering 33.2% of the daily energy requirement.
   Rather than the correction marked with an asterisk above, one could also take into account the fact that 20% of the total glucosidase activity is found in the gut. This would suggest that 80% (= 65.5 μg/day) of glucose present in the original salicin is recovered from the glands, covering 46.6% of the larval daily requirement.

[a] Data from Pasteels et al. (1983) and Keister and Buck (1974).

TABLE 4. MEAN WEIGHTS OF ADULT CHRYSOMELINAE WHEN DEFENSIVE SECRETION OF LARVAE HAS (EXPERIMENTAL) OR HAS NOT (CONTROL) BEEN REMOVED DAILY[a]

Chrysomelinae species	Host-plant species	Defence nature	Mean weights (mg ± sd (*N*))		*P*
			Control	Experimental	
Ph. tibialis	*Salix purpurea*	M	5.89 ± 0.54 (52)	5.63 ± 0.64 (33)	*
Pl. versicolora	*S. purpurea*	M	7.09 ± 1.39 (82)	6.68 ± 1.11 (78)	*
Ph. vitellinae	*S. nigricans*	S	5.41 ± 0.63 (7)	6.25 ± 0.52 (14)	**
Ph. vitellinae	*S. caprea*	none	5.41 ± 0.63 (7)	5.39 ± 0.74 (7)	NS
Ph. vitellinae + reduced salicin		S	5.41 ± 0.63 (7)	5.49 ± 0.08 (13)	NS
Ch. 20punctata	*S. nigricans*	S + X	25.10 ± 5.19 (79)	25.60 ± 3.19 (26)	NS
Ch. 20punctata	*S. caprea*	X	25.20 ± 2.80 (8)	22.60 ± 3.70 (120)	*

[a] Means and standard deviations were calculated for each group (columns 4 and 5).

[b] Abbreviations: M, methylcyclopentanoid monoterpenes; S, salicylaldehyde; X, unidentiified compounds; *N*, sample size; *P*, probability levels of difference between experimental and control groups (Student's *t*-test); **, $P < 0.01$; *, $P < 0.05$; NS, $P > 0.05$.

group, which do not lose their secretion, will synthesize at a much lower rate than the experimental group.

Within 12 hr of eclosion, and before feeding, the resulting adults were weighed, and this value was used as a measure of larval growth and, by inference, of adult fitness. In those species secreting autogenous monoterpenes (*Ph. tibialis* and *Pl. versicolora*), the mean weight of the experimental group is significantly lower than that of the control: a daily renewal of the secretion is thus a drain on their metabolism. It is not excluded that this is due to the fluid lost occurring during collection of the secretion in the experimental group. In the species which secrete exclusively salicylaldehyde (*Ph. vitellinae*), the experimental group is significantly heavier than the control. This weight gain is probably associated with the extra glucose obtained from the hydrolysis of salicin. When the larvae of this species are completely deprived of salicin [by raising them on *S. caprea*, which does not contain this compound (Hegnauer, 1973)] or allowed only one fifth of their normal intake, the experimental imaginal fresh weights do not differ from those of the control group feeding on *S. nigricans*. Additionally, preliminary results suggest that when *Ph vitellinae* is reared undisturbed on *S. nigricans* leaves with added glucose, the adult weights are comparable to those of the experimental group on the same plant. The last species (*Ch. 20-punctata*) reared on *S. nigricans*, secretes a mixture of both salicylaldehyde and various compounds which are as yet unidentified but most likely not directly derived from the host plant. Here there is no difference betweeen the control and the experimental groups, suggesting that the glucose recovered from salicin degradation is balanced by the biosynthetic cost of the other compounds. This hypothesis is supported by the finding that in the same species raised on *S. caprea* (i.e., without salicin) the experimental animals weigh significantly less than the controls.

These results show, to our knowledge for the first time, that an autochthonously produced secretion entails an appreciable metabolic cost. This cost is expressed as a loss of weight of the adult, which in many other insects has been demonstrated to be associated with reduced fecundity and fitness (references in Raupp and Denno, 1984). These costs can be avoided by the use of an appropriate plant precursor: where this is a glucoside, as in the present example, the resultant glucose production can convert the loss into a profit. Leaves, because of their high fiber and cellulose content, usually have a low available calorific density (McNab, 1978), and this spin-off metabolic advantage could be of particular relevance to a folivorous insect. Plant glucosides are not, in general, available to herbivores as energy sources because of the potential toxicity of the aglycone; in chrysomeline larvae, it is the localization of the greater part of the degradation process in the external glands which makes this feasible. Lastly, larvae in the field are likely to be frequently disturbed by parasites and predators, or even by the movement of leaves in the wind, and will tend to lose

secretion by contact with these agents. If this is true, the laboratory situation, where the secretion is collected daily, may reflect the natural situation rather closely.

The transformation of salicin to salicylaldehyde does not only provide an inexpensive or even profitable defense for the larvae but also an effective one. Laboratory tests with a generalist predator such as the ant *Myrmica rubra*, often seen exploring the foliage of the host plants of various leaf beetles, show that salicylaldehyde is a potent deterrent, more active than salicin or its aglycone saligenin (Pasteels et al., 1983).

DEFENSE OF EGGS AND NEONATE LARVAE

At hatching, the larvae are clustered and immobile on the leaves. They are thus highly exposed to predation and also to cannibalism. An innate defense might therefore be critical. The observation that, on the one hand, the larvae of some species already produce secretion at hatching, before feeding, and that, on the other hand, some other species do not have "full" glands at the beginning of their larval life, is therefore very interesting. It is only the neonate larvae of those species secreting salicylaldehyde which have functional glands on hatching. No larvae of any species seem to be able to produce monoterpenes at eclosion. Indeed, chemical analysis demonstrated that, paradoxically, only those larvae which depend on salicin normally found in their food seem to be able to produce a defensive secretion before feeding. A possible explanation could be that salicin is sequestered in the eggs and used as a precursor by the neonate larvae. To test this hypothesis, we studied the occurrence of defensive allomones in chrysomelid eggs.

The eggs, often brightly colored, are laid in clusters on the foliage and are thus highly exposed to predation. In the field, survival of the eggs can be as low as 25% (Wade and Breden, personal communication). There might therefore be a strong selective pressure for the eggs to be protected. Indeed, chemical analysis showed that the two isoxazolinone glycosides characteristic of the adult defensive secretion (Pasteels et al., 1982) are present in the eggs of all species studied (Table 5). The occurrence of identical compounds in the eggs and in the adult defensive glands was already reported in the Chrysomelina producing cardenolides (Pasteels and Daloze, 1977) and could represent a general feature of the Chrysomelinae. However, these isoxazolinones were never detected in neonate larvae, and, at least in one species, it was found in the egg shells left after hatching.

Additionally, the eggs of some of the Salicaceae feeders contain salicin, and those species are also those whose larvae secrete salicylaldehyde. Since the salicin was no longer detected in the neonate larvae, it must have been trans-

TABLE 5. PRESENCE (+) OR ABSENCE (−) OF SALICIN AND ISOXAZOLINONES IN EGGS OF SPECIES BELONGING TO SUBTRIBES CHRYSOMELINA AND PHYLLODECTINA[a]

	Host plant	Salicin	Isoxazolinones
Subtribe Chrysomelina			
Ch. populi[b]	Salicaceae	+	+
Ch. tremulae[b]	Salicaceae	+	+
Ch. saliceti[b]	Salicaceae	+	+
Ch. 20-punctata[b]	Salicaceae	+	+
G. viridula	*Rumex*	−	+
Pl. versicolora	Salicaceae	−	+
Subtribe Phyllodectina			
Ph. vitellinae[b]	Salicaceae	+	+
Ph. laticollis	Salicaceae	−	+
Ph. tibialis	Salicaceae	−	+

[a] Abbreviated from Pasteels et al. (submitted).
[b] Species whose larvae produce salicylaldehyde from their defensive glands.

formed into salicylaldehyde. The sequestration of salicin in the eggs explains the fate of this plant toxin in the adult females. It protects the eggs themselves and also provides precursor for protection of the neonate larvae at a very vulnerable life-stage when other species are not protected. The amount of salicin found, on average, in one *Chrysomela* egg is about the LD_{50} dose for an ant (Pasteels et al., 1985).

CONCLUSIONS

One of the challenges of chemical ecology is to interpret chemical results in an ecological context. Ecomones (semiochemicals) often appear as "wisdom" remaining from past history. The selective pressures from which they result are difficult to identify and might even be absent from the biotopes in which the organisms live today.

We would like to suggest a possible scenario for the evolution of chemical defense in the larvae of leaf beetles. The secretion of methylcyclopentanoid monoterpenes in species feeding on herbs could be a response to the pressure of numerous predacious arthropods (or parasitoids) exploring the foliage. These compounds, classified as volatile irritants, are well known ant repellents. The larvae of these beetle species are rather small and inconspicuous, feeding on the lower surface of the leaves. Larval defense is not associated with aposematism, and preliminary experiments suggest that birds are only slightly re-

pelled by their secretions. The chemical diversity of the secretion might prevent adaptation by predacious arthropods.

Shifts of host plants occurred during the evolution of chrysomeline beetles, and one of these shifts had a drastic influence on chemical defense: the shift to salicaceous species, with leaves rich in phenolglycosides. It has occurred at least three times (Pasteels et al., 1984), and it frequently results in the use of salicin as a precursor of the defensive secretion.

The utilization of salicin offers multiple advantages for chrysomelid beetles. First, it allows them to exploit a set of host plants otherwise well protected from generalist herbivores by phenolglycosides. Second, it provides the larvae with an inexpensive defense, an effective deterrent against generalist predators. Third, the recovered glucose contributes significantly to the larval energy budget and is positively correlated with an increased relative growth rate of the larvae. Fourth, the adults sequester salicin in the eggs at concentrations which are toxic for ants. Fifth, neonate larvae of the species producing salicylaldehyde are protected from hatching onwards, whereas other species secreting monoterpenes are not so protected.

The larvae of species feeding on Salicaceae produce a much larger volume of secretion than those synthesizing monoterpenes (three or four times larger for larvae of equal sizes). This is probably made possible by the relative availability of the salicylaldehyde derived from the host plant. Such abundant production of a volatile irritant deters predaceous birds as well as arthropods. These larvae (e.g., *Chrysomela* spp.) are large, gregarious, and aposematic, and several species feed on the upper surface of the leaf. The utilization of salicin has led to chemically homogenous secretions. This is probably advantageous against predacious birds. It reinforces the aposematic signal and can be considered as a case of chemical mimicry.

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ALLELOPATHIC POLYACETYLENES FROM *Centaurea repens* (RUSSIAN KNAPWEED)

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Abstract—The allelopathic weed Russian knapweed (*Centaurea repens*) was found to contain polyacetylenes VIII–XIV in the roots. Dose vs. response of the root length elongation against lettuce, alfalfa, barnyard grass, and red millet showed IX to be active. Closely related isomers were not active. Examination of the soil surrounding the Russian knapweed roots revealed the presence of IX in sufficient concentration to have an appreciable effect on the surrounding plant community.

Key Words—Allelopathy, polyacetylenes, Russian knapweed, *Centaurea repens, Compositae*

INTRODUCTION

It has been recognized for some time (Fletcher, 1963) that Russian knapweed (*Centaurea repens* L., Compositae), a rapidly spreading noxious weed, exhibits many of the characteristics of an allelopathic plant species. Earlier work (Stevens, 1982) showed the presence of a number of sesquiterpene lactones, many of which were shown to have some phytotoxic activity (Stevens and Merrill, 1985). However, both the concentrations of these sesquiterpene lactones in the plant and their toxicity to test species left some doubt as to whether these were responsible for the total phytotoxicity of Russian knapweed to the surrounding plant community.

Many polyacetylenic compounds have been isolated from composites (Bohlmann et al., 1973) but relatively few have been shown to be phytotoxic. Specifically, matricaria ester (I, ME), dehydromatricaria ester (II, DME), lachnophyllum ester (III, LE), and the two matricaria ester derivatives IV and V

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have been shown to have activity against rice seedlings (Kawazu et al., 1969; Kobayashi et al., 1974, 1980; Numata et al., 1973; Ichihara et al., 1976, 1978) (Figure 1). Since some of these compounds were found in the soil, it was concluded that the C_{10} polyacetylenes are probably allelopathic substances of ecological importance (Kobayashi et al., 1980). With the exception of the two

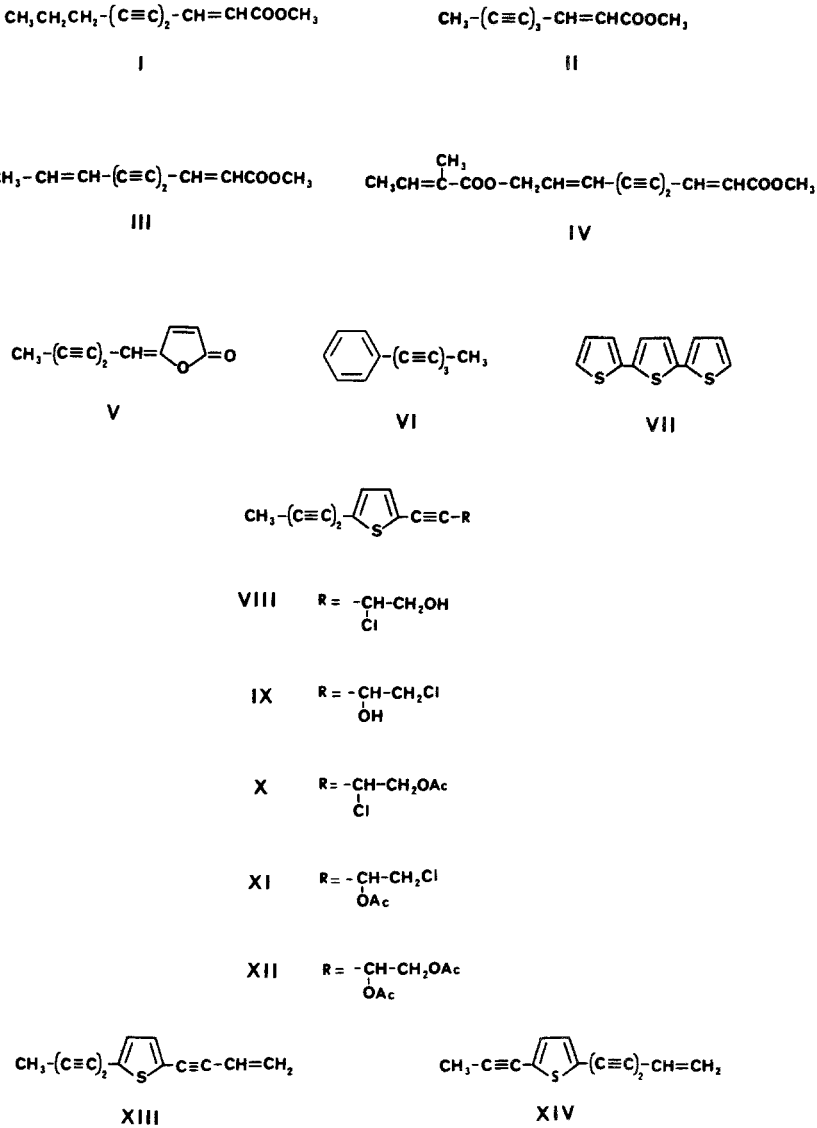

FIG. 1. Structures of Polyacetylenes

polyacetylenes phenyheptatriyne (VI, PHT) and α-terthienyl (VII), which have been found in the leaves of the tropical weed *Bidens pilosa* and the roots of common marigold *Tagetes erecta*, respectively (Campbell et al., 1982), there appear to be no data which would suggest that other polyacetylenes are phytotoxic. It must be recognized, however, that the vast majority of polyacetylenes described in the literature have never been tested for biological activity, particularly phytotoxicity.

METHODS AND MATERIALS

Extraction and Isolation. Roots of Russian knapweed were collected throughout its growing season near Discovery Bay (Hwy. 4 between Brentwood and Stockton), California. The severed roots were immediately placed on Dry Ice then ground in a rotating plate mill with Dry Ice to approximately $\frac{1}{8}$-in. particle size. The Dry Ice–root mixture was then slurried with ether–Skelly-F (1 : 2) in a larger beaker and allowed to warm to room temperature. Light was excluded to prevent any photodecomposition of the polyacetylenes. After warming to room temperature, the mixture was filtered and the solvent reduced in volume on a rotary evaporator at approximately 25°C.

Final separation of the polyacetylenes was accomplished by a combination of column chromatography on silica gel with benzene and preparative TLC (silica gel) using a variety of solvents, i.e., ether–Skelly-F (1 : 9), benzene, and cyclohexane–acetone (4 : 1). Polyacetylenes were collected from the preparative plates by scraping the bands from the plate and extracting the silica gel with ether. In all instances, care was taken to exclude light and keep the temperature of the solvents below 30°C.

Isolation of Polyacetylenes from Soil. Soil (375 g wet) from around the roots of Russian knapweed was sieved to remove organic material then placed in a chromatography column. Ether–Skelly-F (1 : 2) was percolated through the column and collected (2 : 1). The solvent was evaporated under reduced pressure and the residue chromatographed on preparative plates (20 × 20 cm. × 0.5 mm). Bands were scraped off and extracted with ether.

Identification of Compounds. Isolated compounds were analyzed by UV and [^{1}H]NMR spectroscopy and compared with reported values.

Bioassay. The seedling inhibition assays were carried out as follows: Seeds of lettuce (*Lactuca sativa*, black-seeded Simpson), barnyard grass (*Echinochloa crusgalli*), alfalfa (*Medicago sativa*), and red millet (*Panicum miliaceum*) were germinated on 0.5% agar (Bacto-Agar, Difco Laboratories, Detroit, Michigan) in a growth chamber set at 16-hr, 68°F days and 8-hr, 58°F nights. Twelve seedlings of each species were placed on 0.5% gels (20 ml agar/19 cm Petri dish) which had previously been slurried with the prescribed amount of polyacetylene in ether. In our tests, the concentrations were: 0, 5, 10, 20, 40, and

80 ppm. The polyacetylenes were added to the still-fluid gel at approximately 30°C to avoid thermal decomposition. Controls had only solvent incorporated into the gels. The seedlings were then placed in the dark at 20°C for 24 hr and root length measured to the nearest millimeter. Root growth studies with ME were carried out for 48 hr. Of the 12 seedlings whose root length were measured, the longest and shortest were discarded and the remaining 10 were used in the statistical analyses. In each case two replicates were run.

Data Analysis. The results of the seedling growth assays were analyzed separately using the Washington, D.C., Computing Center facilities and Statistical Analysis System (SAS Institute, Inc., Cary, North Carolina, 1982). The transformed data were subjected to Cochran's test for homogeneity of variances of all treatments and the least significant difference (LSD) test for differences between all treatment means.

RESULTS AND DISCUSSION

Five polyacetylenes, VIII–XII, obtained in pure form from Russian knapweed have been identified. Table 1 shows their respective UV and NMR spectra along with the literature reference. In addition to those listed in the table, com-

TABLE 1. UV AND NMR DATA OF POLYACETYLENES VIII–XII

Compound	UV(ether), λ_{max}[a]	NMR($CDCl_3$), 90 MHz, δ values(TMS)	Reference
VIII	344, 324, 270, 250, 206	2.04(s,3H,Me), 3.92(d,1H,J = 6), 3.97(d,1H,J = 5), 4.88(dd,1H, J = 5,6), 7.12(s,2H)	Bohlmann, 1965
IX	342, 322, 250, 236, 210	2.00(s,3H,Me), 3.70(d,1H,J = 6) 3.71(d,1H,J = 4), 4.79(dd,1H, J = 4,6), 7.02(d,1H,J = 3.5)	Bohlmann, 1970
X	342, 324, 270, 250, 236, 210	2.00(s,3H,Me), 2.08(s,3H,COMe), 4.37(d,1H,J = 6), 4.38(d,1H, J = 5), 4.91(dd,1H,J = 5,6), 7.08(s,2H)	Bohlmann, 1965
XI	342, 322, 250, 238	2.00(s,3H,Me), 2.10(s,3H,COMe), 3.73(d,2H,J = 6), 5.78(t,1H, J = 6), 7.06(s,2H)	Bohlmann, 1970
XII	342, 322, 248, 210	2.00(s,3H,Me), 2.07(s,3H,COMe), 2.09(s,3H,COMe), 4.22(dd,1H, J = 5,12), 4.34(dd,1H,J = 7,12), 5.80(dd,1H,J = 5,7), 7.02(s,2H)	Bohlmann, 1965

[a]Intensities were similar for all compounds [i.e., 342(2×10^4), 322(2.2×10^4), 250(8×10^3), 210(1.7×10^4)].

pounds XIII and XIV were isolated as a mixture. Lack of material, coupled with their instability, precluded separating the two isomers; however, they were readily identified by comparing the UV and NMR spectra of the mixture with those reported (Bohlmann et al., 1965).

Each of the isolated polyacetylenes is a C_{13} derivative, containing one thienyl group which can formally be envisioned (Bohlmann et al., 1973) as the addition of hydrogen sulfide across two triple bonds. These substances have not previously been found in other Composites. Compound VIII has been found in a number of *Echinops* spp. (Bohlmann et al. 1965) and *Pluchea dioscorides* (Bohlmann et al., 1973). Likewise, IX has been found in the roots of *Eclipta prostata* L. (Bohlmann et al., 1973) while X has been isolated from both *Echinops* spp. and *Centaurea cristata* (Bohlmann et al., 1966). Although a number of *Centaurea* species have been investigated for polyacetylenes (Bohlmann et al., 1973), VII, IX, and X represent new compounds to this genus. Bohlmann (Bohlmann et al., 1965) reported the presence of the diacetate XII in *Echinops sphaerocephalus* L.; however, it was only inferred to be present and was not isolated, nor were any data given. Its presence in Russian knapweed thus confirms it as a natural product.

The presumed intermediate oxidation products between the unsaturated compounds XIII, XIV, and the chlorohydrins and chlorohydrin acetates, viz., the epoxides, have not been found in spite of their presence in other *Centaurea* species (Bohlmann et al., 1973).

In all cases, with the exception of XIII and XIV, the isolated polyacetylenes are optically active, which rules out the possibility of their being hydrolysis products of XIII. As a typical example, IX showed $[\alpha]_{589}$ ($CHCl_3$) = +17.5°; $[\alpha]_{578}$ = +18.9°; $[\alpha]_{546}$ = +20.6°; $[\alpha]_{436}$ = +37.9°.

Root length assay of VIII–XII has shown activity for only IX, with the results of lettuce, alfalfa, barnyard grass, and red millet tabulated in Table 2. The mean root length of the control for lettuce seedlings was 10.6 mm (starting length was approximately 3 mm), while at 10 ppm the root length was 6.9 mm. Fifty percent reduction in root length occurs at 12 ppm. The results of alfalfa, barnyard grass, and red millet were all similar, indicating that IX effectively inhibits root length elongation of grasses as well as broadleaf plants.

The possibility of synergism among the isolated polyacetylenes was tested by comparing the activity of the crude extract with that of IX. Allowing for concentration differences, it was found that IX accounted for all of the activity with no synergistic effect from other compounds.

Matricaria ester (ME, I), the first reported phytotoxic polyacetylene, was likewise tested under the same conditions against lettuce seedlings. Matricaria ester closely approximates the activity of IX with a 50% reduction in root length elongation occurring at 10 ppm. Little is known of the mode of action of the matricaria ester polyacetylenes. In a review (Numata et al., 1973) it was stated that addition of 5 ppm of either indoleacetic acid (IAA) or gibberelin (GA_3)

TABLE 2. EFFECT OF IX ON ROOT GROWTH OF WEED AND CROP SEEDLINGS

Seedling	Conc. of IX (ppm)	Mean root length (mm)[a]	
Lettuce	0	10.6	a
	10	6.9	b
	20	4.8	c
	40	3.7	d
	80	3.2	d
Barnyard grass	0	20.7	a
	5	16.3	b
	10	8.7	c
	20	4.7	d
	40	6.0	d
	80	5.0	d
Alfalfa	0	17.8	a
	5	14.6	a
	10	14.4	a
	20	10.1	b
	40	5.0	c
	80	5.1	c
Red millet	0	15.4	a
	5	19.1	a
	10	13.3	a
	20	6.1	b
	40	5.2	b
	80	2.0	c

[a] Means associated with a given test seedling with different letters are significantly different at the $\alpha = 0.05$ level according to the Least Significant Difference (LSD) test performed on transformed values. Original values are given.

failed to nullify the effects of 20 ppm of an ME derivative. It was concluded that the inhibitory effects were not based on a hormonal mechanism. The mechanism of plant growth inhibition by both matricaria esters and the thienyl compounds must await further studies.

To lend credence to the hypothesis that IX participates in the allelopathic action of Russian knapweed, the soil immediately surrounding the plant was extracted and found to contain 4–5 ppm of this particular compound. The analysis was based on actual material isolated, and hence represents a minimum amount because of the instability of the compound. The actual concentration in

the soil may be considerably higher than 4–5 ppm. Extrapolation of the data from the bioassay to field conditions, gives a 30% reduction in root growth at 4 ppm. This is certainly sufficient to have an appreciable effect on the surrounding plant community (Kobayashi et al., 1974).

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CUTICULAR HYDROCARBONS OF GREGARIOUS AND SOLITARY LOCUSTS *Locusta migratoria cinerascens*

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Abstract—The cuticular hydrocarbons of *Locusta migratoria cinerascens*—larvae and adults, males and females, gregarious and solitaries—have been investigated by combined gas chromatography–mass spectrometry. The hydrocarbons comprise 52–78% of the cuticular lipids and are divided into *n*-alkanes (28.7–47.3%), 3-, 4-, and 5-methylalkanes (11.3–15.8%), internally branched monomethylalkanes (13.7–19.9%), and internally branched dimethylalkanes (19.8–35.9%) with seven or nine methylenes between the two branch points. While the sexual dimorphism does not seem to be reflected in the cuticular hydrocarbon composition, clear quantitative variations favoring the longest chain alkanes have been observed between gregarious and solitary locusts, thus revealing a new phase character in these insects.

Key Words—Locusts, *Locusta migratoria*, cuticular hydrocarbons, mass spectrometry, phase polymorphism, Orthoptera, Acrididae.

INTRODUCTION

The cuticular lipids play a part in the regulation of water evaporation in insects and also protect them from the penetration of insecticides and microorganisms (Beament, 1964; Ebeling, 1964; David, 1967; Hadley, 1981). These cuticular lipids contain a large proportion of hydrocarbons. Some of these hydrocarbons act as pheromones (Carlson et al., 1978; Blomquist and Jackson, 1979; Howard and Blomquist, 1982; Jallon, 1985) or can be factors of chemotaxonomic differentiation (Blomquist et al., 1976; Lockey, 1976, 1984; Jackson, 1981).

These are the reasons for researchers' sustained interest in the chemical analysis of cuticular hydrocarbons from numerous species (Nelson, 1978;

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Blomquist and Jackson, 1979). Their reviews show that cuticular hydrocarbons are generally homologous series of *n*-alkanes and branched alkanes, mono-, di-, or trimethylated. Monomethylalkanes with terminal (2-, 3-, 4-, 5-methyl) and internal (7- to 23-methyl) branches, have been identified in three species of crickets (Hutchins and Martin, 1968), cockroaches (Tartivita and Jackson, 1970; Jackson, 1972), ants (Lok et al., 1975; Nelson et al., 1980), and bark beetles (Lockey, 1982). The 2-methylalkanes which do not occur in most of the insects already studied but are present in large proportions in three species of crickets (Blomquist et al., 1976) may permit taxonomic assignment. Di- and trimethylalkanes with isoprenoid spacing have been described in various insects (Nelson, 1978). Other polymethylalkanes with 1, 5, 7, 9, or 11 carbon atoms between branch points have also been identified (Nelson et al., 1980, 1981, 1984).

So far, the cuticular hydrocarbons of nine acridids have been studied: *Schistocerca vaga* (Nelson and Sukkestad, 1975), *Schistocerca gregaria* (Lockey, 1976), *Schistocerca americana* (Jackson, 1982), *Melanoplus sanguinipes* and *packardii* (Soliday et al., 1974) reanalyzed along with *differentialis* (Nelson et al., 1984), *Melanoplus bivittatus femurrubrum* and *dawsoni* (Jackson, 1981), and *Locusta migratoria* (Lockey, 1976). In 1976, Lockey suggested that the hydrocarbon compositions of different locusts are closer together if they belong to the same subfamily. Thus, a very fine chemical analysis appears to be the first condition for the use of the hydrocarbon profiles as additional characters in the insects' taxonomic grouping. Since Lockey (1976) has only studied the major components, we undertake here a new study of cuticular alkanes of a subspecies of the migratory locust: *Locusta migratoria cinerascens*.

The migratory locust is well known for its ability to change its kind of life, crowded or gregarious (gregaria) and isolated or solitary (solitaria) "phases." Thus it is interesting to know if the behavioral, morphological, and physiological changes of both phases (Uvarov, 1921, 1966) could lead to changes in the chemical composition of cuticular hydrocarbons.

Chemical stimulations can come into play at different ages of the locust's life (Gillett, 1975; Loher, 1960; Norris, 1970). However, the origin of these chemical pheromones is still under discussion and their nature remains to be specified.

In this study we decided to compare cuticular hydrocarbons at larval and adult ages of both sexes, from gregarious and solitary locusts of the *Locusta migratoria cinerascens* species.

METHODS AND MATERIALS

Insects Used. Last larval instar and mature adult, male and female, gregarious and solitary insects were used. They were raised at the Insect Biology Laboratory. The strain was *Locusta migratoria cinerascens* from Sardinia. Gre-

garious locusts were bred in groups of 200 individuals in cages 40 × 40 × 60 cm. Solitary locusts were maintained from birth in individual 1-liter containers in a separate breeding room. Each room was submitted to a regular change of air 12 times an hour. The photo- and thermoperiods were 12/12 hr with temperatures of 25 ± 1°C (night) and 35 ± 1°C (day). Separate evaluation of characters such as behavior (Nicolas, 1972; Gillett et al., 1972), morphometrics (Nicolas, 1973; Minato et al., 1973), pigmentation, and fecundity (Nicolas, 1972) had shown that, under our laboratory conditions, the locusts isolated from birth are conspicuously different from the crowded ones bred simultaneously.

All locusts were fed on fresh corn shoots and bran every day of the week with no interruption.

The same numbers of the animals of both sexes used were: 23 gregarious, last-instar larvae, two to three days old; 5 gregarious, mature adults, 34 days old; 10 solitary, last-instar larvae, two to three days old; and 21 solitary, mature adults, 21 days old.

All the solitary locusts used were light beige as this color was close to that of the white paper surrounding them to keep them separated. Each insect was killed by freezing at −20°C, then extracted by stirring during 10 min in hexane (5 ml/adult and 3 ml/larva). After evaporation of the solvent, hydrocarbons were separated from the weighed extracted cuticular lipids by thin-layer chromatography (Merck 10 × 20 plates of silica gel 60 F254 with concentration zones) and elution with nanograd hexane. From this weighed fraction of hydrocarbons, branched ones were separated with 5 Å Linde molecular sieves (as described by O'Connor et al., 1962).

Combined gas chromatography–mass spectrometry (GC-MS) was used to identify the components of the different extracts. Mass spectra were obtained on a Nermag R10-10 spectrometer associated with a PDP8 calculator (Digital Equipment Instrument) and coupled to a Girdel 31 chromatograph with a Ros injector. Fractions were temperature programmed from 200 to 300°C at 3°/min with isotherm at 300° on a capillary column either 10 or 25 m long, 0.32 mm wide, coated with CpSil 5 CB Chrompack. The carrier gas was helium and inlet pressure 0.25 bar. Fractions were analyzed by GC-MS either by electronic impact or by positive chemical ionization. In electronic impact, ionization voltage was 70 eV and the temperature of ion source was 110°C for hydrocarbons up to C_{40}. In these conditions, the molecular peak is always present. The temperature of the ion source was 290°C for more condensed hydrocarbons (C_{40} to C_{53}), but then only the ion M-15 is present. Mass scanning was carried out from atomic mass unit (amu) 100 to 600 or 750. In these conditions and without amplification (the sensitivity for the masses examined being better) spectra of branched hydrocarbons often have a base peak which corresponds to a characteristic fragmentation at a methyl branch. Positive chemical ionization was performed using methane generating an internal source pressure of 0.2 torr; ioni-

zation voltage was 90 eV, temperature of ion source 150°C, and mass scanning started at 100 amu.

The mass spectra of alkanes were interpreted according to the criteria proposed by McCarthy et al. (1968), Nelson et al. (1972), Nelson (1978), and Pomonis et al. (1978, 1980). Integration of chromatographic peaks was carried out with a Hewlett Packard integrator coupled to a Varian 3700 chromatograph with the 25-m column mentioned above (see GC-MS), programmed from 40 to 300°C at 4°/min and isothermal at 300° with a flow rate of helium being 18 cm/sec; an "on column" injector was used.

Retention indices (Ettre, 1964) were calculated with the 25-m column, using the *n*-alkanes from C_{22} to C_{37} identified without ambiguity by GC-MS and present in all total hydrocarbonated fractions.

All the bar charts (Figure 14–18) were constructed from the *n*-alkane percentage and the branched alkane percentage columns shown in Table 2 (%/N. and %/B.).

RESULTS

The hydrocarbons comprise 52–78% of all the cuticular lipids (Table 1). The smallest percentages were obtained for the solitary locusts. Our results, qualitatively in agreement with those of Lockey (1976) for the described compounds, exhibit quantitative differences; however, it must be mentioned that Lockey's analyses concern wings rather than total insect extracts. The chromatographic profile of a partial but complex hydrocarbon fraction (up to nonatriacontane) is presented in Figure 1 (25-m column).

Total (A) and corresponding branched (B) fractions (up to tripentacontane) are presented in Figure 2 (10-m column). Table 2 summarizes all the identified alkanes with their percentage for the eight groups of insects studied.

To perform analysis, correlations were carried out with results of electronic impact and chemical ionization in mass spectrometry on the one hand, and difference of retention index (*dI*) in gas chromatography between branched alkanes and *n*-alkanes with the same carbon number, on the other hand. It is known that *dI*s are related to the position of the methyl branch for a monomethylalkane (Mold et al., 1966) and to the number of methyl branches in a polymethylalkane (Nelson and Sukkestad, 1970). These correlations show, in agreement with Lockey (1976), that the fractions studied appear to consist of four classes of alkanes: class A, *n*-alkanes; class B, terminally branched monomethylalkanes; class C, internally branched monomethylalkanes; and class D, dimethylalkanes.

Class A: n-Alkanes. Their mass spectra are characteristic and their presence is in agreement with the comparison between the chromatographic profiles obtained before or after the separation on molecular sieves (Figure 2).

TABLE 1. ALKANE CONSTITUENTS OF CUTICULAR LIPIDS OF *Locusta migratoria cinerascens*[a]

	MGL	FGL	MSL	FSL	MGA	FGA	MSA	FSA
One insect extract weight (mg)	0.3	0.3	0.3	0.3	1.5	1.7	1.3	1.7
Hydrocarbons/cuticular lipids (weight %)	72	77	52	57	78	78	64	66
Relative surfaces (%):								
Class A	45.6	47.3	43.2	39.1	28.7	30.6	38.7	32.8
Class B	12.5	11.3	12.8	12.4	13.7	12.7	15.8	12.9
Class C	17.6	18.8	16.6	19.9	15.2	16.7	13.7	16.6
Class D	22.0	19.8	24.8	25.3	35.9	35.6	26.7	32.8
Unidentified components	2.2	2.7	2.6	3.1	5.8	4.4	5.1	4.9

[a] MGL: male gregarious larvae; FGL: female gregarious larvae; MSL: male solitary larvae; FSL: female solitary larvae; MGA: male gregarious adults; FGA: female gregarious adults; MSA: male solitary adults; FSA: female solitary adults; Class A: *n*-alkanes; class B: terminally branched monomethylalkanes; class C: internally branched monomethylalkanes; class D: dimethylalkanes.

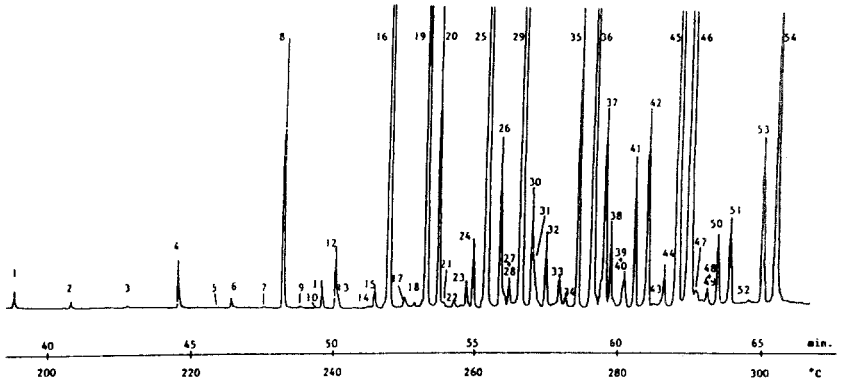


FIG. 1. Partial gas chromatogram of a hydrocarbon fraction of male solitary fifth-instar larvae of *Locusta migratoria cinerascens*. CpSil 5 CB Chrompack capillary column of 25 m × 0.32 mm, temperature programmed from 40 to 300° at 4°/min and held at 300°; "on-column" type injector.

In electronic impact conditions (70 eV, starting mass scanning: 100 amu), the molecular peak $M^{+}\cdot$ is base peak. Sixteen linear alkanes, from C_{22} to C_{37}, have been identified; *n*-nonacosane in gregarious and *n*-hentriacontane in solitary locusts are the most important. The percentage of these total *n*-alkanes decreases from larval to adult ages (Table 1) and more strongly in gregarious locusts.

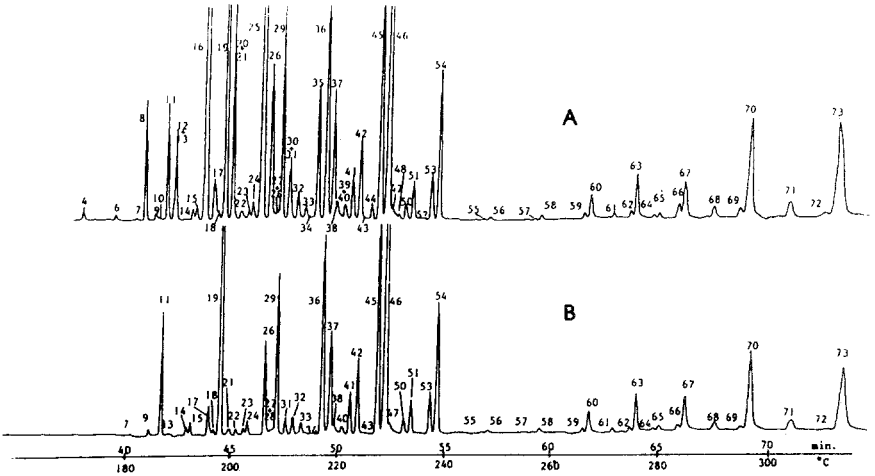


FIG. 2. Gas chromatographic analysis of the total (A) and branched (B) hydrocarbon fractions of male gregarious mature adults, *Locusta migratoria cinerascens*. Same conditions as Figure 1 with a column of 10 m × 0.32 mm.

TABLE 2. CUTICULAR HYDROCARBONS OF *Locusta migratoria cinerascens*

GC Peak No.	Retention indices (I)	MGL[a]			FGL			MSL			FSL		
		%/T[b]	%/N	%/B	%/T	%/N	%/B	%/T	%/N	%/B	%/T	%/N	%/B
1	2200							tr.	tr.		tr.	tr.	
2	2300	tr.	tr.		0.1	0.2		tr.	tr.		tr.	tr.	
3	2400	tr.	tr.		0.1	0.2		tr.	tr.		tr.	tr.	
4	2500	0.3	0.7		1.0	2.1		0.2	0.5		0.3	0.8	
5	2572	tr.		tr.	tr.		tr.						
6	2600	0.2	0.4		0.2	0.4		tr.	tr.		0.1	0.3	
7	2672	tr.		tr.	tr.		tr.						
8	2700	4.5	9.8		4.0	8.5		1.3	3.0		1.2	3.0	
9	2733	0.4		0.7	0.3		0.6						
10	2758	tr.		tr.									
11	2772	2.1		3.9	1.7		3.2	0.2		0.4	0.2		0.3
12	2800	2.0	4.4		1.8	3.8		0.4	0.9		0.4	1.0	
13	2806				0.3		0.6						
14	2858	0.1		0.2	tr.		tr.						
15	2872	0.2		0.4	0.1		0.2	tr.		tr.	tr.		tr.
16	2900	21.3	46.6		22.6	47.7		9.5	22.0		8.7	22.3	
17	2933	1.3		2.4	1.6		3.0	0.2		0.4	0.2		0.3
18	2950	0.1		0.2	0.2		0.4	tr.		tr.			
19	2972	7.3		13.4	6.3		12.0	2.9		5.1	3.6		6.0
20	3000	3.0	6.6		3.1	6.6		2.9	6.7		2.6	6.6	
21	3006	tr.		tr.	tr.		tr.						
22	3033	0.2		0.4	0.3		0.6	tr.		tr.			
23	3058	0.1		0.2	0.1		0.2	0.1		0.2	0.1		0.2
24	3072	0.3		0.6	0.3		0.6	0.4		0.7	0.3		0.5
25	3100	12.4	27.1		12.4	26.2		20.7	47.9		18.1	46.3	
26	3133	3.0		5.5	4.1		7.8	1.2		2.1	1.6		2.6

TABLE 2. Continued

GC Peak No.	Retention indices (I)	MGL[a]			FGL			MSL			FSL		
		%/T[b]	%/N	%/B	%/T	%/N	%/B	%/T	%/N	%/B	%/T	%/N	%/B
27	3150	tr.		tr.	tr.		tr.	0.2		0.4	0.2		0.3
28	3158	tr.		tr.	0.1		0.2						
29	3172	2.1		3.9	1.7		3.2	7.7		13.6	6.9		11.4
30	3200	0.5	1.1		0.4	0.8		1.1	2.5		1.1	2.8	
31	3206	0.2		0.4	0.2		0.4	0.1		0.2	0.2		0.3
32	3233	0.5		0.9	0.5		0.9	0.5		0.9	0.6		1.0
33	3258	0.2		0.4	0.6		1.1	0.2		0.4	0.3		0.5
34	3272	tr.		tr.	0.2		0.4	tr.		tr.	0.1		0.2
35	3300	1.3	2.9		1.4	3.0		5.8	13.4		5.1	13.0	
36	3333	5.3		9.7	5.8		11.0	5.5		9.7	7.1		11.8
37	3360	2.1		3.9	2.0		3.8	2.2		3.9	2.2		3.7
38	3372	tr.		tr.	tr.		tr.	0.7		1.2	0.5		0.8
39	3400	tr.	tr.		tr.	tr.		0.3	0.7		0.3	0.8	
40	3406	tr.		tr.	tr.		tr.	0.2		0.4	0.2		0.3
41	3433	0.8		1.5	0.8		1.5	1.0		1.8	1.1		1.8
42	3460	1.4		2.6	1.1		2.1	1.6		2.8	1.4		2.3
43	3472							0.2		0.4			
44	3500	0.1	0.2		0.2	0.4		0.9	2.1		0.8	2.0	
45	3533	4.8		8.8	4.6		8.7	6.6		11.6	7.8		12.9
46	3560	9.4		17.3	8.2		15.6	10.6		18.7	10.4		17.2
47	3572	tr.		tr.	tr.		tr.	0.2		0.4	0.2		0.3

48	3600	tr.	tr.		tr.	tr.		0.1	0.2		0.1	0.3	
49	3606							tr.		tr.	0.1		0.2
50	3633	0.7		1.3	0.3		0.6	0.6		1.1	0.5		0.8
51	3660	0.5		0.9	0.4		0.8	0.6		1.1	0.5		0.8
52	3700	tr.	tr.		tr.	tr.		tr.	tr.		0.3	0.8	
53	3733	0.6		1.1	0.5		0.9	1.0		1.8	1.2		2.0
54	3760	1.7		3.1	1.6		3.0	2.5		4.4	2.1		3.5
55	>3800	tr.		tr.	tr.		tr.	tr.		tr.	tr.		tr.
56	·	tr.		tr.	tr.		tr.	tr.		tr.	tr.		tr.
57	·	tr.		tr.	tr.		tr.	tr.		tr.	tr.		tr.
58	·	tr.		tr.	tr.		tr.	tr.		tr.	tr.		tr.
59	·	tr.		tr.	tr.		tr.	tr.		tr.	tr.		tr.
60	·	0.2		0.4	0.2		0.4	0.4		0.6	0.4		0.7
61	·	0.2		0.4	0.2		0.4	0.2		0.4	0.2		0.3
62	·							0.2		0.4	0.2		0.3
63	·	0.3		0.6	0.3		0.6	0.4		0.6	0.4		0.7
64	·	tr.		tr.	tr.		tr.	tr.		tr.	tr.		tr.
65	·							0.2		0.4	0.2		0.3
66	·	0.2		0.4	0.3		0.6						
67	·	0.3		0.6	0.2		0.4	0.5		0.8	0.8		1.2
68	·	0.2		0.4	0.2		0.4	0.4		0.6	0.5		0.7
69	·	0.4		0.8	0.6		1.2	0.5		0.8	0.6		0.9
70	·	2.4		4.6	2.1		4.1	2.6		4.5	3.2		5.2
71	·	0.6		1.2	0.4		0.8						
72	·	0.2		0.4	0.3		0.6	0.4		0.6	0.5		0.7
73	·	3.9		7.5	3.7		7.2	3.8		6.6	4.3		7.0

TABLE 2. Continued

GC Peak No.	Retention indices (I)	MGA			FGA			MSA			FSA		
		%/T	%/N	%/B	%/T	%/N	%/B	%/T	%/N	%/B	%/L	%/N	%/B
1	2200	tr.	tr.		tr.	tr.		0.3	0.8		0.2	0.6	
2	2300	tr.	tr.		tr.	tr.		0.1	0.3		tr.	tr.	
3	2400	tr.	tr.		tr.	tr.		0.2	0.5		0.1	0.3	
4	2500	0.1	0.3		0.2	0.7		0.4	1.0		0.2	0.6	
5	2572												
6	2600	tr.	tr.		0.1	0.3		0.4	1.0		0.2	0.6	
7	2672	tr.		tr.	tr.		tr.						
8	2700	1.4	4.9		2.5	8.2		0.9	2.3		0.9	2.7	
9	2733	0.2		0.3	0.3		0.4						
10	2758												
11	2772	1.6		2.2	2.4		3.5	tr.		tr.	0.4		0.6
12	2800	0.9	3.1		1.2	3.9		0.6	1.6		0.5	1.5	
13	2806	0.3		0.4	0.2		0.3						
14	2858	0.2		0.3	0.2		0.3						
15	2872	0.2		0.3	0.2		0.3	tr.		tr.	tr.		tr.
16	2900	12.7	44.3		14.6	47.7		8.4	21.7		8.4	25.6	
17	2933	0.7		1.0	1.0		1.4	0.1		0.2	0.2		0.3
18	2950	0.2		0.3	0.1		0.2	tr.		tr.	tr.		tr.
19	2972	7.4		10.2	6.0		8.6	2.8		4.6	3.4		5.1
20	3000	1.9	6.6		1.8	5.9		2.4	6.2		2.1	6.4	
21	3006	tr.		tr.	0.1		0.2	tr.		tr.	tr.		tr.
22	3033	0.2		0.3	0.2		0.3	tr.		tr.	tr.		tr.
23	3058	0.2		0.3	tr.		tr.	0.2		0.3	0.2		0.3
24	3100	0.3		0.4	0.3		0.4	0.4		0.7	0.3		0.4
25	3100	9.1	31.7		8.1	26.5		15.7	40.6		13.2	40.2	
26	3133	2.0		2.8	2.3		3.3	0.5		0.8	0.9		1.3

27	3150	tr.		tr.	tr.		tr.	0.1		0.2	0.1		0.2
28	3158	tr.		tr.	0.2		0.3						
29	3172	2.8		3.9	2.4		3.5	9.5		15.5	6.7		10.0
30	3200	0.5	1.7		0.4	1.3		1.7	4.4		1.3	4.0	
31	3206	0.4		0.6	0.4		0.6	0.3		0.5	0.4		0.6
32	3233	0.5		0.7	0.5		0.5	0.4		0.7	0.4		0.6
33	3258	0.3		0.4	0.3		0.4	0.4		0.7	0.4		0.6
34	3272	tr.		tr.	tr.		tr.	0.2		0.3	0.2		0.3
35	3300	1.5	5.2		1.2	3.9		5.9	15.2		4.4	13.4	
36	3333	4.2		5.8	4.5		6.5	3.6		5.9	4.7		7.0
37	3360	3.0		4.1	3.5		5.0	2.1		3.4	2.9		4.3
38	3372	0.3		0.4	0.2		0.3	1.7		2.8	0.8		1.2
39	3400	0.2	0.7		0.3	1.0		0.4	1.0		0.2	0.6	
40	3406	0.1		0.2	tr.		tr.	0.3		0.5	0.4		0.6
41	3433	0.8		1.1	0.9		1.3	0.9		1.5	1.2		1.8
42	3460	2.0		2.8	2.2		3.2	1.6		2.6	2.4		3.6
43	3472	tr.		tr.	0.3			tr.		tr.	tr.		tr.
44	3500	0.2	0.7		0.2	0.7		0.9	2.3		0.7	2.1	
45	3533	5.1		7.1	5.2		7.5	6.3		10.3	7.2		10.7
46	3560	12.8		17.7	13.6		19.6	10.5		17.1	14.6		21.7
47	3572	0.2		0.3	0.1		0.2	0.5		0.8	0.4		0.6
48	3600	0.2	0.7		tr.	tr.		0.2	0.5		0.3	0.9	
49	3606							tr.		tr.	tr.		tr.
50	3533	0.5		0.7	0.7		1.0	0.6		1.0	0.6		0.9
51	3660	0.9		1.2	1.0		1.4	0.8		1.3	1.0		1.5
52	3700	tr.	tr.		tr.	tr.		0.2	0.5		0.1	0.3	
53	3733	1.0		1.4	1.1		1.6	1.3		2.1	1.4		2.1
54	3760	3.6		5.0	3.8		5.5	2.6		4.2	3.3		4.9
55	3760	tr.		tr.	tr.		tr.	tr.		tr.	tr.		tr.
56	>3800	tr.		tr.	tr.		tr.	tr.		tr.	tr.		tr.
57	·	tr.		tr.	tr.		tr.	tr.		tr.	tr.		tr.
58	·	0.2		0.3	tr.		tr.	tr.		tr.	tr.		tr.
59	·	0.2		0.3	tr.		tr.	0.2		0.3	tr.		tr.

TABLE 2. Continued

GC Peak No.	Retention indices (I)	MGA			FGA			MSA			FSA		
		%/T	%/N	%/B	%/T	%/N	%/B	%/T	%/N	%/B	%/L	%/N	%/B
60	·	0.8		1.2	0.6		0.9	0.7		1.1	0.4		0.6
61	·	0.2		0.3	0.3		0.4	0.2		0.3			
62	·	0.3		0.5	0.2		0.3	0.3		0.5	0.2		0.3
63	·	1.4		2.2	0.9		1.3	1.0		1.6	0.8		1.2
64	·	tr.		tr.	tr.		tr.	tr.		tr.	tr.		tr.
65	·	0.3		0.5				0.4		0.7	0.2		0.3
66	·	0.6		0.9	0.2		0.3	0.5		0.8	0.5		0.7
67	·	1.4		2.2	1.3		1.9	1.3		2.1	1.0		1.5
68	·	0.6		0.9	1.8		2.6	0.6		1.0	0.5		0.7
69	·	0.5		0.8	0.2		0.3	0.5		0.8	0.8		1.2
70	·	4.4		6.3	2.9		4.1	3.0		4.9	2.3		3.4
71	·	0.8		1.2	0.2		0.3	0.6		1.0	0.8		1.2
72	·	0.5		0.8	0.2		0.3	0.5		0.8	0.7		1.0
73	·	6.4		9.4	6.4		9.1	3.8		6.1	4.5		6.7

GC Peak No.	Retention indices (I)	Carbon No.	Class	Hydrocarbons[c]
1	2200	22	A	*n*-Docosane
2	2300	23	A	*n*-Tricosane
3	2400	24	A	*n*-Tetracosane
4	2500	25	A	*n*-Pentacosane
5	2572	26	B	3-Methylpentacosane
6	2600	26	A	*n*-Hexacosane
7	2672	27	B	3-Methylhexacosane
8	2700	27	A	*n*-Heptacosane
9	2733	28	C	13°- and 15-Methylheptacosanes (1)
10	2758	28	B	5-Methylheptacosane
11	2772	28	B	3-Methylheptacosane
12	2800	28	A	*n*-Octacosane
13	2806	29	?	? (2)
14	2858	29	B	4-Methyloctacosane
15	2872	29	B	3-Methyloctacosane
16	2900	29	A	*n*-Nonacosane
17	2933	30	C	11°-, 13-, and 15-Methylnonacosanes (3)
18	2950	30	B	5-Methylnonacosane
19	2972	30	B	3-Methylnonacosane
20	3000	30	A	*n*-Triacontane
21	3006	31	?	? (2)
22	3033	31	C	11°-, 12-, 13-, 14-, and 15-Methyltriacontanes (4)
23	3058	31	B	4-Methyltriacontane
24	3072	31	B	3-Methyltriacontane
25	3100	31	A	*n*-Hentriacontane
26	3133	32	C	11- and 13°-Methylhentriacontanes

TABLE 2. Continued

GC Peak No.	Retention indices (I)	Carbon No.	Class	Hydrocarbons[c]
27	3150	32	B	5-Methylhentriacontane
28	3158	32	B	Dimethylhentriacontane (5)
29	3172	32	B	3-Methylhentriacontane
30	3200	32	A	*n*-Dotriacontane
31	3206	33	?	? (2)
32	3233	33	C	12-, 13°- and 14-Methyldotriacontanes (6)
33	3258	33	B	4-Methyldotriacontane (7)
34	3272	33	B	3-Methyldotriacontane
35	3300	33	A	*n*-Tritriacontane
36	3333	34	C	11- 13°-, and 15-Methyltritriacontanes
37	3360	35	D	11, 21-, and 13,21-Dimethyltritriacontanes
38	3372	34	B	3-Methyltritriacontane
39	3400	34	A	*n*-Tetratriacontane
40	2406	35	?	? (2)
41	3433	35	C	12-, 13°-, 14-, and 5-Methyltetratriacontanes (8)
42	3460	36	D	12,20-, 12,22-, and 13,21-Dimethyltetratriacontanes
43	3472	35	B	3-Methyltetratriacontane
44	3500	35	A	*n*-Pentatriacontane
45	3533	36	C	13°-, 15-, and 17-Methylpentatriacontanes
46	3560	37	D	13,21-, and 13,23-Dimethylpentatriacontanes
47	3572	36	B	3-Methylpentatriacontane
48	3600	36	A	*n*-Hexatriacontane
49	3606	37	?	? (2)
50	3633	37	C	12-, 13-, 14°-, and 15-Methylhexatriacontanes (9)
51	3660	38	D	12,22- and 13,23-Dimethylhexatriacontanes
52	3700	37	A	*n*-Heptatriacontane
53	3733	38	C	13°-, 15-, 17-, and 19-Methylheptatriacontanes

54	3760	39	D	13,21- and 13,23-Dimethylheptatriacontanes
55	>3800			? (10)
56	·			? (10)
57	·			? (10)
58	·			? (10)
59	·			? (10)
60	·			? (10)
61	·			? (10)
62	·			? (10)
63	·	47	D	13,21-Dimethylpentatetracontane
64	·			? (10)
65	·			? (10)
66	·			? (10)
67	·	49	D	13,21-Dimethylheptatetracontane
68	·			? (10)
69	·			? (10)
70	·	51	D	13,21-Dimethylnonatetracontane
71	·			? (10)
72	·			? (10)
73	·	53	D	13,21-Dimethylhenpentacontane

[a] See footnote to Table 1 for abbreviations.

[b] %/T: percentages (±0.1%), scaled to 100, of total alkanes. %/N: percentages (±0.1%), scaled to 100, of normal alkanes. %/B: percentages (±0.1%), scaled to 100, of branched alkanes. tr.: <0.1%.

[c] °: major isomer. (1) 11- and 13°-methylheptacosanes for MGA and FGA. (2) The retention indice and the carbon number agree with a 3,X-dimethylalkane (Nelson et al., 1980), but the very small percentage of this product did not allow us to get a good spectrum. (3) 11°- and 13-methylnonacosanes for MSL and MGA; 9-, 11°-, and 13-methylnonacosanes for FGA. (4) 10-, 11°-, 12-, and 13-methyltrialontanes for MGA and FGA; 11°-, 12-, 13-, and 14-methyltriacontanes for FGL. (5) Branch points unidentified. (6) 12°-, 13-, and 14-methyldotriacontanes for FGL; 12-, 13°-, and 14-methyldotriacontanes for MSA; 11-, 12°-, 13°-, and 14°-methyldotriacontanes for MGA. (7) + 12,20-dimethyldotriacontane for MGA and MGA; + unidentified dimethyldotriacontane for the other insects. (8) 11-, 12-, 13°-, 14-, 15-, and 16-methyltetratriacontanes for FGL and FSL; 12-, 13°-, 14-, 15-, 16-, and 17-methyltetratriacontanes for MSL. (9) 13°-, 14°-, and 15-methylhexatriacontanes for MGL, FGL, and FSL; 12-, 13°-, 14-, 15-, 16-, 17-, and 18-methylhexatriacontanes for MSL. (10) The small percentage of this component did not allow us to identify it; present on the gas chromatograms after separation on molecular sieves, it is quite probably a branched alkane.

Class B: Terminally Branched Monomethylalkanes. 5-, 4- and 3-methylalkanes have been identified. The *dI*s between these alkanes and the *n*-alkanes with the same carbon number are, respectively, 50, 42, and 28. The first two series account for less than 1% of the different analyzed fractions. According to the biosynthesis of these compounds (Blomquist and Jackson, 1979), 5-methylalkanes are even hydrocarbons (5-methylnonacosane and 5-methylhentriacontane) and 4-methylalkanes are odd hydrocarbons (4-methyloctacosane and 4-methyltriacontane). 3-Methylalkanes are by far the most abundant products of this class: they account for 12% of the total hydrocarbon fraction with a little more for the male solitary adult locusts (15.8%). They range from 26 to 36 carbon numbers; among them, those with an even number of carbons are the most abundant. 3-Methylnonacosane is the major one of the series for gregarious locusts and 3-methylhentriacontane for solitary ones.

Their mass spectra (from 100 amu) give the following characteristic fragments: 5-methylalkanes: M-57 (base peak) and M-85; 4-methylalkanes: M-43 (base peak) and M-71; 3-methylalkanes: M-29 (base peak) and M-57.

Class C: Internally Branched Monomethylalkanes. They account for 13–20% of total hydrocarbon extracts and range from C_{28} to C_{38} (described here but probably up to C_{52}). The *dI* with same number linear hydrocarbons is 67. The predominant branch points are 11, 12, and 13 for odd methylalkanes (the minor ones) and 11 and 13 for even methylalkanes (the major ones).

In fact, each chromatographic peak corresponds to a mixture of odd or even monomethylalkanes, isomers for the position of the branch point. The mass spectrometry of these derivatives has been described by McCarthy et al. (1968) and Nelson et al. (1972). This has enabled us to establish the structures proposed in Table 2.

The mass spectrum of peak 41 of Figure 2 suggests a mixture of 12-, 13-, 14-, and 15-methyltetratriacontanes (Figure 3). In the same way, peak 45 of

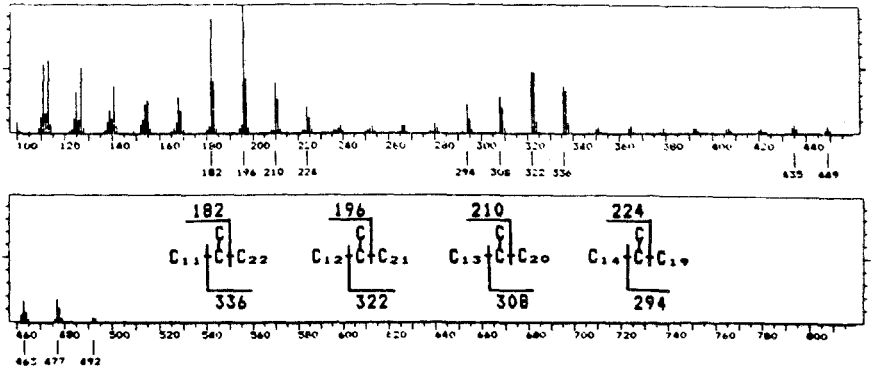


FIG. 3. Mass spectrum of GC peak 41 (Figure 2): 12-, 13-, 14-, and 15-methyltetratriacontanes.

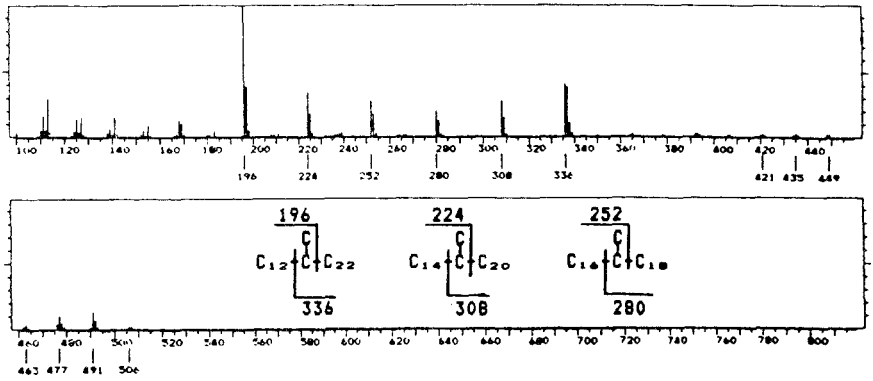


FIG. 4. Mass spectrum of GC peak 45: 13-, 15-, and 17-methylpentatriacontanes.

Figure 1 is interpreted as a mixture of 13-, 15-, and 17-methylpentatriacontanes (Figure 4). In all cases, the base peak of these spectra recorded from 100 amu is the secondary even ion of the weaker molecular mass of the major isomer (*m/z* 196 for 13-methyl isomer in Figures 3 and 4).

Some qualitative differences appear between insect categories. For example, to the isomers identified in peak 41 of Figure 2 (Figure 3), are added 16- and 17-methyltetratriacontanes in peak 41 of Figure 1 (Figure 5). Probably, these qualitative differences come from quantitative variations between monomethylalkanes inside the same chromatographic peak of the various insect categories analyzed.

Class D: Dimethylalkanes. The percentages of these products are close to those of monomethylalkanes (8–19% of total hydrocarbon extracts), and range from C_{33} to C_{53}. After C_{39}, only four odd dimethylalkanes, C_{47} to C_{53}, have been proposed, the others being minor ones. The *dI* of 140 and the molecular

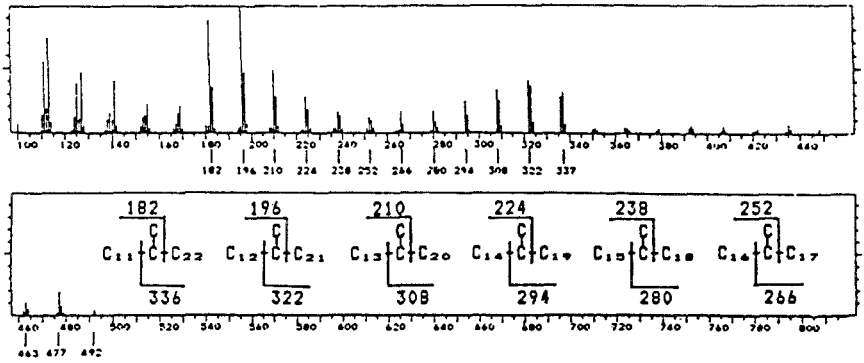
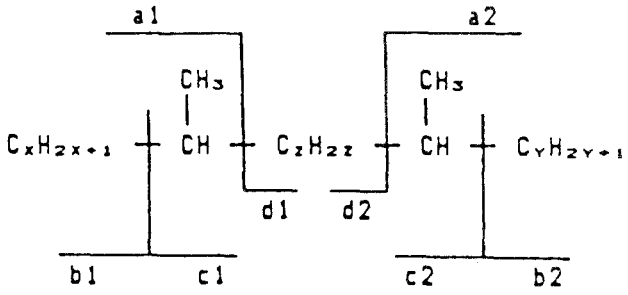


FIG. 5. Mass spectrum of GC peak 41 (Figure 1): 12-, 13-, 14-, 15-, 16-, and 17-methyltetratriacontanes.



SCHEME 1.

masses, determined by chemical ionization, agree with internally branched dimethylalkanes.

The mass spectrometry, more complex than for monomethylalkanes, was used to suggest structures. According to the Nelson school (Nelson et al., 1972; Pomonis et al., 1978, 1980), internally dimethylalkanes undergo two more fragmentations than monomethylalkanes (Scheme 1).

The spectra of 15,19-dimethylpentatriacontane (Sonnet, 1976) (Figure 6) and 13,23-dimethylheptatriacontane (Carlson et al., 1984) (Figure 11B) are presented as examples. Note the relative importance in Figure 6 of m/z 266/267 (even ion major one) which correspond to a pair of ions $d - 1/d$. The other doublet $d - 1/d$ is superimposed with m/z 294/295. Pomonis et al. (1980) mention this rupture with a greater intensity and a major odd ion, $d >> d - 1$, for 9,14-dimethylheptacosane.

As in the case of the monomethylalkanes, each chromatographic peak of the dimethylalkanes corresponds to a mixture of isomers. We present here our suggestions for identifications and the corresponding spectra of some of them (Figures 7–11).

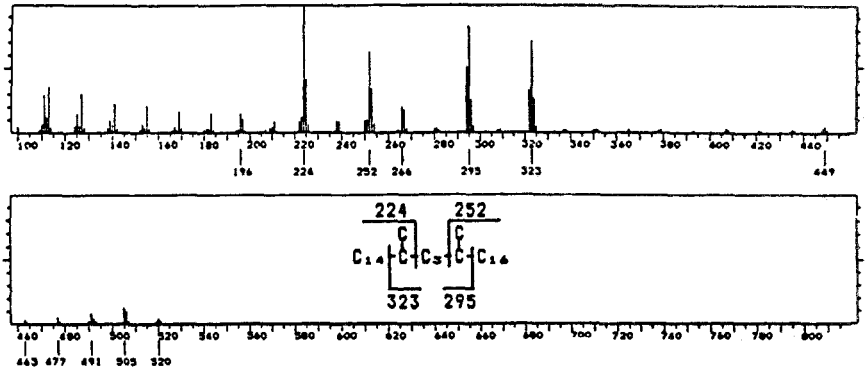


FIG. 6. Mass spectrum of 15,19-dimethylpentatriacontane.

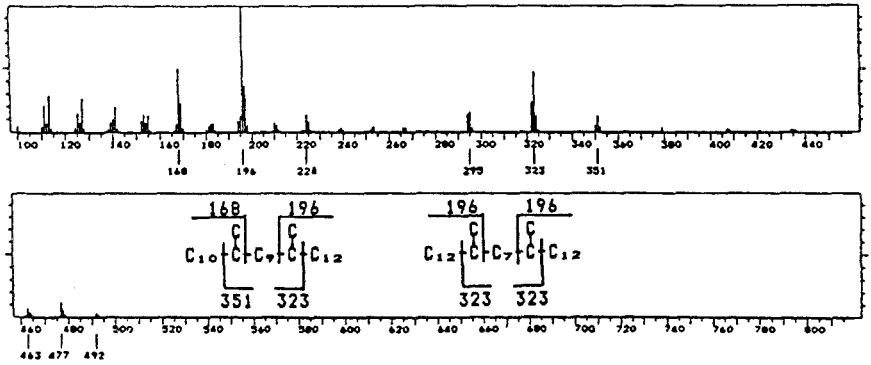


FIG. 7. Mass spectrum of GC peak 37: 11,21- and 13,21-dimethyltritriacontanes.

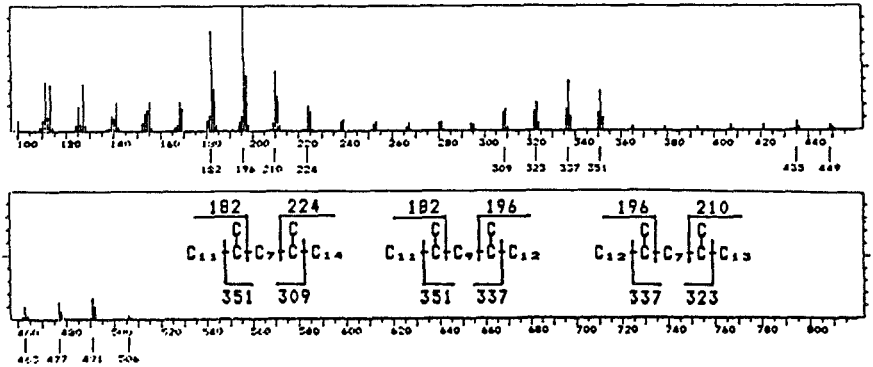


FIG. 8. Mass spectrum of GC peak 42: 12,20-, 12,22-, and 13,21-dimethyltetratriacontanes.

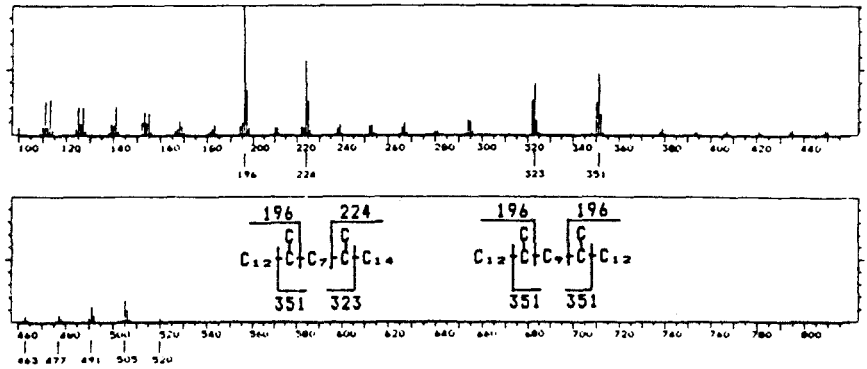


FIG. 9. Mass spectrum of GC peak 46: 13,21- and 13,23-dimethylpentatriacontanes.

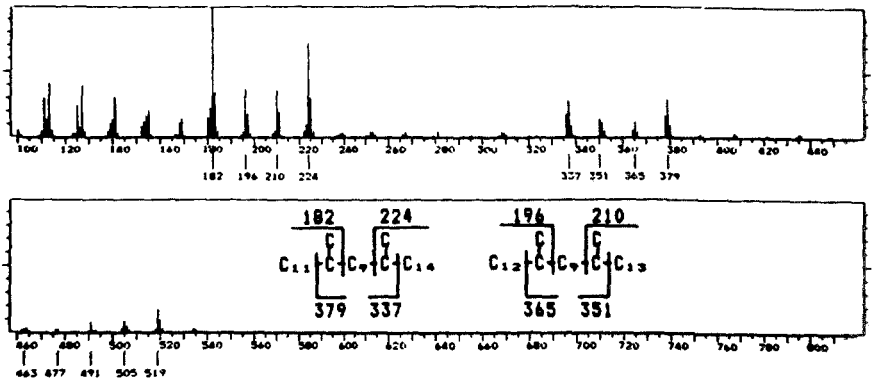


FIG. 10. Mass spectrum of GC peak 51: 12,22- and 13,23-dimethylhexatriacontanes.

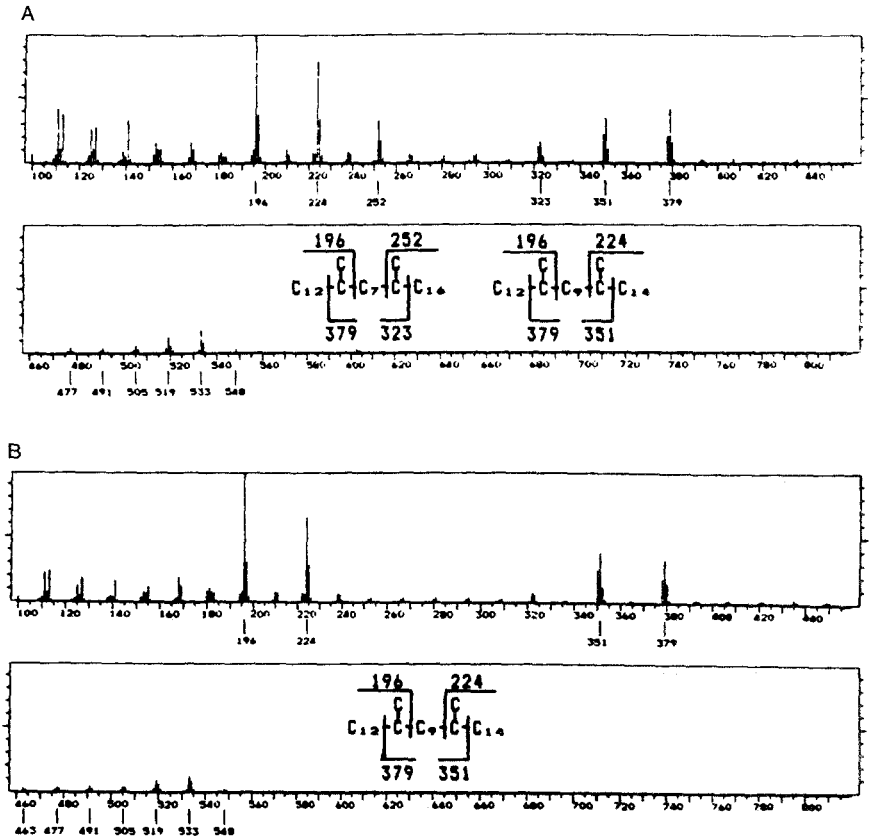


FIG. 11. (A) Mass spectrum of GC peak 54: 13,21- and 13,23-dimethylheptatriacontanes. (B) Mass spectrum of 13,23-dimethylheptatriacontane.

The spectrum of Figure 7 (chromatographic peak 37), corresponds to a mixture of dimethyltritriacontanes (M = 492). According to the pairs of ions **168**/169, **196**/197, 322/**323**, and 350/**351** (major ions in heavy type), we propose 11,21-dimethyltritriacontane. Moreover, the increased intensities of ions at *m/z* **196**/197 and 322/**323** suggest the presence of the symmetric 13,21-dimethyltritriacontane. Finally, the ions at *m/z* **224**/225 and 294/**295** suggest a third isomer with a branch point at carbon 19, but it is difficult to say if it is 11,19- or 13,19-dimethyltritriacontane. The results mentioned further on might be in favor of 11,19 (seven carbons between branch points). We must note that the ion at *m/z* 294 also corresponds to the ion $d - 1$ of the two precedent isomers.

The mass spectrum of Figure 8 (chromatographic peak 42) was interpreted as a mixture of 12,20-, 13,21-, and 12,22-dimethyltetratriacontanes. The two first isomers are sufficient for the attribution of the eight major fragments observed, but the fact that the ratios 351/309 and 337/323 are positive implies the presence of the third isomer.

In the same way, the mass spectrum of Figure 9 (chromatographic peak 46) agrees with the one of 13,21-dimethylpentatriacontane (first proposed by Lockey, 1976) but the ion at *m/z* 351 (>323) suggests the presence of symmetric 13,23-dimethylpentatriacontane (minor one).

For the mass spectrum of Figure 10 (chromatographic peak 51), if we put together the two major even ions at *m/z* 182 and 224 and the two odd ones at *m/z* 337 and 379, we suggest 12,22-dimethylhexatriacontane. The 13,23-dimethylhexatriacontane agrees with other even fragments at *m/z* 196 and 210 and odd fragments at *m/z* 351 and 365. It should be noted that the intensity of the ion at *m/z* 350, greater in this particular case than the ion at *m/z* 351, is partially due to an ion $d - 1$ of the isomer 12,22.

The mass spectrum of peak 54, Figure 11A, in comparison with the spectrum of 13,23-dimethylheptatriacontane (Figure 11B), proves the presence of this hydrocarbon in product 54. But the positive intensity ratio 379/351, on the one hand, and the presence of ions at *m/z* 323 and 252, on the other, can be interpreted as the fragmentation pattern of isomer 13,21.

It should be emphasized that all the dimethylalkane structures suggested here have seven or nine carbons between branch points. Such series are reported, among others, in the hemolymph of the Japanese beetle *Popilla japonica* Newman (Nelson et al., 1975), with seven methylene groups, and in the cuticular hydrocarbons of the house fly *Musca domestica* (Nelson et al., 1981) and of the grasshoppers *Melanoplus differentialis*, *sanguinipes*, and *packardii* (Nelson et al., 1984), with nine methylene groups.

DISCUSSION

The differential bar charts presented in Figures 12 to 16 concern the insect population described in the experimental part. However, similar analyses have

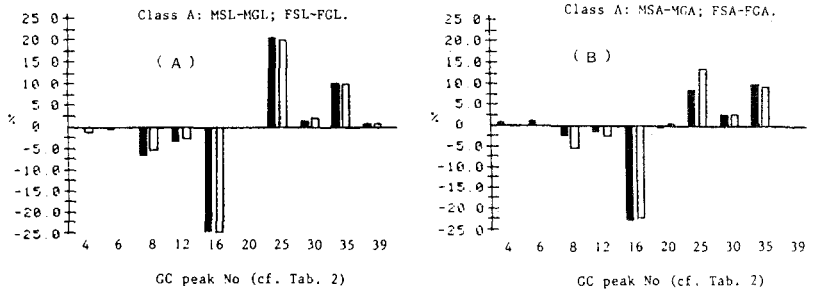


FIG. 12. (A) %MSL − %MGL (black bar chart); %FSL − %FGL (white bar chart). (B) %MSA − %MGA (black bar chart); %FSA − %FGA (white bar chart). MSL: male solitary larvae; MGL: male gregarious larvae; FSL: female solitary larvae; FGL: female gregarious larvae; MSA: male solitary adult; MGA: male gregarious adult; FSA: female solitary adult; FGA; female gregarious adult.

been carried out on at least one other insect population. In all cases, similar typical schemas are observed, even if slight variations in absolute identified hydrocarbon percentages occur from one population to another.

The differential bar charts in Figures 12 to 14 reveal that there is a general tendency for the cuticular hydrocarbons of solitary locusts to be more condensed than those of the gregarious ones. This emerges from the comparison of their hydrocarbon profiles and is valid for both ages and sexes. For the products belonging to classes A, B, and C, we can find a compound representing the variation mean: *n*-triacontane (No. 20) for class A (Figure 12A and B), 3-methyltriacontane (No. 24) for the 3-methylalkanes of class B (Figure 13A and B), and the methyltetratriacontanes (No. 41) for class C (Figure 14A and B). On the other hand, the relative percentages of dimethylalkanes are not modified much by phase changes.

Concerning the evolution of cuticular hydrocarbon composition during the locusts' transformation from larvae to adults, not many changes are observed

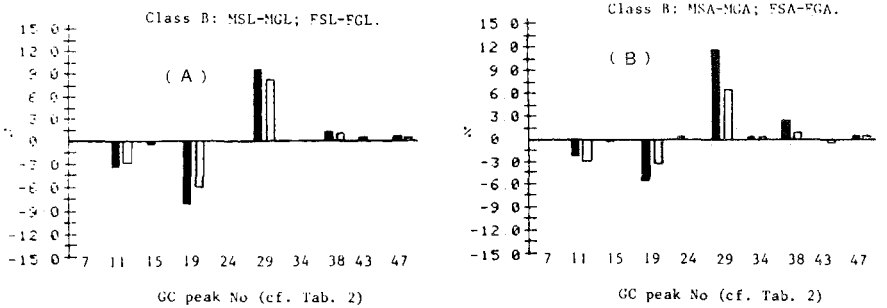


FIG. 13. (A) %MSL − %MGL (black bar chart); %FSL − %FGL (white bar chart). (B) %MSA − %MGA (black bar chart); %FSA − %FGA (white bar chart).

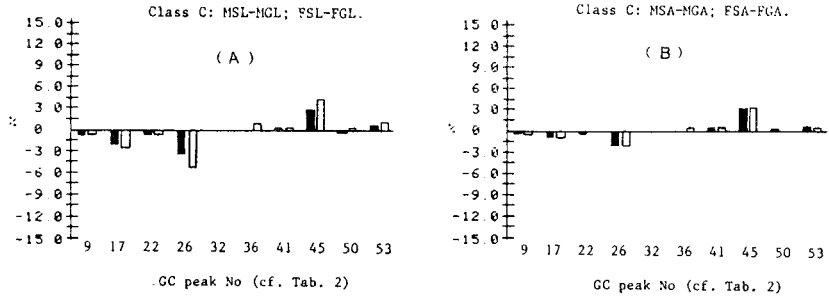


FIG. 14. (A) %MSL − %MGL (black bar chart); %FSL − %FGL (white bar chart). (B) %MSA − %MGA (black bar chart); %FSA − %FGA (white bar chart).

in the *n*-alkanes and the 3-methylalkanes. A decrease in the relative percentages of monomethylalkanes can be observed (Figure 15A and B) in both gregarious (−11.6% for the females and −11.1% for the males) and solitary insects (−8.5% for the females and −6.9% for the males). The variations of dimethylalkanes are presented in Figures 16A and B. They show a clear increase in these compounds in gregarious (+9.4%) and solitary (+8.5%) females. In the males, the tendency seems weaker (+3.0% for gregarious) or reversed (−2.3% for solitary ones). The presence of long-chain branched alkanes, in greater quantities in adults than in larvae, has already been described in *Schistocerca americana* (Jackson, 1982).

The differences between the sexes are slight but seem to be greater in the case of the solitary locusts, especially for dimethylalkanes. This very weak sexual dimorphism leads to the hypothesis of a probable absence of any sexual contact cuticular pheromone of a hydrocarbon nature in these insects.

The differences observed between gregarious and solitary insects show the importance of the role of the cuticle in phase dimorphism; this can be added to the underlying pigmentary tegumental phase dimorphism, which was the only one known until now (Uvarov, 1966; Albrecht, 1967). This new phase char-

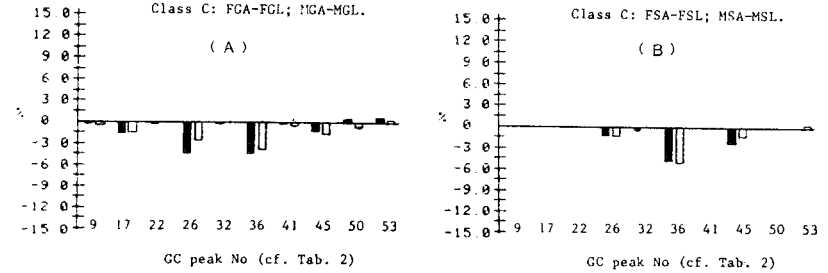


FIG. 15. (A) %FGA − %FGL (black bar chart); %MGA − %MGL (white bar chart). (B) %FSA − %FSL (black bar chart); %MSA − %MSL (white bar chart).

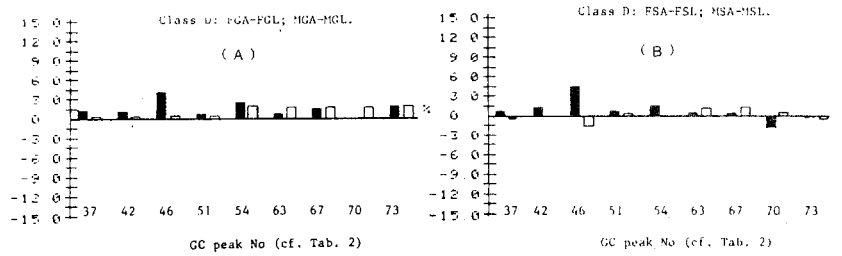


FIG. 16. (A) %FGA − %FGL (black bar chart); %MGA − %MGL (white bar chart). (B) %FSA − %FSL (black bar chart); %MSA − %MSL (white bar chart).

acter is now to be taken into account in biosynthesis problems, on the one hand, and in all taxonomic research with the aim of identifying species according to their hydrocarbon profiles, on the other hand: the kind of life—gregarious or solitary—quantitatively changes the chemical composition of the cuticle. Further research into the possible biological role of these phase change modifications remains to be done.

Acknowledgments—We thank D.A. Carlson for samples of 15,19-dimethylpentatriacontane and 13,23-dimethylheptatriacontane; we thank Professor J. Jullien and J.M. Péchiné for helpful discussions.

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WESTERN AVOCADO LEAFROLLER, *Amorbia cuneana* (WALSINGHAM), (LEPIDOPTERA: TORTRICIDAE) Discovery of Populations Utilizing Different Ratios of Sex Pheromone Components[1]

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Abstract—The most effective lure for male *Amorbia cuneana* (Walsingham) in Orange, Ventura, and Riverside counties of California was previously found to be a 1:1 ratio of (*E*,*E*)-10,12- and (*E*,*Z*)-10,12-tetradecadien-1-ol acetates. In subsequent field tests in San Diego and Santa Barbara counties, this lure was ineffective. Analysis of sex pheromone glands (SPG) of female *A. cuneana* from these two counties showed the *EE*:*EZ* ratio to be about 1:9 and synthetic lures of this composition were highly attractive in these areas. Analysis of the SPG of a number of females from both areas showed that there were three population types: two in the low ratio areas possessed 37 and 58% *EZ*, and the third in the high ratio areas possessed 89% *EZ*.

Key Words—*Amorbia cuneana*, *Amorbia essigana*, sex pheromone, sex pheromone component ratios, Lepidoptera, Tortricidae, (*E*,*E*)-10,12-tetradecadien-1-ol acetate, (*E*,*Z*)-10,12-tetradecadien-1-ol acetate.

INTRODUCTION

Amorbia cuneana (Walsingham) is a pest of avocados and citrus in California (Ebeling, 1959). It is native to North America (Walsingham, 1879). At one time it was thought there was another *Amorbia* species in California, *Amorbia essigana* (Busck) (Busck, 1929). Subsequently, *A. essigana* was placed in syn-

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onymy with *A. cuneana* (Powell, 1983). In previous work, McDonough et al. (1982) reported the structure and synthesis of the sex pheromone of *A. cuneana*, a mixture of (*E*,*E*)- and (*E*,*Z*)-10,12-tetradecadien-1-ol acetates. Subsequently, field tests to determine optimum component ratios, dosages, lure longevity, and other parameters associated with use of the lure were carried out (Hoffmann et al., 1983). The field tests were conducted in Orange, Ventura, and Riverside counties of California. However, when the lure was later tested in San Diego and Santa Barbara counties, few or no male *A. cuneana* were captured even though light traps showed high populations were present. We therefore undertook this study to determine the reason for these results.

METHODS AND MATERIALS

Insects. Amorbia cuneana females analyzed in this study were collected as larvae or pupae from avocado groves in San Diego, Santa Barbara, and Ventura counties in California during the summer and autumn of 1981.

Pupae were sexed, and the females were sent to Yakima, Washington, where they were placed on moist vermiculite and kept at 17°C, 63% relative humidity in a photoperiod of 14 : 10 light–dark. Emerged females were caged daily and offered a diet of beer, sucrose, and ascorbic acid (Calkins and Sutter, 1976). Larvae were taken to the UC South Coast Field Station in Irvine, California, where they were reared on *Trichoplusia ni* diet (Shorey, 1963) at 26.7°C in a photoperiod of 14 : 10 light–dark. Upon pupation, the specimens were collected and sent to Yakima where they were treated in the same manner as previously described.

Females analyzed in the mating studies were the progeny of single adult pairs collected as pupae from a commercial avocado grove in Ventura County. Rearing and handling procedures were the same as previously described, with the exception that the progeny of each mated pair were kept separate, allowing for determination of the range in isomer ratios of the sex pheromone in single matings.

Determination of Ratio of Pheromone Components in Females and Field-Aged Septa. Females (2–4 days old) were collected ca. 8 hr after the beginning of scotophase and refrigerated 10–30 min before dissection. The abdomen was squeezed to extrude the terminal segments and the tip was excised so as to include all of the abdominal segment not covered by scales. Each tip was extracted separately in 10 μl of heptane for about 33 min.

The extracts of individual female abdominal tips were analyzed on a Quadrex glass capillary column (56 m × 0.25 mm ID) coated with methyl silicone (SE 30®) and operated in the splitless mode with the following program: 45 sec delay on inlet purge; 2 min at 80°C; 32°C/min to 190°C.

Septa from the field aging test were extracted in dichloromethane for 1 hr

by shaking with a mechanical shaker. The dichloromethane was evaporated, and the residue was dissolved in heptane and analyzed in the same manner as the abdominal tip extracts.

Preparation of Lures. The synthetic pheromone consisting of a mixture of *EE* and *EZ* isomers was prepared as reported previously (McDonough et al., 1982). Lures of given *EE:EZ* compositions in red rubber septa (West Co., Phoenixville, Pennsylvania) were prepared from the purified isomers. The isomers were purified by high-pressure liquid chromatography (Hewlett-Packard chromatograph model 1084B), using an ultraviolet detector and a 10-μm particle size silica with a bonded coating of octadecyl (RP-18) in a column 20 cm $\times$ 4.6 mm ID. Fractions were eluted with 77% MeOH in water.

Field Tests. Tests were conducted in commercial avocado groves in Santa Barbara and San Diego counties. Pherocon® 1C traps were used with an extra coating of Stickem Special® applied to the trap bases to improve trap efficiency. Lures were rubber septa impregnated with 0.2 mg of 98.5%, 90.0%, 80.0%, and 50.0% of the *EZ* component of the pheromone. These septa were impaled on No. 17 straight pins hung from the top inside center of the traps.

Traps were hung on peripheral branches of the trees 2–3 m off the ground and spaced no closer than 27 m within and between trap rows. A randomized complete block design was used. Four treatments replicated five times were used in the Santa Barbara County study. Traps were checked every two days and rotated one position within the block each time they were checked to minimize bias in trap-catch due to location. Traps were checked eight times so that each treatment was at each location twice during the study.

The test in San Diego County was conducted in a commercial avocado grove near San Luis Rey. It was conducted in the manner described above except that the distance between traps was about 50 m and four replicates were used.

Trap catch data were analyzed using ANOVA and DMRT ($P = 0.05$).

RESULTS AND DISCUSSION

Following our initial unsuccessful trapping experiments in San Diego and Santa Barbara counties, pupae of *A. cuneana* were obtained from these areas, reared to adults, and female sex pheromone glands (SPG) were extracted. An obvious difference in extracts of female SPG from those we had analyzed previously (McDonough et al., 1982; Hoffmann et al., 1983) was in the ratio of the (*E*,*E*)-10,12- and (*E*,*Z*)-10,12-tetradecadien-1-ol acetates.

Determination of the *EZ* isomer content (as a percent of the total of *EE* + *EZ*) from 16 virgin female *A. cuneana* from San Diego County gave an average value of 86.6 $\pm$ 5.1 SD%. All values except one were in the range 82.0–93.0%; the exception was 72.1%. The *EZ* content in 11 females from Santa

Barbara County averaged 89.7 ± 2.4% (range 86.7–92.7%). Accordingly, lures with high ratios were tested and compared with a low-ratio lure (50% *EZ*) in these areas.

The results of the Santa Barbara test are summarized in Table 1. At the termination of the test, the lures were extracted and the isomer content was determined. This procedure was necessary because of the ease with which conjugated dienes in rubber septa can isomerize outdoors (Davis et al., 1984; Guerin et al., 1983). After 19 days, *EZ* percentages had decreased to levels that were significantly different from the ratios found in females of this area. The final ratios found in the lures indicate that during the test those of initial values of 98.5 and 90.0% *EZ* were changing mainly throughout the range found in females, while the 80% lure would have been at best marginal at the beginning of the test and considerably below the values found in females during much of the test. From this standpoint then, the trap catches indicate a male preference only for those ratios found in the females of this region.

A similar field test was conducted in San Diego County (Table 2). The lures were not analyzed at the end of this test. Less isomerization would be expected here than in the first test because of the shorter test period (8 days). Again there was a preference for the higher ratios of *EZ*. The 50% isomer again caught very low numbers, while the catch of the other treatments increased with increasing percentages of *EZ*.

In our earlier study (Hoffmann et al., 1983), we reported that *EZ* percentages from individual female *A. cuneana* from Riverside County averaged 54.8 ± 10.2% *EZ* (range 33.7–69.6%). Subsequent examination of these data indicated that most of the values appeared to be grouped around two values. To determine if this were in fact the case, *EZ* percentages of female progeny from seven singly mated pairs were determined.

TABLE 1. TEST OF EFFECT OF (*E,E*)-10,12- AND (*E,Z*)-10,12- TETRADECADIEN-1-ol ACETATE RATIOS (PERCENT *EZ* AS PERCENT OF *EE* + *EZ*) ON TRAP CATCH OF MALE *A. cuneana* IN SANTA BARBARA COUNTY, CALIFORNIA[a]

Percent *EZ*		No. of males caught[b]	
Initial	Final	Total	Per trap–day
98.5	78.1 ± 1.6	1403	14.8 a
90.0	59.3 ± 2.7	926	9.7 a
80.0	55.9 ± 4.8	39	0.4 b
50.0	not analyzed	24	0.3 b

[a] Because the conjugated diene structure isomerizes, values of the percent *EZ* are listed both at the beginning and at the end of the test. Test was conducted from 8/24/82 to 9/11/82 (19 days). There were five replicates of each ratio.

[b] Means followed by the same letter are not significantly different; ANOVA and DMRT ($P = 0.05$).

TABLE 2. TEST OF EFFECT OF (*E,E*)-10,12- AND (*E,Z*)-10,12- TETRADECADIEN-1-ol ACETATE RATIOS (PERCENT *EZ* AS PERCENT OF *EE* + *EZ*) ON TRAP CATCH OF MALE *A. cuneana* IN SAN DIEGO COUNTY, CALIFORNIA[a]

Initial percent EZ	No. of males caught[b]	
	Total	Per trap–day
98.5	332	10.4 a
90.0	152	4.8 b
80.0	83	2.6 bc
50.0	14	0.4 c

[a] The test was conducted from 10/14/82 to 10/22/82 (8 days).
[b] Means followed by the same letter are not significantly different; ANOVA and DMRT ($P = 0.05$).

The ratios of the pheromone components in females from the singly mated pairs from a low ratio area are summarized in Table 3. The *EZ* ratios fall into two well-defined groups with small standard deviations: one at about 37% *EZ* and the other at about 58% *EZ*. It is noteworthy that the 37 and 58% *EZ* populations share the same geographical area while the 88% *EZ* population appears to inhabit its own distinct areas.

A summary of all *EZ* percentages determined in single females from this and our preceding study (Hoffman et al., 1983) is shown in Figure 1. The *EZ* values clearly occur in three groups: at 37.2 ± 3.9% *EZ* and 58.3 ± 3.0% *EZ* for the population responding to the low *EZ*% lure, and 88.5 ± 3.2% for the population responding to the high *EZ*% lure. The greatest number of individual females possessed the 37.2% *EZ* lure, and the distribution of values about the

TABLE 3. PERCENT OF (*E,Z*)-10,12- AND (*E,E*)-10,12-TETRADECADIEN-1-ol ACETATES FOUND IN PROGENY FROM SINGLE MATED PAIRS

Single mated pair	No. of female progeny	No. of females having given values of *EZ*% ± SD
A	12	12 (36.8 ± 1.7%)
B	8	2 (35.6 ± 2.2)
		6 (59.5 ± 3.2)
C	13	13 (36.7 ± 1.5)
D	8	8 (36.4 ± 0.9)
E	14	8 (37.0 ± 1.9)
		6 (57.9 ± 1.9)
F	9	6 (35.4 ± 1.6)
		3 (55.1 ± 0.7)
G	16	16 (37.1 ± 1.3)

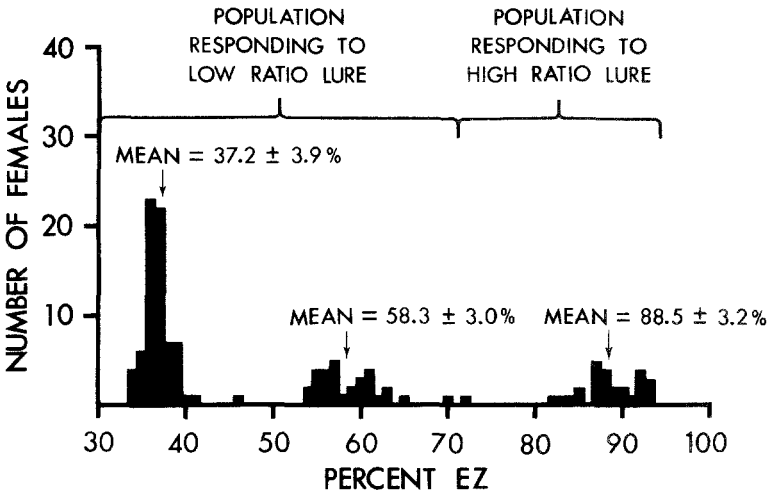

FIG. 1. Number of female *A. cuneana* having a given percent of *EZ* (as a percent of *EE* + *EZ*).

mean resembles a normal distribution. Presumably, the distribution about the 58.3 and 88.5 values would also resemble a normal distribution if a greater number of insects had been analyzed. The relative proportion of the females having the 37.2 and 58.3% *EZ* values (71 and 29 females, respectively) does not necessarily represent the actual relative proportions in field populations. The sampling was not extensive enough to ensure a statistical sample.

The phenomenon of three groups of values of *EZ*% in *A. cuneana* suggests a situation similar to the one found for the European corn borer, *Ostrinia nubilalis* (Hübner) as reported by Klun and Maini (1979), Klun et al. (1973, 1975), Kochansky et al. (1975), and Cardé et al. (1975). The sex pheromone of *O. nubilalis* is (*E*)- and (*Z*)-11-tetradecen-1-ol acetates. Originally two varieties of *O. nubilalis* were found to exist in nature: one in which females used a 4:96 *Z:E* ratio (New York strain), and the other in which they used a 97:3 ratio (Iowa strain). The males of each variety responded only to the ratios found in females of the same variety. Subsequently, geographic areas were discovered in which three varieties coexisted: males responded to *Z:E* ratios of 1:1 as well as 97:3 and 3:97. In those areas where males responded to the 1:1 ratio, native females were found that contained a 35:65 *Z:E* ratio. Laboratory breeding experiments between the 97:3 and 4:96 varieties yielded only the 35:65 variety. Breeding of the 35:65 variety gave the 97:3, 35:65, and 4:96 in an approximately 1:2:1 ratio. Klun and Maini (1979) concluded that the *Z:E* ratio was determined by a single pair of alleles with one allele exhibiting incomplete dominance. Although a similar situation might exist for *A. cuneana*, it is not possible to know, with our present information, which genotypes produce the

observed phenotypes of *A. cuneana*. Breeding experiments are needed to answer this question. From a practical standpoint lures containing a 1:1 *EE:EZ* and a 1:9 *EE:EZ* ratio should be adequate for monitoring the low and high ratio *A. cuneana* populations.

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BENEFICIAL ARTHROPOD BEHAVIOR MEDIATED BY AIRBORNE SEMIOCHEMICALS
I. Flight Behavior and Influence of Preflight Handling of *Microplitis croceipes* (Cresson)[1]

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Abstract—Oriented responses of *Microplitis croceipes* (Cresson) to airborne odors of actively feeding *Heliothis zea* (Boddie) larvae were observed in a flight tunnel. The behavior of *M. croceipes* prior to and during sustained, oriented flights was videotaped and analyzed in detail. Preflight exposure of the parasitoid to feces and other components of the plant–host complex were found to be vital in effective flight behavior, while maturation of the parasitoid had little effect. The increased frequency of oriented flight that resulted from preflight exposure of a plant–host complex persisted for at least 24 hr.

Key Words—Hymenoptera, Braconidae, *Microplitis croceipes*, parasitoid behavior, habitat location, *Heliothis zea*, Lepidoptera, Noctuidae, flight tunnel, female flights, preflight handling.

SERIES PREFACE[5]

Improved methods for harnessing beneficial entomophagous arthopods are considered of priority importance as a means of providing effective, safe, and eco-

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nomically sound technology for combating the losses of agricultural products to insect pests. Current approaches for using these biological control agents include: (1) importing and establishing new species of beneficial parasitoids and predators from foreign countries; (2) encouraging maximum abundance and efficiency of these beneficial arthropods in crops or other target areas by providing food, shelter, attractive odors, etc.; and (3) mass production and release of beneficial arthropods into the target areas.

A central problem underlying all approaches for biological control is the inability to ensure retention and sustained activity of the biological agents in the target area. This problem exists in the case of many pests where the beneficial insects are naturally present in high numbers and/or technology for their mass production and release is available. A prerequisite for resolving this problem is a knowledge of factors and mechanisms governing the means by which they find and attack their hosts and prey.

Trail odors from the pest insects, cues from the host plants, and other biological communication mediators are vital to the host- or prey-finding process (Lewis et al., 1982a). A considerable amount of information has begun to accumulate regarding the role of these factors. For example, substantial progress has been made toward elucidating close-range foraging behavior of entomophages mediated by contact chemicals (Weseloh, 1981). However, major gaps in knowledge exist which seriously constrain employment of biological control (Greany et al., 1984). Of particular significance is the lack of information regarding the foraging behavior mediated by volatile semiochemicals. The work of Hagen et al. (1976) on the response of *Chrysopa carnea* Stephens to volatile tryptophan by-products from honeydew of aphids, the occurrence of volatile kairomones in prey searching by predatory mites (Sabelis and Van de Baan, 1983; Sabelis and Dicke, 1985), reports of Lewis et al. (1982b, 1985) and Noldus and van Lenteren (1985) regarding the use of moth sex pheromones by *Trichogramma* spp. to locate host eggs, and various reports regarding host plant and habitat preferences by entomophages (Altieri, 1983; Vinson, 1981) demonstrate the importance of airborne semiochemicals to host and prey selection by entomophages.

Volatile allelochemicals have been shown to be an important tool for beneficial arthropods in microhabitat selection and niche segregation (Vet, 1983, Vet and van Opzeeland, 1984; Vet et al., 1984) and distant prey selection (Dicke and Groeneveld, 1985). Yet, essentially no information is available about the behavioral mechanisms involved in entomophage responses to the volatile semiochemicals. Additionally, little is known about the chemical nature, role, or interactions of components from different sources (plants and host insects) that comprise the total semiochemical system. Only a few structures of volatile allelochemicals emitted from host plants or fermentation products of host-associated frass have been elucidated (Greany et al., 1977; Dicke et al., 1984; Elzen et al., 1984).

Natural-enemy foraging behavior, as mediated by volatile semiochemicals, is the subject of a set of collaborative studies planned by J.C. van Lenteren and coworkers at the Agricultural University, Wageningen, The Netherlands, and J.H. Tumlinson, W.J. Lewis and coworkers, ARS, USDA, Gainesville, Florida, and Tifton, Georgia, respectively. These investigations will be the subject of this series of reports. The studies are designed to involve both generalist and specialist entomophages and those attacking different stages of insect pests, especially the egg and larval stages. Also, the research will involve entomophages from the European and United States agricultural ecosystems. It is hoped that these joint investigations will provide an impetus for breaking through this central barrier to biological control.

INTRODUCTION

The primary constraint in deciphering long-range host-searching behavior of entomophagous arthopods has been the lack of effective bioassay techniques. Much research has been conducted with various olfactometers, but these systems confine the parasitoids, making flight and related behavioral expressions virtually impossible (Kennedy, 1977; Vet et al., 1983). Moreover, olfactometers are inappropriate for insects that only orient to odors after initiation of flight. Previous attempts to use greenhouses or flight tunnels for behavioral response studies of parasitoids to airborne semiochemicals also have generally failed because the insects typically exhibit disoriented behavior under captive conditions.

These characteristics have prohibited bioassays of oriented flight behavior and other responses of entomophages to airborne stimuli. Gross et al. (1975) demonstrated that preflight exposure to kairomones could be used to override the tendency of *Microplitis croceipes* (Cresson) to disperse. Preflight exposure of two *Trichogramma* spp. and *M. croceipes* to the respective kairomones from moth scales and larval feces of their common host *Heliothis zea* (Boddie) increased rates of parasitization. It was postulated that the higher parasitization resulted from more consistent orientation to the host. This paper reports behavioral responses of *M. croceipes* to airborne semiochemicals in a flight tunnel, describes their flight characteristics, and evaluates the influence of preflight handling.

METHODS AND MATERIALS

Insects. Larval *H. zea* were reared on artificial diet using the method of Burton (1969). The *M. croceipes* were obtained from the Insect Biology and Population Management Research Laboratory, Tifton, Georgia. Parasitoids of both sexes were allowed to emerge and mate in acrylic cages (30 × 30 × 17

cm) at 28°C, 50–70% relative humidity, and a 16-hr photophase (Lewis and Burton, 1970). Mating occurs as soon as both sexes are present (Bryan et al. 1969; Lewis and Burton, 1970). Age of females used for experiments varied between 1 and 5 days.

Odor Source. The following kind of target was prepared to approximate the composition of cues present in a natural plant–host complex. Larvae were placed on cowpea leaves in the late afternoon the day prior to experimentation to ensure that larval feces came from pea leaves only. A water-filled vial (9.5 cm height, 6 dm diam.) that contained a cowpea leaf with an eating and defecating *H. zea* larva (plant–host complex) was placed in the middle of the intake end of the test section of the flight tunnel.

Flight Tunnel. A variable-windspeed flight tunnel was constructed to study the flight behavior of parasitic Hymenoptera (Figure 1). The tunnel has a 50 × 50-cm cross-section and is an assembly of three sections: intake, test, and exhaust, which has a venturi shape. Air enters the intake made of 6-mm-thick plywood by passing through a standard dust filter. Inside is a honeycomb of plastic soda straws (197 mm length, 6 mm diam.) packed loosely in a regular linear pattern. Two sheets of aluminum window screen (7 × 7 mesh/cm^2) hold the straws in place.

The test section is 120 cm long with a glass top and cast acrylic walls and floor, all 6 mm thick. A black and white pattern on poster board is supported underneath the floor of the test section by a sheet of cast acrylic to provide a visual reference for flying insects. Nylon mosquito netting (13 × 13 mesh/cm^2) covers both open ends of the test section. One side wall is hinged at the top to allow access to the test section; foam weather-strip tape is used to seal this portal when closed. Aluminum angle braces (2 × 2 cm) reinforce the edge joints of glass and acrylic. In addition, these braces are mounted at the ends of each section as flanges to permit fastening tunnel sections together. Overhead

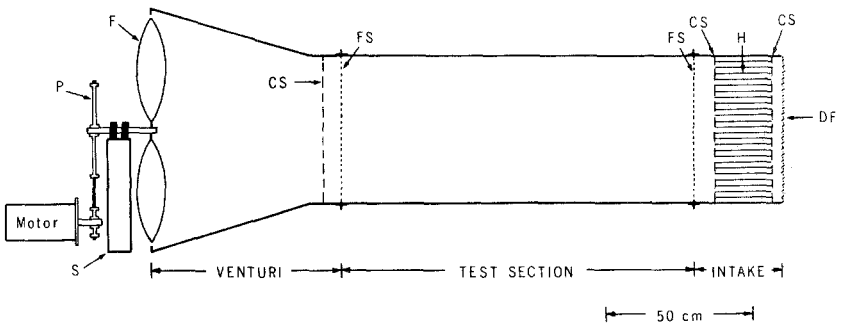

FIG. 1 Lateral section of the flight tunnel: H, honeycomb of 6-mm-diam. soda straws; CS, course screen (7 × 7 mesh/cm^2); FS, fine screen (13 × 13 mesh/cm^2); DF, dust filter; F, fan blade; and S, fan support.

lighting is provided by eight flood lamps arranged in two rows, or for video recording by two fluorescent bulbs. Light intensity in the flight tunnel varied between 3229 and 4090 lux for the flood lamps and between 2260 and 3014 lux for the fluorescent tubes.

The plywood venturi opens the cross-section from 50 × 50 cm to 80 × 80 cm over 55 cm, and internal baffles transform the section from a square to an octagon at the fan end. One sheet of aluminum window screen is mounted near the upwind end of the venturi. A three-blade aluminum fan, shrouded within 1 cm of the 76-cm-diam. blades, is powered by a variable-speed motor (model 4Z248A, 0.2 kW = $\frac{1}{4}$ hp, Dayton Electric Mfg. Co., Chicago, Illinois). The fan draws air through the intake into the test section. Screens and mosquito netting in the flight tunnel aid in achieving laminar air flow while the honeycomb removes some turbulence before air enters the test section.

Flight tunnel performance was assessed using both the smoke of a ventilation smoke tube (Mine Safety Appliance Co., Pittsburgh, Pennsylvania) and a hot-wire anemometer (model AB-27, Hastings Airmeter, Hastings Raydist, Hampton, Virginia). Air flow through the test section is approximately laminar as judged by viewing smoke plumes. Windspeed using the calibrated motor speed dial can be varied between 7 ± 1 cm/sec and 200 ± 3 cm/sec and was maintained at 23 ± 2 cm/sec, measured at the center of the flight tunnel during all experiments.

Flights were recorded on video tape from the center top with a RCA® TC 2055/C camera and a Panasonic® NV-8959 recorder. Tapes from sustained flights only were replayed in slow motion or frame by frame for analysis.

Preflight Handling. Standard exposure cups were prepared by placing one medium-sized cowpea leaf (ca. 3.45 cm^2) and one, third-instar *H. zea* larva in a 30-ml plastic cup in the late afternoon, allowing it to eat and defecate during the night. The next day, just prior to an experiment, each *M. croceipes* female was transferred with a clean 1-dram vial (6 cm height, 1.5 cm diam.) from the rearing cage and allowed to contact the material in the exposure cup during a 2-min period and sting the larva once. If a parasitoid could not find the host within 2 min, the larva was picked up carefully with forceps and presented to the female, which always resulted in a sting. If a female stung more than once, she was discarded. Immediately after this preflight exposure procedure, the parasitoid was transferred to the flight tunnel in a 1-dram vial. The vial was placed open end up at the downwind end of the flight tunnel, straight downwind from the target. Time from introduction into the tunnel until initiation of flight (latency), time from initiation of flight until landing on the target (navigation time), and type of flight were recorded for each female.

Observation time per individual was limited to a maximum of 10 min and stopped after a female landed on the target. Females that did not take off within 5 min were discarded. The number of no take-offs did not exceed 4% of the number of tested females for any experiment. For experiments in which a par-

asitoid was given a second flight trial immediately after landing, it was recaptured in the vial within 3 sec, before it could sting the larva. If oviposition could not be prevented, the parasitoid was excluded from the second trial and the target was replaced by one with an unparasitzed larva.

Flight Initiation and Orientation. The behavior of female *M. croceipes* exposed to the plant–host complex in the flight tunnel was observed. All females had the standard preflight exposure to a plant–host complex. Observations were restricted to the behavior expressed on the 1-dram release vial and in flight. During these observations, we defined a classification of discrete behavioral events. Relationships between these behavioral events during flights and on the 1-dram vial were described. Four categories of flight were used to evaluate the effect of different preflight exposures.

Next, characteristics of sustained flight toward a plant–host complex were determined and described. Video recordings were made only of the first flights of individuals, eight of which were used for the analysis.

Experiment 1. Flight responses to undamaged plants with and without associated infestation odors were observed. The responses of nine females provided with the standard preflight exposure were tested individually in three sequential trials. The female was recaptured after each trial and released at the introduction point for the next trial. The stimuli were given in the following order: (1) noninfested cowpea leaves, (2) noninfested cowpea leaves with the odor of a plant–host complex added, and (3) noninfested cowpea leaves again with infestation odors removed. The second treatment was achieved by placing the plant–host complex outside the flight tunnel, out of sight of the insects, at the intake end. In this way the insect perceived visual cues of the noninfested cowpea leaves in every trial, while it was exposed to additional olfactory cues of the plant–host complex only in the second.

Experiment 2. Five types of preflight exposure cups were used to determine the effect of preflight exposure to individual components of the plant–host complex on the initiation and characteristics of flight of *M. croceipes* females. In addition to the standard exposure cups containing a plant–host complex and empty control cups, these cups contained either an artificially damaged cowpea leaf, a larva, or larval feces. Leaves were artificially damaged by three pricks with a pair of forceps just prior to use so the damage would be fresh when presented to parasitoids. Larvae and larval feces were obtained from standard exposure cups and transferred to clean ones just prior to use. Latency, navigation time, and flight type were recorded for the first and second flights of each individual tested. Only females that landed on the target after the first flight were given a second trial. To determine whether maturation influenced the flight behavior of female *M. croceipes*, behavioral data of individuals with a preflight exposure to the larval feces or the plant–host complex were combined into three age groups.

Experiment 3. Retention of the effect of preflight exposure of a parasitoid to a plant-host complex was assessed. First flight responses were tested for females either 0 or 24 hr after their preflight exposure procedure.

Statistical Analysis. The number of females that exhibited a certain flight type after a given preflight exposure were analyzed with Chi-square test. Multiple comparisons of mean times spent to certain behavior were performed using the Duncan's multiple range test. A *t*-test was used when only two mean times needed to be compared. Latencies were transformed to log (x + 1) to stabilize variance. The 0.05 level was set for the rejection of all null hypotheses.

RESULTS AND DISCUSSION

Description of Behavioral Responses. The behavior of female *M. croceipes* was observed from time of introduction into the flight tunnel and first contact with the wind until landing on the target. The typical sequence of the behavioral events observed during these studies was diagrammed (Figure 2). The left side of the diagram represents the preflight behavior performed on the 1-dram release vial while the right side shows the behavior performed by *M. croceipes* in flight.

Responses of the females to the odor sources were defined using the following classification of behavioral events.

1. Standing still: standing still on the substrate without moving antennae.
2. Standing still while drumming: standing still while drumming the substrate with antennae.
3. Standing still while preening: standing still while preening antennae, legs, or both sides of wings.
4. Standing still upwind oriented: standing still while sticking antennae in a vertical plane perpendicular to the wind direction.
5. Walking while drumming: Walking while drumming the substrate with antennae.
6. Initiation of flight pose: sitting on hind legs while making "walking" movements with front legs in the air and sometimes temporarily spreading wings (Figure 3A).
7. Initial zigzagging: making sideways excursions mainly in the horizontal plane and perpendicular to the wind direction soon after take off.
8. Straight flight: flying directly into the main wind direction.
9. Recurrent zigzagging: sideways excursions recurring after a period of straight flight. These excursions are similar to the initial zigzagging, except the movements are less restricted to the horizontal plane.

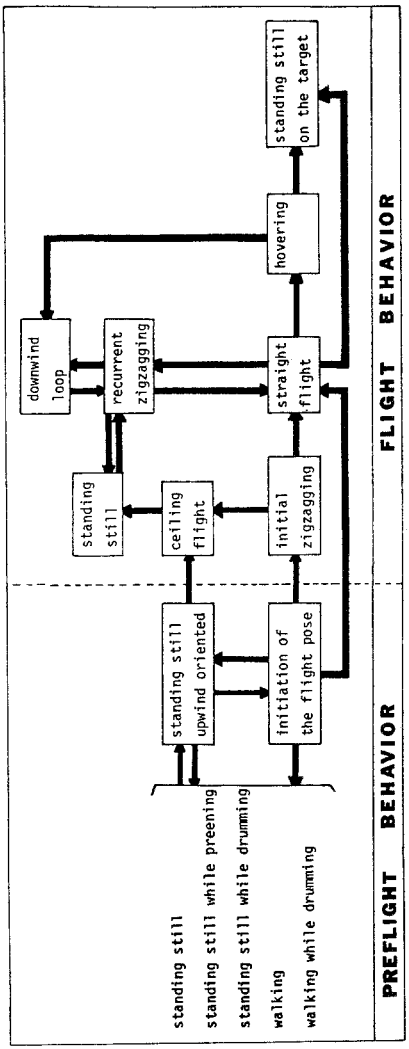

FIG. 2 Schematic representation of the behavioral events and transitions as exhibited by *Microplitis croceipes* responding to *Heliothis zea* larvae feeding on cowpeas.

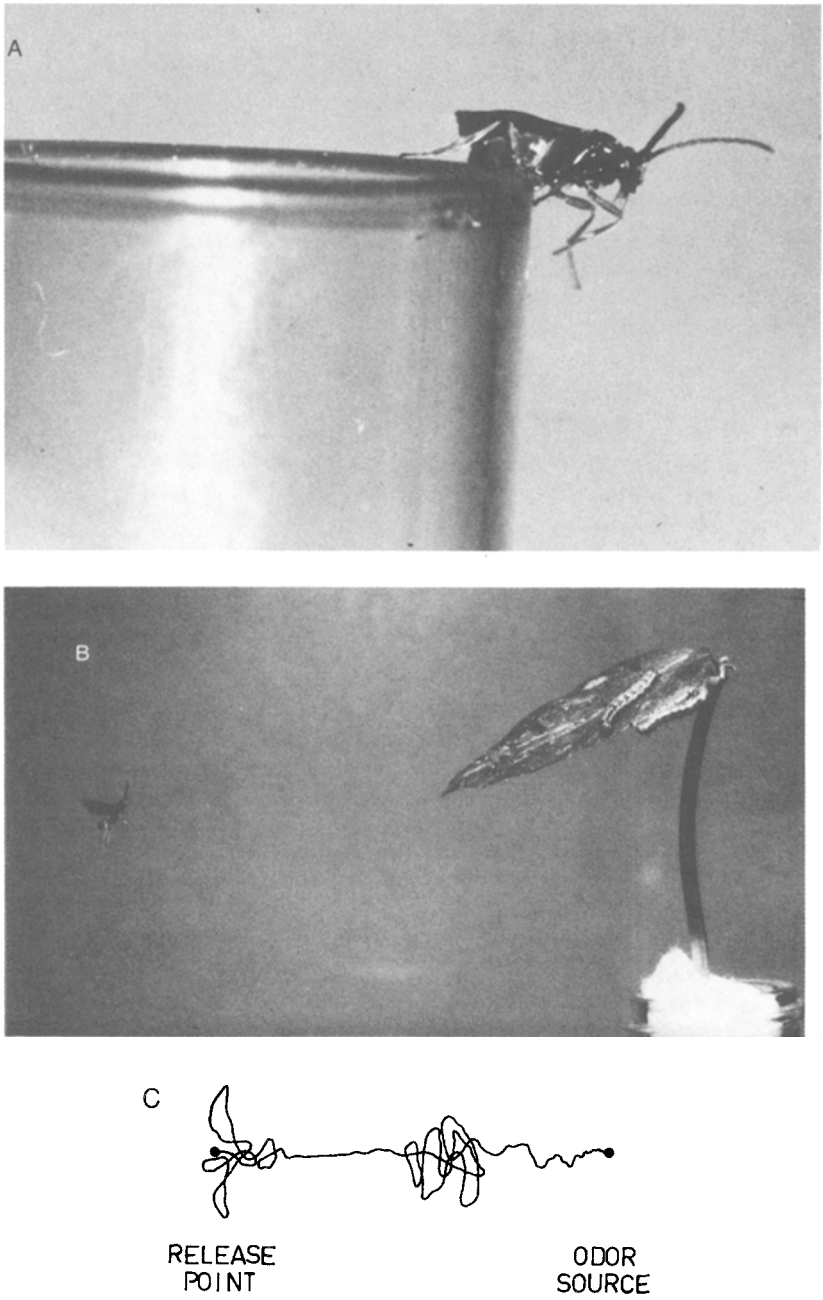

FIG. 3 Behavior of female *Microplitis croceipes* in the flight tunnel. (A) Initiation of the flight pose. (B) Hovering flight. (C) Top view of a sustained flight track.

10. Hovering: keeping stationary while flying at approximately 10 cm downwind from the target (Figure 3B).
11. Downwind loop: a looping maneuver, resulting in a return to an earlier downwind position.
12. Ceiling flight: a strong phototactic, "nonplume-oriented" response that results in the female being in contact with the ceiling of the flight-tunnel where she continues her wing beating.

Figure 2 represents the typical sequence of the described behaviors. Quantifications of these behaviors will be presented in subsequent reports of this series.

Preflight Behavior. Individuals that reached the open end of the 1-dram vial responded by taking an upwind oriented pose for several seconds. This action was followed either by walking, walking while drumming, or by the initiation of the flight pose. The process of intermittent upwind orientation and walking on the 1-dram vial was often repeated before flight was initiated

Flight Behavior. Four types of flight were distinguished; sustained flights, interrupted flights, temporary oriented flights, and non-target-oriented flights.

Sustained flights can involve all steps beginning with an initiation of the flight pose and ending with standing still on the target except a ceiling flight. A typical sustained flight (Figure 3C) begins with initial zigzagging, during which a little "ground" is lost and/or the parasitoid rises slightly above what is presumedly the odor plume. Subsequently there is a dramatic decrease in the extent of the sideways excursions followed by a straight approach toward the target. Sometimes this straight approach is interrupted by a brief recurrent zigzagging that occurred mostly at a 40-cm distance downwind from the target and consisted of sideways excursions smaller than the initial zigzagging. After resuming straight flight or continuing recurrent zigzagging, hovering takes place often at a 10-cm distance downwind from the target. From this point, quick darts were performed toward the target, ending with a landing on the target. Females allowed to fly a second time spent less time on both types of zigzagging.

Interrupted flights differ from sustained flights by the occurrence of a very wide recurrent zigzagging, which begins soon after the onset of straight flight, and sometimes include a downwind loop. These very wide zigzaggings lead to a landing on the sidewalls of the flight tunnel. Within a minute, an initiation of flight pose occurs again, after which flight continues first with a zigzagging and on to oriented flight.

Temporary oriented flights are the same as interrupted flights except that after the landing there is no further flight activity within 10 min.

Non-target-oriented flights include a ceiling flight that occurs immediately after release or after a brief initial zigzagging flight.

Experiment 1. No females had a sustained flight to undamaged plants only, compared to 89% that had one when the odor of a plant–host complex was added. Four of nine tested females flew beyond the cowpea leaves and landed on the screen at the upwind end of the flight tunnel when odor of a plant–host complex was provided behind the screen. Mean latencies of the first two trials (15.6 ± 2.8 sec ISE and 7.9 ± 3.7 sec ISE) were not significantly different. However, after response to the plant–host complex odors (second trial), there was a significantly longer mean latency in the third trial (102.0 ± 48.3 sec ± SE). Comparision of navigation times was not relevant, since sustained flights were only exhibited in second trials and not in the first and third.

Experiment 2. There are several factors in a plant–host complex that might contribute to the preflight experience of a female and hence influence the percentage of target-oriented responses in the flight tunnel. Contact with feces is known to elicit an intense searching response and initiate host-oriented flight (Lewis and Jones, 1971; Gross et al., 1975). When the female encounters a larva, she will generally oviposit. The deposition of an egg might have a different effect on searching behavior than contact with cues that give information about the presence of larvae. This experiment tested the respective effects of preflight exposures to individual components of a plant–host complex on subsequent flight behavior.

Exposures to larval feces or the full plant–host complex was the most effective preflight preparation, based on the percentage of resulting sustained flights. Preflight exposure to larval feces or a plant–host complex also significantly decreased the percentage of nontarget oriented flights (Figure 4). Stinging a larva without contacting other host-related cues did not increase the percentage of sustained flights as much as exposure to larval feces or a plant–host complex. After an *M. croceipes* female has parasitized a larva in a cup, there is usually a short postoviposition period (< 10 sec), during which the female does not search for other hosts. After this period, she continues walking and, upon encountering feces, she is reinforced to search. This reinforcement by feces apparently is important for the parasitoid to continue host searching. However, the effect of preflight exposure to a full plant–host complex reduced the percentage of non-target-oriented flights even more than feces, which suggests that the feces is not the only cue responsible for the behavioral change. Occurrences of temporary oriented flights did not differ significantly with the type of preflight exposure, whereas interrupted flights were observed only after preflight exposure to a larva, feces, or the plant–host complex.

Mean latencies of females exposed to feces or the plant–host complex were significantly longer than those of females exposed to either a damaged leaf, a larva, or nothing (control) (Table 1). This was mainly because females that were exposed to feces or the plant–host complex did not restrict themselves to an upwind orientation pose, they took off like the control group but searched

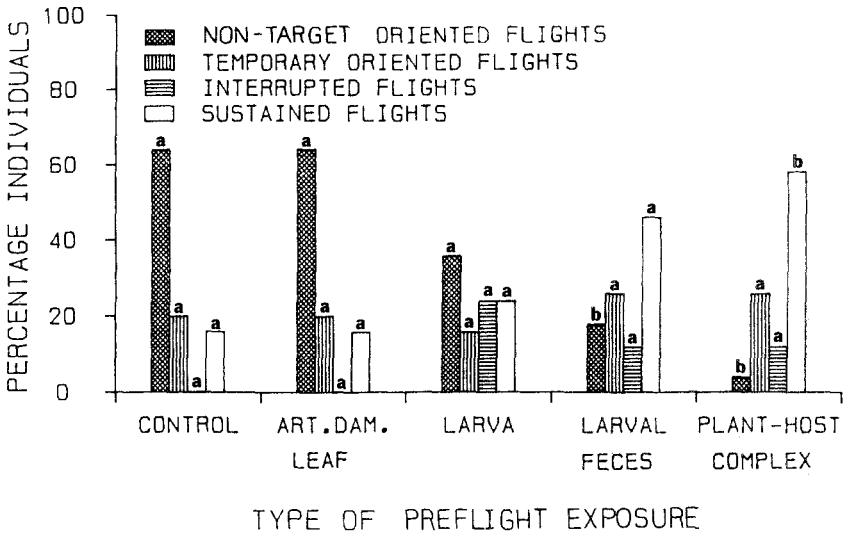

FIG. 4 Percentages of female *Microplitis croceipes* that exhibited various flight responses when provided the indicated preflight exposure. Bars within groups associated with different letters are significantly different (chi-square, $P < 0.05$).

TABLE 1. FLIGHT PERFORMANCE OF FEMALE *M. croceipes* PROVIDED VARIOUS PREFLIGHT EXPOSURES[a]

Preflight exposure	*N*	Sustained flights (%)	Flight characteristics	
			Mean latency ± SE (sec)	Mean navigation time (sec)
Control	25	16.0 a	30.6 ± 11.1 a	8.8 ± 3.2 a
Artificially damaged leaf	25	16.0 a	28.2 ± 11.7 a	10.0 ± 5.4 a
Larva	25	24.0 a	16.9 ± 3.4 a	11.0 ± 2.3 a
Larva feces	24	46.0 a	49.5 ± 12.7 b	15.9 ± 3.9 a
Plant-host complex	24	58.0 b	45.4 ± 11.8 b	12.3 ± 1.9 a

[a] Percentages (chi-square) and means (Duncan's multiple-range test) within columns not associated with the same letter differ significantly ($P < 0.05$).

the vial for a longer time. There were no significant differences in mean navigation times of females that had a sustained flight, which indicated that preflight exposure influences only the initiation of sustained flight.

If exposure to hosts and/or host-associated trail odors is vital for obtaining host-oriented responses of female *M. croceipes*, how do they find a host the first time under natural circumstances? Artificially reared parasitoids are separated from the host before they emerge and thus do not contact host cues before testing. In the field, however, *M. croceipes* emerge in close proximity to the host larva. Although these larvae are unsuitable for oviposition, they may give newly emerged female parasitoids basic information that increases their host selection efficiency significantly.

Second flights were more efficient than first flights. The percentage of sustained flights was significantly higher (74% vs. 52%) and the navigation times significantly shorter (2.6 ± 0.5 sec ± SE, $N = 31$ vs. 13.2 ± 2.1 sec ± SE, $N = 48$). Furthermore, second flights involved more straight flight than first ones. Little time was spent on initial zigzagging and only recurrent zigzagging was observed. Lewis (unpublished data) observed that *M. croceipes* females in field experiments repeatedly flew to visual structures associated with host cues, while Jones (personal communication) observed that *M. croceipes* males, when repeatedly responding to female sex pheromones, recognize visual structures in a flight tunnel. This might be a case of associative learning, and the results discussed above lend additional evidence in that direction.

Maturation did not influence the response of exposed females (Table 2). Data from females with a preflight exposure to larval feces and plant–host complex were pooled and rearranged in three age groups. There were no significant differences in the percentages of sustained flights among the three age groups.

TABLE 2. EFFECT OF AGE ON FLIGHT OF FEMALE *Microplitis croceipes*[a]

Flight characteristics	Response per age class		
	1–2 ($N = 29$)	3 ($N = 11$)	4–5 ($N = 8$)
Sustained flights (%)	68.9 a	45.5 a	75.0 a
Mean latency ± SE (sec)	50.9 ± 12.5 a	50.5 ± 15.9 a	30.9 ± 11.9 a
Mean navigation time ± SE (sec)	15.6 ± 2.8 a	8.6 ± 1.7 a	12.7 ± 2.7 a

[a]Observations of females with preflight exposure to larval feces and plant–host complex were pooled. Percentages (chi-square) and means (Duncan's multiple-range test) across columns not associated with the same letter differ significantly ($P < 0.05$).

TABLE 3. EFFECT OF 24-HR DELAY FROM PREFLIGHT EXPOSURE TO PLANT–HOST COMPLEX UNTIL TESTING ON RESPONSE OF FEMALE *Microplitis croceipes*[a]

	Preflight history and resultant response		
Flight characteristics	Control ($N = 15$)	Immediately after exposure ($N = 15$)	24 hr after exposure ($N = 15$)
Sustained flights (%)	20.0 a	73.0 b	66.7 b
Mean latency ± SE (sec)	7.6 ± 2.4 a	10.9 ± 2.3 a	10.8 ± 1.7 a
Mean navigation time ± SE (sec)	9.7 ± 1.8 a	10.1 ± 1.7 a	10.8 ± 1.7 a

[a] Percentage (chi-square) and means (Duncan's multiple-range test) across columns not associated with the same letter differ significantly ($P < 0.05$).

Experiment 3. Parasitoids exposed to a plant–host complex 24 hr before their first flight were more responsive than those that were not exposed (Table 3). This indicates that preflight exposure causes a prolonged behavioral change.

CONCLUSIONS

The results of this study demonstrate that oriented flight responses of *M. croceipes* to airborne host odors can be studied under laboratory flight tunnel conditions.

Characteristics of the behavioral responses prior to take off and during flight could be observed adequately. Thus, this system is efficient for elucidating behavioral mechanisms of parasitoids mediated by host-associated volatiles. Such experimental tests have not been developed previously for study of parasitoid host-searching behavior.

The nature of the flight responses of *M. croceipes* was strongly affected by preflight exposures to host-related materials. Prior exposure to host feces was the single most important experience for obtaining sustained flight responses to the target. However, preflight exposure to combinations of the plant–host complex was more effective than any of the single components. Influence of the preflight exposure on the flight characteristics lasts for at least 24 hr without any intermediate encounter to such materials. These findings demonstrate the importance of airborne, as well as contact, semiochemicals in the host-searching behavior of *M. croceipes* and show the value of the flight tunnel system as an approach for elucidating the mechanisms of the host-foraging behavior of *M. croceipes*.

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STUDIES ON IDENTIFICATION AND SYNTHESES OF INSECT PHEROMONES XXII.[1]
Sex Pheromone of Poplar Twig Clearwing Moth *Paranthrene tabaniformis* Rott—Structure and Synthesis

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Abstract—(*E,Z*)-3,13-Octadecadien-1-ol (1a) was identified as the sex pheromone from the poplar twig clearwing moth, *Paranthrene tabaniformis*, females by gas chromatography–mass spectrometry analysis, synthesis, and laboratory bioassays. In the field tests, a trap baited with 200 μg synthetic 1a caught more male moths than two live female moths.

Key Words—*Paranthrene tabaniformis*, poplar twig clearwing moth, sex pheromone, Lepidoptera, Aegeriidae, (*E,Z*)-3,13-octadecadien-1-ol.

INTRODUCTION

3,13-Octadecadien-1-ol (1), its acetate, or the mixture of their geometrical isomers have been identified as sex pheromones, or shown to be sex attractants of many species of clearwing moth (Lepidoptera: Aegeriidae) (Tumlinson, 1979; Tumlinson et al., 1974). Exceptions are *Vitacea polistiformis* which uses (*E,Z*)-2,13-octadecadienyl acetate as its sex pheromone (Schwarz, et al., 1983), and *Synathedon tipuliformis*, for which this compound is a sex attractant (Voerman et al., 1984). Although it was reported (Du et al., 1981; Nielsen and Purrington, 1978) that males of the poplar twig clearwing moth *P. tabaniformis* were

[1] Studies on the identification and syntheses of insect pheromone XXI. Stereoselective synthesis of all the possible optical isomers of the mosquito oviposition attractant pheromone. Lin Guo-Qiang, Xu Hai-Jian, Wu Bi-Chi, Guo Guang-Zhong, and Zhou Wei-Shan, *Tetrahedron Lett.* 26:1233–1236, 1985.

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attracted by a synthetic compound (*E*,*Z*)-3,13-octadecadien-1-ol (1a) by screening tests, the structure of the sex pheromone of this insect remained undetermined. Since *P. tabaniformis* is a primary pest of forests in northern China, the structure determination of the sex pheromone of this insect would facilitate the use of traps for monitoring purposes and would be the first step in the exploration of a possible pest control method. We now describe the structure determination and synthesis of the sex pheromone of the poplar twig clearwing moth.

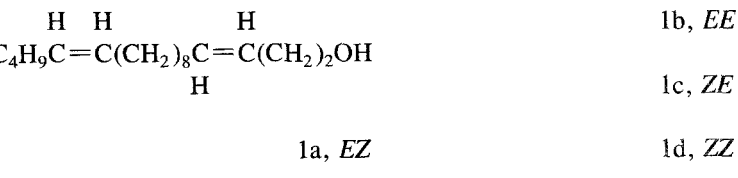
METHODS AND MATERIAL

Pheromone Collection. Moths used in this study were reared on an artificial diet. Details of the insect diet, rearing technique, and the bioassay procedure will be described elsewhere by some of the authors. The ovipositor areas of 1- to 2-day-old unmated females (50 FE) were clipped and extracted with CH_2Cl_2. The extract was filtered over glass wool to remove the solids and then most of the solvent was removed under dry N_2 to an appropriate small volume for analysis.

Instruments for Structure Analysis. Mass spectra were obtained on a Finnigan 4021 GC-MS instrument equipped with a SE-54 capillary column (30 m × 0.25 mm), split ratio 10:1. The column temperature was programmed from 100 to 200°C at 10°C/min. Helium was normally used as the carrier gas. Mass fragmentography was conducted on a Shimadzu QP-1000 GC-MS instrument, FFAP column (50 m × 0.3 mm), column temp 220°C, split ratio 50:1.

Bioassay. EAG (electroantennagram) equipment was home-made according to Roelofs and Comeau (1971). Field tests were conducted in the Beijing area during the adult flight seasons in 1983 and 1984. Delta-type adhesive traps were used and hung on trees about 1.5 m above the ground. Traps were charged with 200–600 μg synthetic lure, using red rubber sleeve stoppers as dispensers.

Synthesis



Many synthetic routes to (1) have been published (Uchida et al., 1978, 1979; Voerman, 1979; Ebata and Mori, 1979; Doolittle et al., 1980; Gardette et al., 1983; Abrams et al., 1983; Vinczer et al., 1984). In this paper, the triple-bond migration method (Zhang et al., 1983; Zhang and Zhou, 1983) was employed, and all the isomers (1a–d) were synthesized from propargyl alcohol (2) in an overall yield of 37%, as illustrated mainly by the preparation of 1a ac-

cording to Scheme 1. The geometric purities of 1a–d were all more than 95%, and these were used in EAG and field tests.

[^{1}H]NMR spectra were recorded in $CDCl_3$ solution with TMS as internal standard on Varian XL-200 and EM-360L spectrometers. Capillary gas chromatographic (CGC) analyses were performed on a Varian 3700 model instrument equipped with DEGS column (40 m × 0.28 mm) at a column temp of 180°C with split ratio of 40:1. Infrared spectra refer to films and were measured on a Perkin-Elmer 577 spectrometer. Mass spectra were obtained with Finnigan 4021 model mass spectrometer with electron impact source. All boiling points were uncorrected.

RESULTS AND DISCUSSION

Structural Determination

The pheromone extract was injected into a GC-MS (SE-54 column) followed by another injection of the synthetic compound 1a for comparison. The mass spectrum and the retention time (R_t) of the natural pheromone were found to be identical with those of the synthetic 1a (Figure 1), indicating the presence of 1 in the female ovipositor area extract. To determine the double bond configuration, the extract was mass fragmentographed (MF) on GC-MS using *m/z* 266 (M^+) and 248 (M-18) (Figure 2B). The four mixed geometric isomers 1a–d were also mass fragmentographed under the same conditions. The synthetic isomers 1a–d gave four virtually baseline-separated peaks on MF with R_t of 18.12, 17.40, 17.05, and 16.42 min for (*ZZ*), (*ZE*), (*EZ*) and (*EE*) isomers. The natural pheromone was shown to be identical to 1a with R_t of 17.05 min (Figure 2A, B). Other evidence was obtained by coinjection of the pheromone extract with 1a on MF. It showed that only the peak with R_t 17.05 min was enhanced (Figure 2C). Further support was obtained by bioassays. The EAG of the synthetic isomers 1a–d were determined at concentration of 1 and 0.1 μg, and the responses in mV were: 1a (1.5, 0.5), 1b (0, 0), 1c (0, 0), and 1d (1.5, 0.5) (Figure 3). Thus both 1a and 1d gave strong EAG responses, while the other two appeared rather weak. However, in the field tests, only 1a was shown to be very attractive to male moths. The trap baited with 200 μg of 1a captured more males than those baited with two live females. In one of the competition tests, traps baited with 200 μg of synthetic 1a captured eight males per trap, while traps baited with one or two females caught 1.5 males per trap (Table 1). The other three isomers are ineffective (Table 2) and seemed to have neither an inhibitory nor synergistic effect toward the sex pheromone in the preliminary binary system tests (Table 3). Thus 1a was established as the sex pheromone of *P. tabaniformis*.

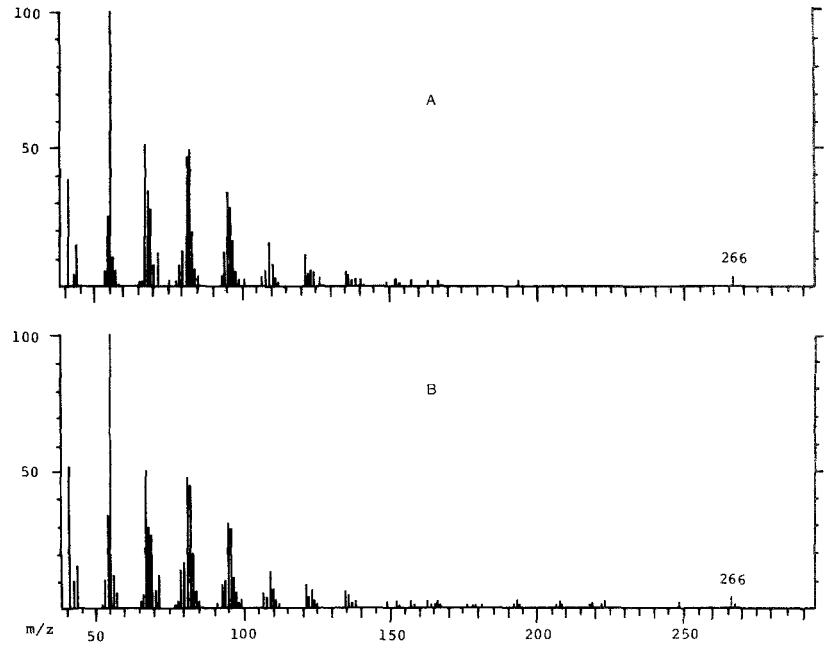
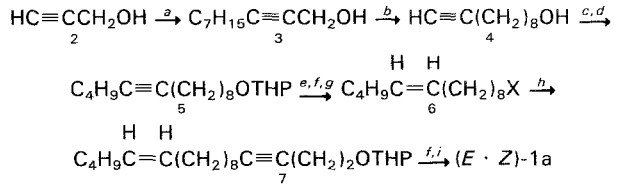


FIG 1. Mass spectra of the sex pheromone of *P. tabaniformis* and synthetic compound 1a. (A) Pheromone from the extract of females, (B) compound 1a.



6a X = OTHP

b X = OH

c X = Br

SCHEME 1. *a*. Li-liq. NH_3, *n*-$C_7H_{15}Br$; *b*. NaH-1,2-PDA or 1,2-EDA; *c*. DHP, HCl; *d*. *n*-BuLi/*n*-BuBr; *e*. P-2 Ni, H_2; *f*. TsOH-CH_3OH; *g*. Py-TsCl, LiBr-acetone; *h*. LiC≡CCH_2CH_2OTHP-HMPT; *i*. LAH-Diglyme.

Synthesis

The procedure chosen to prepare 1a is outlined in Scheme 1.

Dec-3-yn-1-ol (3). Reaction of dilithio-2 [prepared from 2 (1.59 mol) and Li (3.18 mol)] with *n*-heptyl bromide (279 g, 1.56 mol) in liq. NH_3 (1500 ml) at −40°C for 3 hr gave 3 (190.2 g) in 80% yield, bp 78–86°C/2 mm.

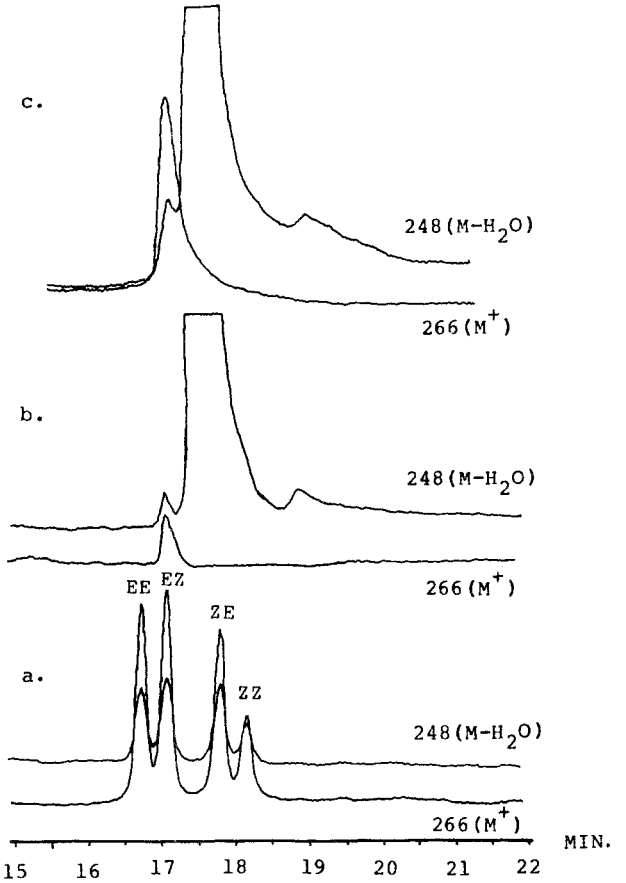


FIG. 2. MF of the sex pheromone of *P. tabaniformis* and synthetic compounds 1a–d. (A) Synthetic compounds 1a–d, (B) pheromone from the extract of the females, (C) coinjection of the pheromone from the extract and compound 1a.

Dec-9-yn-1-ol (4). 1,2-Propylenediamine (100 ml) was added dropwise to NaH (3.7 g, 154 mmol), followed by heating at 70°C for 1.5 hr. After addition of 3 (4 g, 30 mmol), the mixture was stirred at 70°C for 2 hr. The reaction was quenched by addition of aqueous saturated NH_4Cl and extracted with ether. After work-up, 4 (3.3 g) was obtained in 83% yield, bp 135–137°C/23 mm.

The migration of the triple bond in 3 to produce 4 could be monitored by GC (column SE-54, 40 m × 0.25 mm, 170°C, split ratio 70:1), R_t: 3.88 min (R_t: 2.90 min for 3), IR, ν_{max}: 3300(OH), 2130(C≡C) cm^{-1}. NMR, δ: 1.35[12H, br. $(CH_2)_n$], 1.91(1H, OH), 2.13(2H, m, $CH_2C\equiv$), 3.57(2H, m, $C\underline{H}_2OH$).

1-Tetrahydropyranyloxy-9-tetradecyne (5). To a stirred, ice-bath-cooled mixture of 4 (41 g, 0.266 mol) and 0.5 ml HCl, excess dihydropyran (27 g)

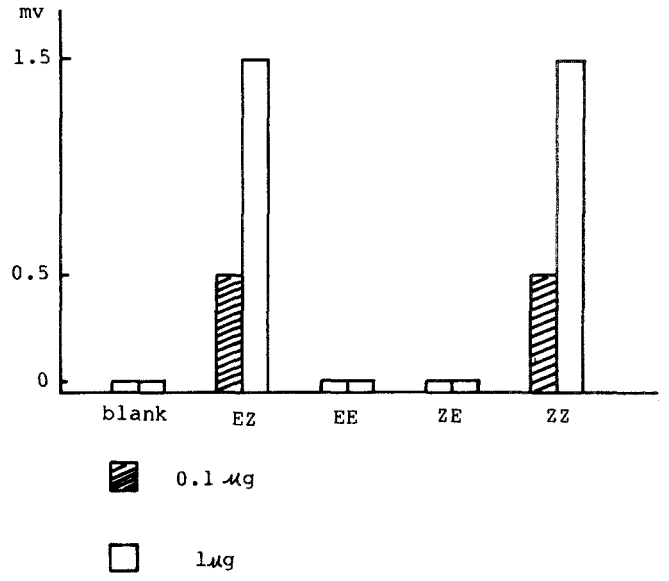


FIG. 3. EAG responses to the synthetic 1a–d.

TABLE 1. COMPETITION RESPONSE OF SYNTHETIC 1a WITH FEMALE MOTHS IN FIELD TEST (JULY 10–28, 1984)

Treatment	Replicate						Catches of male moths	
	I	II	III	IV	V	VI	Total	Average
1a (200 μg)	13	6	11	6	6	6	48	8
2 female moths	3	1	1	1	1	2	9	1.5
1 female moth	1	1	1	4	0	1	9	1.5

TABLE 2. RESPONSE OF *P. tabaniformis* TO TRAPS BAITED WITH SYNTHETIC 1a–d (JULY 4–28, 1984)

Samples	Replicate					Catches of male moths	
	I	II	III	IV	V	Total	Average
1a (*EZ*) (200 μg)	4	5	6	55	16	86	17.2
1b (*EE*) (200 μg)	0	0	0	0	0	0	0
1c (*ZE*) (200 μg)	0	0	0	0	0	0	0
1d (*ZZ*) (200 μg)	0	0	0	0	0	0	0

TABLE 3. BINARY RESPONSE OF SYNTHETIC 1a WITH OTHER ISOMERS (1b–d) IN FIELD TESTS (JULY 10–28, 1984)

Treatments	Replicate					Catches of male moths	
	I	II	III	IV	V	Total	Average
1a (*EZ*) (200 μg) 1d (*ZZ*) (25 μg)	0	1	10	1	2	14	2.8
1a (*EZ*) (200 μg) 1c (*ZE*) (25 μg)	1	2	0	1	0	4	0.8
1a (*EZ*) (200 μg) 1b (*EE*) (25 μg)	0	4	0	5	0	9	1.4
1a (*EZ*) (200 μg)	1	1	3	13	4	22	4.4

was added dropwise followed by stirring for 7 hr at ambient temperature. $NaHCO_3$ (4.5 g) was added, and the mixture was stirred for 1 hr. Upon filteration and evaporation of low boiling impurities, a quantitative yield of crude product was obtained. To the solution of tetrahydropyranyl ether of dec-9-yn-1-ol (30 g, 0.12 mol) obtained above in diglyme (100 ml), a solution of *n*-BuLi in ether (0.15 mol) was added dropwise at 0°C. After evaporation of the ether, *n*-butyl bromide (22 g, 0.16 mol) was added, and the stirred mixture was heated at 140°C overnight. The mixture was poured into ice-water, extracted with ether, and worked up in the usual manner to furnish 5 (29.5 g) in 83.6% yield, bp 137–141°C/0.01 mm.

(Z)-9-Tetradecen-1-ol (6b). Catalytic hydrogenation (Brown and Ahuja, 1973) of 5 (40.7 g, 0.137 mol) over P-2 Ni, and removal of the protecting group (with 11 g TsOH, 280 ml CH_3OH, stirred at room temperature for 8 hr) yielded 6b (33.4 g) in 92.1% yield, bp 107–110°C/0.01 mm. GC: SE-54 column (40 m × 0.25 mm), 180°C, split ratio 70:1, R_t 7.64 min. IR, ν_{max}: 3330(OH), 3010, 1660, 730 (*cis*-CH=CH) cm^{-1}. NMR, δ: 0.89 (3H, t, J = 7, CH_3), 1.31[16H, br, $(CH_2)_n$], 1.87–2.19 (4H, m, CH_2C=), 3.60 (2H, t, J = 6, $C\underline{H}_2OH$), 5.07–5.67 (2H, m, CH=CH).

*1-Bromo-*cis*-9-tetradecene (6c).* The crude tosylate of 6b [prepared from 6b (9 g, 42 mmol) by treatment with pyridine (50 ml) and TsCl (10 g) at 10°C for 2.5 hr] was refluxed with LiBr (6.2 g, 71 mmol) in acetone (60 ml) for 4 hr to afford the corresponding bromide 6c (9.3 g) in 94.4% yield, bp 88–96°C/0.01 mm.

(Z)-1-Tetrahydropyranyl-octadec-13-en-3-yne (7). To a solution of 6c (13.7 g, 47.9 mmol) in HMPT (50 ml), a solution of $LiC{\equiv}CCH_2CH_2OTHP$ (50.8 mmol) in THF (50 ml) was added. The temperature was kept at 0–10°C overnight to yield 7 (12.5 g) in 94.7% yield.

(E,Z)-3,13-Octadecadien-1-ol (1a). Decetrahydropyranylated 7 (4.9 g) was treated with $LiAlH_4$ (3 g) and diglyme (60 ml) (Rossi and Carpita, 1977) at 140°C for 9 hr to yield a crude product which was further purified by flash column chromatography over SiO_2 (eluted with 10% ethyl acetate–*n*-hexane) to give 1a (3.96 g) in 80.8% yield. The overall yield of 1a from 2 was 37%. Calcd. for $C_{18}H_{34}O$: C, 81.13, H, 12.86. Found: C, 81.00, H, 13.19. IR, ν_{max}: 3300(OH), 3010, 960, and 730 (*trans*- and *cis*-CH=CH) cm^{-1}. NMR, δ: 0.83 (3H, t, J = 7, CH_3), 1.26 [16H, br, $(CH_2)_n$], 1.47–2.3 (8H, m, CH_2C=), 3.60 (2H, t, J = 7, $\underline{CH_2}OH$), 5.24–5.6 (4H, m, CH=CH). MS, *m/z*: 266 (M^+, 1.6%), 248 (M-H_2O, 0.5%), 54 (100%). CGC(DEGS) analysis showed that 1a contained (*EZ*) 95.1%, (*EE*) 2.9%, (*ZZ*) 1.1%, (*ZE*) 0.9% with R_t of 25.25, 24.12, 27.68, and 26.36 min.

The other three isomers (1b–d) were synthesized in a similar manner to 1a with the only difference being the use of $LiAlH_4$-diglyme treatment in place of partial hydrogenation over P-2 Ni to introduce a *trans* double bond at C_{13} or to introduce the *cis* configuration at position C_3. Compound 1b contained (*EE*) > 95%, (*ZZ*) 3%, (*ZE*) 1%, (*EZ*) 1%; 1c, (*ZE*) 95.2%, (*EE*) 3.5%, (*ZZ*) 1.3%; 1d, (*ZZ*) 95.2%, (*ZE*) 2.7%, (*EZ*) 1.0%, (*EE*) 1.1%. All compounds (1b–d) were fully characterized by IR, NMR, and MS spectrometry.

CONCLUSION

The chemical and biological data presented in this paper are strong evidence that (*E,Z*)-3,13-octadecadien-1-ol (1a) is a sex pheromone of *P. tabaniformis*. In addition to 1a, all the other geometric isomers have been synthesized. The preliminary data indicated significant attractiveness of the *EZ* isomer alone; the other isomers were inactive and seemed to have neither inhibitory nor synergistic effects toward the *EZ* isomer. Synthetic 1a has been used for monitoring and in a large-scale control program for *P. tabaniformis* since 1983.

In the field tests, one of the synthetic isomers, 1d-(*ZZ*), although inactive toward *P. tabaniformis*, was highly attractive to the poplar large hornet moth *Aegeria apiformis* Clerk that also belongs to Aegeriidae. The structure identification of the sex pheromone of *A. apiformis* is under way in cooperation with Zhang Zhi-Yong and his coworker of Shan-Xi Agricultural University.

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FIELD STUDIES ON CHEMICALLY MEDIATED BEHAVIOR IN LAND HERMIT CRABS: Volatile and Nonvolatile Odors[1]

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Abstract—Land hermit crabs, *Coenobita rugosis*, were tested in the field in Costa Rica for behavioral responses to odors. Volatile odors associated with horse feces, fruit, and honey attracted crabs within minutes. Odors from dead gastropod flesh were not immediately attractive, but after aging, odors from a variety of flesh sources attracted crabs. Crabs fed actively upon the materials that attracted them. Feeding behavior was stimulated by components of fruit juice and fresh gastropod flesh juices of less than 10,000 daltons, honey, a 0.5 M sucrose solution, and a saturated solution of tyrosine. Twenty additional amino acid solutions tested at 0.1 M concentration were weak feeding stimulants at best. Chemical cues controlled feeding behavior, but not shell acquisition; *C. rugosis* were not differentially attracted to flesh odors or to living gastropods whose shells they occupied.

Key Words—Chemoreception, Crustacea, odors, *Coenobita rugosis.*

INTRODUCTION

With the exception of chemoreception studies using components of crude oil, kerosene, and drilling muds (reviews, Sutterlin, 1974; Atema, 1976; Epifanio, 1979; Pearson and Olla, 1979, 1980; Pearson et al., 1981) and ammonium (Carter and Steele, 1982; Zimmer-Faust et al., 1984a), there are few journal reports of responses of decapod crustaceans to volatile odors. In water, solubility rather than volatility is a major consideration (Atema, 1982). Separation

[1] Contribution No. 002. T.H. Curran Marine Field Station.

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of responses to volatile and nonvolatile odors is especially difficult with aquatic organisms because water carries nonvolatile water-soluble cues as well as volatile water-soluble cues. In addition, volatiles may be transported absorbed to particulates, in miceles and crystals (Epifanio, 1979). Thus, in water, volatile and nonvolatile cues can function in distance chemoreception, while in air, under most atmospheric conditions, only volatile molecules can function in distance chemoreception.

Feeding responses of aquatic organisms in general (Bardach, 1975; Atema, 1982) and in crustaceans specifically (Bardach, 1975; Ache et al., 1976; Ache, 1977, 1982; Fuzessery et al., 1978; Pearson et al., 1979; Robertson et al., 1980, 1981; Zimmer-Faust et al., 1984a,b; Carr and Derby, 1986; Derby and Atema, 1982a–c; Derby and Ache, 1984) have focused on crude aqueous extracts, polar, nonvolatile compounds (proteins, amino acids, nucleotides, sugars) or secondary plant compounds (Derby et al., 1984). That volatile components contribute to crustacean feeding responses has been demonstrated, but not fully assessed.

Because air is the transport medium, responses of terrestrial crustaceans to volatile components can be easily separated from responses to nonvolatile compounds in complex mixtures. We chose the land hermit crab, *Coenobita rugosis*, for field studies because morphological, behavioral and natural history information suggested land hermit crabs might detect volatile substances. Studies of *Coenobita* antennules (crustacean sensory structures) indicate that they have arthropod olfactory reception morphology (Ghiradella et al., 1968). Antennal flicking, a way of maximizing sampling of the environment (Schmitt and Ache, 1979) is an obvious feature of land hermit crab behavior. Finally, in the field we observed the omnivorous nature (e.g., Hazlett, 1981; Page and Willason, 1983) and apparent olfactory abilities of land hermit crabs. Hermit crabs arrive at and feed upon distinct food islands such as feces, carrion, and fruit within minutes of their deposition. Kurta (1982) investigated first arrivals and total number of *C. compressus* coming within 12.5 cm of 30- to 35-mm-diameter turtle eggs and did not demonstrate olfactory ability. However, Kurta pointed out that the high density of crabs in the study area could have resulted in lack of sensitivity of his test measures and that social facilitation (Wilson, 1975) of responses confounded the interpretation of data with respect to olfaction. We designed our tests to minimize confounding variables and maximize assay sensitivity with respect to Kurta's observations.

The relative ease of chemical separation of volatile from nonvolatile substances in air, coupled with the omnivorous habits of land hermit crabs should provide information applicable to studies of olfaction in Crustacea in general. Understanding the chemoreceptive abilities of land hermit crabs as well as the chemistry of the volatile attractant compounds should broaden our perspectives on the potential for experimentation with volatile water-soluble compounds in olfaction studies with other terrestrial, semiterrestrial, and aquatic crustaceans.

Additionally, we can make comparisons of the factors of biological importance for aquatic and terrestrial hermit crabs. For example, shells are of major importance to intertidal and subtidal populations of most hermit crabs studied (for review see Hazlett, 1981). Populations are limited by shell availability (Vance, 1972) and fitness of individuals is affected by shell fit (Bach et al., 1976). Aquatic hermit crabs have highly specialized behaviors associated with locating and procuring shells (McClean, 1974; Hazlett, 1981; Rittschof, 1980a; Wilber and Herrnkind, 1982, 1984; Gilchrest, 1982). Specific shell acquisition behaviors are mediated by nonvolatile molecules released from the flesh of dead gastropods (Rittschof, 1980a,b), and these cues are instrumental in the location of new shells by crabs (Wilber and Herrnkind, 1984). In field tests, aquatic hermit crabs do not respond to food (McClean, 1975; Rittschof, 1980a; Gilchrest, 1982), although they do in the laboratory (Hazlett, 1971a,b). Thus, shell acquisition behavior is not associated with feeding. In this regard, we asked the general question: How do land hermit crabs compare to local aquatic hermit crabs with respect to responses to odor cues from flesh food sources and flesh sources associated with the potential availability of gastropod shells?

METHODS AND MATERIALS

Study Area. The study area was a 15 × 3-m crescent-shaped stretch of sand at the base of a bluff approximately 0.8 km west of West Esterillos, Costa Rica. The area is the mouth of a creek during the rainy season, but at the time of the study, April 1985, the creek was dry. The sand was damp at the base of the bluff due to slight seepage. The damp ground was covered with driftwood and the fallen trunks of several large trees. Three mature almond trees bearing and dropping fruit shaded the area after 0900 hr. Dry beach sand stretched from the fallen trees toward the Pacific Ocean for approximately 4 m to the mean high water mark. Land hermit crabs, *Coenobita rugosis*, were in evidence at all times. They were especially abundant in the daytime in the damp sand feeding on almonds. In the daytime, however, about 70% of the crabs could be found in refuges under and within the rotting logs.

At night, the crabs left the shelter of the logs and ranged between the bluff and the surf. Experiments (described below) were conducted near (olfactory tests) or in areas (taste tests) heavily traveled by crabs at all times. Most studies were conducted on dry sand along an irregular line 30 cm to the Pacific Ocean side of the fallen logs.

Tests of Attraction by Volatile Compounds. In tests for attraction of crabs by volatile odors, behavioral assays used pitfalls to eliminate touch and vision as means of sensory input about test substances. Attractive volatile odors were defined operationally as those that evoked investigation by crabs. Two types of assays were conducted. Pitfalls in both assays were 115-ml polypropylene bot-

tles with their tops cut off. The pitfalls were buried flush with the sand in a single line at approximately 1.0-m intervals along the line of logs mentioned above. Fallen almonds were removed from the vicinity within 2 m of the pitfalls. Daytime assays were conducted for 40 min each, between 1200 and 1700 hr. We reduced the likelihood that random movements by the crabs would be mistaken for positive responses by: (1) placing pitfalls in areas near to, but not directly in regions of, high crab activity and (2) by choosing a target diameter for determining a positive response of 5 cm. Visual cues were eliminated by placing odor sources in pitfalls. Social facilitation (Wilson, 1975; Kurta, 1982) was eliminated by collecting responding individuals immediately. Test substances were placed in every other pitfall (4–6 of each type), and every crab that touched a pitfall was captured, recorded, and held until the experiment was completed. Each test was repeated at least twice and on different days. Nighttime assays were initiated at 2000 hr and terminated at 0400 hr the following morning. Flesh responses and responses to living gastropods were tested at night. The number of crabs in control and experimental pitfalls (those containing potential attractants) were counted.

Different endpoints for the assays were used because crabs did not fall into the traps in the daytime and the crabs were disturbed by lights at night. We assumed vision was not a factor at night because crabs fell into traps. In both types of assays, the null hypothesis that there was an equal response to control and experimental pitfalls was tested with the G statistic with one degree of freedom (Sokal and Rohlf, 1981). Because of the relatively large number of tests performed, the null hypothesis was rejected only with probabilities of $P < 0.01$. Because of the large number of crabs in the area and the impossibility of individual marking, only within-test comparisons were made.

Test Substances—Volatile Odors. The origins of all test subtances containing volatile odors were complex mixtures of volatile and nonvolatile compounds. Separation of volatile from nonvolatile compounds was by the pitfalls described above. In all tests, winds were light, approximately 5.0 km/hr or less. Excreta, fermentation products, fruit, honey, living gastropods, and dead flesh odors were tested for attractiveness to crabs. Excreta included fresh horse feces (about 10 g/pitfall) and human urine (20 ml/pitfall). Fermentation products were a solution containing actively fermenting baker's yeast growing for 3 hr in a 0.25 M sucrose solution and a 5% acetic acid solution (20 ml/pitfall). Fruits included 5- to 10-g samples of banana, coconut, mango, and cashew fruit. Honey was diluted 1 : 5 with boiled sea water and tested at 20 ml/pitfall.

Living flesh included 0.1–0.3 g (wet tissue weight) of the coiled gastropods *Nerita scabricosta*, *Nerita funiculata*, and *Planaxis planicostata*. Living snails were kept from escaping and were protected from hermit crabs by small bags made of plastic screening. Dead flesh included 0.3- to 3.0-g samples of the coiled gastropods *Nerita scabricosta* and *Acanthina brevidentata*, the limpets *Siphonaria gigas* and *Fissurella virescens*, 0.1–0.3 g of the flesh of an

unknown aquatic hermit crab, and 4.0–5.0 g of an unknown holothurian. The coiled gastropods are utilized for protection by *Coenobita*, while the limpets are unsuitable for this purpose. Night observations at low tide revealed *C. rugosis* frequented the habitat of the living gastropods. All animals were killed by freezing for 4–8 hr prior to use. After thawing, the flesh was allowed to stand for 1 hr to "age" before using it in an experiment (see Rittschof, 1980b).

Tests of Feeding Stimulation. Tests of feeding stimulation used feeding movements (placement of chaelae to the mouthparts) as an endpoint. Test solutions including a boiled seawater control were presented to the crabs either soaked into paper towels (paper towel assay) or loaded into segments of dialysis tubing (10,000-dalton cutoff) which were placed along paths heavily traveled by crabs. In order to control for differences in crab distribution, three types of data were collected: (1) the number of crabs walking within 2.5 cm of the test solution but not contacting it, (2) the number of crabs walking within 1.5 cm of this solution and contacting it, and (3) whether or not a crab fed after contacting the test solution. The former data allow consideration of any volatile attractants in the test solution. The latter measures whether the test solution actually stimulated feeding. Experiments with honey, melon juice, and seawater were conducted for 40 min. All other tests were conducted until 15 crabs had contacted a particular test solution. In the latter, 20–30 min were required for a total of 15 crabs to contact a test solution.

Test Substances—Nonvolatile Odors. Sources of nonvolatile compounds were complex mixtures and seawater solutions of nonvolatile compounds. Mixtures included melon juice, honey, and homogenates of limpet flesh. Nonvolatile solutions were cane sugar and 21 L-amino acids (Sigma Chemical Corporation, St. Louis, Missouri, kit No. LAA-21). These were tested for induction of crab feeding responses. Melon (honeydew) juice was from the rind of a locally grown melon. Honey was local honey diluted 1 : 5 with boiled seawater. Sugar solutions were from locally processed cane sugar and were made up in boiled seawater at 0.1 and 0.5 M concentrations. Limpet homogenates were 3 g fresh limpet flesh (*Siphonaria gigas*) macerated in 18 ml of boiled seawater with stainless-steel dissecting scissors. The limpet macerate was mixed several times and allowed to stand for 5 min before the light brown solution was decanted and used in experiments. No more than 30 min elapsed between the killing of a limpet and the completion of testing of its juices. The 21 L-amino acids were dissolved in boiled seawater to a final concentration of 0.10 M.

Tests with Local Hermit Crabs. Tests of responses of *Clibinarius* spp. and tidal pool *Calcinius* spp. were performed to determine their responsiveness to flesh from coiled and uncoiled gastropods. Tests were patterned after Rittschof (1980b). Frozen and thawed gastropod flesh (5 g/container) were placed in perforated polypropylene bottles and the bottles with and without flesh placed at 1-ml intervals in tidal pools. Aquatic crabs touching the containers were removed and counted.

TABLE 1. RESPONSES OF *Coenobita rugosis* TO WASTE AND FERMENTATION ODORS

Odor source	Number responding		G	Significance
	Control	Treatment		
Horse feces	2	58	65.0	<.01
Human urine	7	15	2.9	NS
Yeast	8	17	3.2	NS
Acetic acid	4	0	—	NS

RESULTS

Responses to Volatile Odors. When tested with waste and fermentation odors, land hermit crabs responded significantly only to the odor of fresh horse feces (Table 1). Although fermenting yeast, human urine, and 5% acetic acid all have distinct odors to humans, crabs were not significantly attracted to these odors in the assay interval. In contrast, *C. rugosis* responded significantly to the odor of honey and several kinds of fruits (Table 2). *C. rugosis* did not respond to freshly killed *Siphonaria gigas* (Table 2).

In overnight assays, odors from the flesh of animals were highly attractive to hermit crabs (Table 3). This was true even when the flesh was of limpets and holothurians, organisms unsuitable as shell sources. In contrast, tests of two genera of intertidal hermit crabs showed these crabs responded specifically to odors of dead gastropods with shells of a type that could be occupied and not to odors from limpet flesh (Table 4). There was no evidence that *C. rugosis* was attracted to living snails (Table 5).

Tests of Feeding Responses. The distinction between attraction by volatile compounds and stimulation of feeding responses is illustrated by comparison

TABLE 2. RESPONSES OF *Coenobita rugosis* TO ODORS OF FRUITS, HONEY, AND FRESHLY KILLED SNAILS

Odor source	Number responding		G	Significance
	Control	Treatment		
Cashew	8	61	45.8	<0.01
Mango	3	107	124.3	<0.01
Banana	5	106	112.6	<0.01
Coconut	6	162	180.5	<0.01
Honey	9	26	8.6	<0.01
Siphonaria gigas	0	5	—	NS

TABLE 3. RESPONSES OF *Coenobita rugosis* TO ODORS OF FLESH FROM ANIMALS DEAD FOR OVER AN HOUR[a]

Odor source	Number responding		G	Significance
	Control	Treatment		
Shell sources				
Nertia scabricosta	12	267	287.2	<0.01
Acanthina brevidentata	34	112	46.4	<0.01
Nonshell sources				
Siphonaria gigas	34	104	37.0	<0.01
Fissurella viriscens	34	138	47.4	−0.01
Unknown hermit crab	48	229	128.3	<0.01
Unknown holothurian	48	137	44.5	<0.01

[a] G statistics test H_0 1 : 1 ratio between control and experimental.

TABLE 4. RESPONSES OF INTERTIDAL HERMIT CRABS TO ODORS OF FLESH FROM ANIMALS DEAD FOR OVER AN HOUR[a]

Odor source	Number responding		G	Significance
	Control	Treatment		
Shell sources				
Nerita scabricosta	0	78	107.4	<0.01
Acanthina brevidentata	0	76	104.6	<0.01
Purpura pansa	0	46	63.8	<0.01
Nonshell sources				
Siphonaria gigas	0	1	—	NS
Fissurella virescens	0	4	—	NS

[a] See Table 3 above for explanation of G statistic.

TABLE 5. RESPONSES OF *Coenobita rugosis* TO ODORS OF LIVING SNAILS

Odor source	Number responding		G	Significance
	Control	Treatment		
Nerita scabricosta	71	75	0.1	NS
Nerita funiculata	21	18	0.2	NS
Planaxis planicostata	32	30	0.1	NS

TABLE 6. RESPONSES OF CRABS IN PAPER TOWEL ASSAY

Solution	Touching (*N*)	Feeding (*N*)	Passing w/o touch (*N*)
Boiled seawater	15	0	13
Melon juice	28	28	0
Honey	17	17	0
Sucrose			
0.5 M	15	15	>15
0.1 M	15	1	>15
S. gigas juice	15	15	>15
Tyrosine	15	5	>15
Cisteine	15	1	>15
Glutamic acid	15	1	>15
Hydroxyproline	15	1	>15
Methionine	15	1	>15
Proline	14	1	>15
Threonine	15	1	>15
Valine	15	1	>15
Histidine	15	Repulsion[b]	>15
13 other amino acids (each)[a]	15	0	>15

[a] Alanine, arninine, asparanine, aspartic acid, cysteine (free base) glutamine, glycine, isoleucine, leucine, lysine, phenylalanine, serine, tryptophan.
[b] Crabs contacting backed up immediately.

of the response of crabs to towels soaked in melon juice (a mixture of both types of stimulant) and to 0.5 M sucrose (Table 6). Every crab passing within 2.5 cm of the melon juice-soaked towels was attracted to the towel and stimulated to feed. More crabs actually passed within 2.5 cm of the towel soaked in the 0.5 M sucrose without touching it, than touched it. However, every crab that touched the more concentrated sugar solution showed feeding behavior. In total, 28 different solutions were tested for stimulation of land hermit crab feeding activity with the paper towel assay (Table 6). Two of the complex mixtures tested, melon juices and honey, contained both attractant and feeding-stimulant activity. Two other solutions, 0.5 M sucrose and complex mixture of fresh meat juices from the limpet, *Siphonaria gigas*, elicited feeding activity but did not attract crabs. Dilution of sucrose to 0.1 M resulted in a decrease in feeding stimulation from 100% in the case of 0.5 M to 6%, a level of feeding stimulation observed with several amino acid solutions. Of the 21 amino acid solutions, each tested at 0.1 M concentration, none gave evidence of attractant activity and only eight stimulated at least one crab to feed (Table 6). Tyrosine, not completely soluble in seawater at 0.1 M concentration, was the amino acid most notable as a feeding stimulant, evoking feeding in 5 of 15 cases. Histidine solutions appeared to elicit contact-dependent repellent activity. Every crab

TABLE 7. RESPONSE OF LAND HERMIT CRABS TO SEAWATER AND FLESH JUICES CONTAINED IN DIALYSIS TUBING

Solution	Touching (*N*)	Feeding (*N*)	Passing w/o touch (*N*)
Boiled sea water	15	0	>15
S. gigas juice	15	15	>15

contacting a towel soaked in histidine backed up immediately and did not touch the towel a second time.

The paper towel assay showed that juice from the flesh of *Siphonaria gigas* stimulated feeding responses in land hermit crabs when the crabs encountered the solution. Flesh juice prepared from freshly killed *S. gigas*, encased in 10,000-dalton cutoff dialysis tubing also elicited feeding responses from every crab touching the tubing (Table 7). There was no evidence of volatile attractants as crabs no contacting the tubing passed by it. As was the case with the paper towel assay, crabs were not attracted to the control tubing containing boiled seawater and did not show feeding responses when they contacted it (Table 7).

DISCUSSION

Coenobita rugosis is an omnivorous crab that detects volatile odors and nonvolatile odors of both plant and animal origins. It can use olfaction to locate fruits, honey, feces, and the flesh of dead animals from a distance. When crabs contact the materials emitting the odors (with either their antennules or dactyls), feeding is stimulated. Solutions of sucrose, fresh flesh juices, and saturated solutions of tyrosine were not attractive from a distance, but were feeding stimulants when contacted by crabs. This suggests nonvolatile components are sufficient to cue feeding responses. Unlike its tidepool counterparts that are attracted only to flesh of specific species of gastropods whose shells they occupy, *C. rugosis* are attracted to food and do not exhibit odor-oriented shell acquisition behavior (McClean, 1974). This observation is supported by the fact that *C. rugosis* are not differentially attracted to the flesh of gastropods which are potential shell sources. There is no evidence that *C. rugosis* are attracted to living gastropods.

The possibility that *C. rugosis* can detect conspecifics was not addressed directly in this study. The possibility has been suggested that numbers of crabs captured in nighttime pitfall assays are due to a combination of attraction to odor of test substance and to conspecifics. Examination of the total data set with respect to all control animals and to odor sources found not to be attractive suggests that this is not true (see Tables 3 and 5). Although it is obvious that

crab activity, as measured in control pitfalls, varies markedly from night to night (12–75 animals captured), it is also obvious that responses to empty pitfalls and to substances deemed unattractive (living snails) were similar and did not overlap with frequency of response to what we have concluded are attractive odors. This suggests that there is not a general olfactory attraction to conspecifics.

The terrestrial existence of *C. rugosis* dictates the types of substances that can transmit information over a distance. This is most dramatic in the case of nonvolatile compounds. Where polar, nonvolatile chemicals can transmit specific information over tens of meters to hermit crabs in the water (Rittschof, 1980a,b), these same compounds are only detected upon contact by *Coenobita rugosis*. A biological consequence is the elimination of distance detection of coiled gastropod shells using peptides from snail flesh (Rittschof, 1980b), a major way that aquatic hermit crabs normally acquire shells (Wilber and Herrnkind, 1984). Although it is thought that land hermit crabs obtain access to shells mainly as a result of storm action (Hazlett, personal communication), *C. rugosis* were observed at low tide at night walking in the immediate vicinity of the various species of gastropods used in this study. As shelter of some form is essential for hermit crabs (for review see Hazlett, 1981), the questions of how land hermit crabs find shells and if they use volatile cues in the process are salient ones. If aquatic hermit crabs are starved, it is difficult to separate their shell acquisition responses from feeding responses in the laboratory (Rittschof, unpublished data). Thus, one might expect that well-fed land hermit crabs might exhibit behaviors not observed in this study. In circumstances in which food is in abundance, *C. rugosis* may display chemoreception-dependent shell acquisition behavior as do many aquatic hermit crabs (McClean, 1975; Rittschof, 1980b; Gilchrest, 1982).

In contrast to nonvolatile compounds, volatile compounds can function similarly as distance cues in either aquatic or terrestrial environments. The effectiveness of a volatile molecule in water will be dependent upon the solubility of the compound in water and the sensitivity of the olfactory receptor (Atema, 1982). Given identical receptor sensitivity and because of scale considerations, transport by advection, the effective transmission distance (the distance from the source that the stimulus still evokes a response) of a water-soluble volatile molecule in air and in water would be dependent upon the specific molecule. Because of the greater than 1000-fold slower rate of diffusion in water, depending upon turbulence, the effective transmission distance in water can be greater in water than in air if solubility is high.

Because their feeding responses include frugivory, detritiverory, and carnivory, *C. rugosis* provide a model system for examining and dissecting the relationship between volatile and nonvolatile compounds in crustacean feeding responses common to aquatic crustaceans. In future investigations we will test the hypothesis that soluble volatile compounds that cue feeding responses in

land hermit crabs also function to cue feeding responses in semiterrestrial and aquatic crustaceans.

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PROPAGANDA SUBSTANCES IN THE CUCKOO ANT *Leptothorax kutteri* AND THE SLAVE-MAKER *Harpagoxenus sublaevis*

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Abstract—This paper reports the first discovery of "propaganda substances" in a workerless inquiline ant, the European myrmicine *Leptothorax kutteri* Buschinger. These substances are used by the parasite queen as a chemical weapon for defense against hostile workers of the host species *L. acervorum.* The substances also have an unusual behavioral effect: they cause host workers to attack each other, and they therefore appear to override nestmate recognition in host colonies. Laboratory experiments show that the source of these substances is the Dufour's gland of the *L. kutteri* queen. Our experiments also confirm the hypothesis that the closely related slave-making ant *Harpagoxenus sublaevis* uses its Dufour's gland secretions as a chemical weapon during slave raids and colony foundation. The behavioral effect of these slave-maker secretions is identical to that of *L. kutteri* queens.

Key Words—Dufour's gland, Formicidae, Hymenoptera, inquiline ant, slave-making ant, *Harpagoxenus sublaevis*, *Leptothorax kutteri*, *Leptothorax acervorum*, pheromone, nestmate recognition.

INTRODUCTION

Inquiline ants have the unusual ability to infiltrate colonies of ants of other species and to become accepted by their members. However, compared to our knowledge of the colony infiltration methods of myrmecophiles (e.g., see Hölldobler 1970, 1971; and review of Kistner, 1979), there is relatively little known about how inquiline queens accomplish these feats (see reviews of Wilson, 1971; Dumpert, 1981). For instance, it is not known how the inquiline ant *Leptothorax kutteri* (Buschinger, 1965) manages to infiltrate colonies of its host *Lep-*

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tothorax acervorum. In laboratory introductions the parasite queens are usually attacked by *L. acervorum* workers from the target colony (Buschinger, 1965, and personal communication; Franks, unpublished observations). In such introduction experiments, *L. kutteri* queens display none of the behaviors previously recorded in inquiline foundresses (see Discussion). Instead, this paper demonstrates that they deploy a "propaganda substance" as a defense against the attacks of the target colony workers. *L. kutteri* is the first inquiline in which such substances have been found. Nevertheless, the manner in which the parasite queens follow up this strategem and come to be tolerated by their hosts remains an unsolved problem.

Propaganda substances were first described in slave-making *Formica* from North America (Regnier and Wilson, 1971). On slave raids these ants broadcast mimics of alarm pheromones among resisting host workers, causing them to panic and flee. The substances used by *L. kutteri* have a different behavioral effect. They cause host workers to attack each other, and they therefore appear to override nestmate recognition in host colonies.

In addition, this paper shows that the slave-making ant *Harpagoxenus sublaevis*, another parasite of *L. acervorum* and also a close relative of *L. kutteri*, possesses substances which have a similar effect on *L. acervorum* workers during conflicts between the slave-makers and host colonies, that is during slave raids by *H. sublaevis* workers and in colony foundation by the queens. The most obvious adaptation of *H. sublaevis* females for fighting is their possession of large and secateur-like mandibles, with which they dismember their opponents. The existence of chemical weapons in this species was first suggested by Buschinger (1974a) when he recorded that *L. acervorum* workers may attack each other following contact with slave-raiding or colony-founding *H. sublaevis* females. Similar phenomena have been observed by Alloway (1979) during slave raids by *H. americanus* on its North American *Leptothorax* hosts. We also observed an effect of this type in laboratory introductions of *L. kutteri* queens to *L. acervorum* colonies. In *H. sublaevis* it was suggested (Buschinger, 1974a) that the effect is due to a pheromone from the Dufour's gland which the slave-maker wipes onto its opponent with its sting. The Dufour's gland secretion of *H. sublaevis* has also been proposed to act as a repellent which the slave-makers smear over captured brood in order to deter would-be *L. acervorum* rescuers (Winter, 1979; Buschinger et al., 1980).

Remarkably, in both *H. sublaevis* and *L. kutteri*, the Dufour's gland is hypertrophied (Buschinger, 1974b). Therefore our first experiment was to test under standardized conditions whether or not contact with *L. kutteri* caused *L. acervorum* workers to be attacked by their nestmates. Following a positive result, our second experiment tested the hypothesis that it is the Dufour's gland secretions of *L. kutteri* and *H. sublaevis* that contain the parasites' propaganda substances.

METHODS AND MATERIALS

All the ants used in the experiments came from colonies collected in sourthern Sweden in July 1983. Both *L. acervorum* and *H. sublaevis* are widespread in central and northern Europe, but *L. kutteri* is known only from sites in West Germany, the Alpine region, and Sweden (Buschinger, 1965; Douwes and Buschinger, 1983). The colonies were found in pine forests in hollow dead twigs on the ground. They were maintained in the laboratory, and the experiments were carried out between October 1983 and June 1984.

Experiment 1. Single *L. acervorum* workers were removed from unparasitized colonies and confined in arenas (100 cm^2) with either (1) single newly inseminated *L. kutteri* queens, (2) single *L. acervorum* queens from another nest, or (3) single *L. acervorum* workers from another nest. The behavior of the pairs of ants was then observed. After 60 min the workers were returned to their own nests, and the behavior of their nestmates towards them was recorded.

Experiment 2. We tested the response of *L. acervorum* workers to nestmates treated with extracts of the Dufour's gland of *L. kutteri* queens, *H. sublaevis* females, and, as controls, queens of *L. acervorum*. The *H. sublaevis* females were not classified as workers or queens because in this species most queens are identical in external morphology to workers. The colonies tested in the trials were unparasitized colonies of *L. acervorum*. For every trial, a set of four replicate portions was made from each colony by selecting at random four groups of three workers and housing each group in its own small arena (4 cm^2). We then added to three of these replicate portions a nestmate treated with either (1) an extract of Dufour's gland contents, (2) an extract of poison vesicles, or (3) solvent alone.

The glandular extracts were prepared by first dissecting ants in distilled water. The glands were then removed and crushed whole in liquid paraffin (mineral oil). This solvent was used because, unlike hexane, for example, it was not toxic to the ants. Since the Dufour's gland volumes of *L. kutteri* and *H. sublaevis* are, respectively, about 40 and 60 times that of the *L. acervorum* queen, the *L. kutteri* Dufour's gland extracts contained 6 glands/10 μl of solvent (giving a concentration of 1.5×10^{-2} μl gland content/μl of solvent), and the *H. sublaevis* Dufour's gland extracts contained 4 glands/10 μl of solvent (concentration 1.6×10^{-2} μl gland contents/μl solvent). In the *L. acervorum* queen trials (since here the Dufour's gland is very small), we used single glands crushed in single paraffin droplets (concentration 1.3×10^{-3} μl gland contents/μl solvent). Poison extracts contained the same number of glands as corresponding Dufour's gland extracts. Poison extract was used in these trials as an additional control, to test whether other endogenous substances, in general, could induce hostility.

All the ants which were introduced to the groups of workers were first

marked with paint dots on their heads. The glandular extracts (or liquid paraffin alone) were then applied to the thorax with a pinhead. The average volume applied was 0.50 ± 0.15 μl (determined by weight). Therefore, in the case of the ants treated with Dufour's gland extract, each ant received the equivalent of either 0.29 of an average *L. kutteri* gland, or 0.21 of a *H. sublaevis* gland, or a whole queen *L. acervorum* gland (since here the whole paraffin droplet in which the gland had been crushed was applied). Sixty minutes after the addition of each treated ant to the arena of nestmates, we recorded whether or not it was being bitten. This we defined as an attack. The time interval guaranteed that any attack was not a temporary response to a new object in the arena. Finally, a worker from a different unparasitized *L. acervorum* colony was added to the fourth replicate portion of the trial colony. This worker was also marked with a paint dot, and its reception in the arena was recorded in the same way as that of the treated ants. Only the results from trials in which this alien was attacked were considered valid. This ruled out trials in which the unfamiliar surroundings of the test arena might have lead to a lack of hostility to any introduced worker.

RESULTS

Experiment 1. As regards response of workers to nestmates confined with *L. kutteri* queens or alien conspecifics: the *L. acervorum* workers always fought with the ant with which they were confined, whether it was a queen *L. kutteri*, an alien *L. acervorum* queen, or an alien *L. acervorum* worker. In these fights the *L. kutteri* queens were seen to daub the *L. acervorum* workers with a clear viscous fluid which they released from the tip of the abdomen. This behavior was not observed in *L. acervorum* workers or queens. When the *L. acervorum* workers which had been with the *L. kutteri* queens were returned to their home colonies, all of them were attacked by their nestmates (Table 1). Workers which had been with either worker or queen alien conspecifics were attacked only very rarely when returned to their own nests (Table 1). This suggests that a *L. kutteri* queen can contaminate a hostile host worker with a secretion which causes the worker to be attacked by its nestmates.

Experiment 2. As regards response of workers to nestmates treated with gland extracts: *L. acervorum* workers treated with extracts of the Dufour's gland contents of *L. kutteri* and *H. sublaevis* were significantly more likely to be attacked by their nestmates than were workers treated with either extracts of the contents of the poison vesicles or with liquid paraffin alone (Table 2, columns A and B). Furthermore, extracts of the Dufour's gland substances of *L. acervorum* queens did not induce attack by nestmates at a significantly greater frequency than did the control solvent (Table 2, column C). These results suggest that the hostility-inducing substances of *L. kutteri* and *H. sublaevis* originate in

TABLE 1. RESPONSE OF WORKERS TO NESTMATE CONFINED WITH *L. kutteri* QUEEN OR ALIEN CONSPECIFIC

	Leptothorax acervorum workers[a]	
	Attacked	Not attacked
Exposed to:		
L. kutteri Queen	9	0
L. acervorum Queen	1	8
L. acervorum Worker	0	9

[a] $\chi^2 = 23.2$ $P < 0.001$.

the Dufour's gland and that the active agents in these secretions are either absent or present in only very small amounts in the Dufour's glands of *L. acervorum* queens.

DISCUSSION

The substances used by *L. kutteri* queens when they encounter foreign host colonies and their behavioral effects are, as far as we know, unique among inquilines. Other species of inquiline, in which single queens enter large, mature host colonies, use a variety of techniques, but none of them involve substances which cause host workers to fight one another. For example, the inquiline queen *Anergates atratulus* appears to play dead when met by a worker of the host species *Tetramorium caespitum*. She then clings with her jaws onto the worker's antenna and is dragged into the nest (Wheeler, 1910; Gösswald, 1954). *Epimyrma stumperi* either plays dead in a similar manner, or captures a *Leptothorax tuberum* host worker, rubs it with her forelegs, and grooms herself, apparently acquiring the host's colony odor in this way (Kutter, 1969). Another parasite of *Tetramorium caespitum*, *Teleutomyrmex schneideri*, employs a third type of entry method. She seems to appease the host workers with surface glandular secretions (Stumper, 1950; Gösswald, 1953).

How *L. kutteri* queens penetrate host colonies in the field has never been observed. The possibility cannot be excluded that the parasite queen enters colonies at the founding stage, as suggested in the inquilines *Strumigenys xenos* (Brown, 1955) and *Pogonomyrmex colei* (Rissing, 1983). It is also conceivable that *L. kutteri* queens transfer to daughter colonies during budding of the host colony, as may occur in *Plagiolepis xene* (Passera, 1964). However, the occurrence of single *L. kutteri* queens in isolated *L. acervorum* colonies (Buschinger, personal communication) suggests that at least some parasite colonies are founded by lone *L. kutteri* queens entering mature host colonies. From our

TABLE 2. RESPONSE OF WORKERS TO NESTMATES TREATED WITH VARIOUS EXTRACTS

	(A) *L. kutteri* gland extracts			(B) *H. sublaevis* gland extracts			(C) *L. acervorum* gland extracts		
	Attacked	Not attacked	Total trials	Attacked	Not attacked	Total trials	Attacked	Not attacked	Total trials
Dufour's extract	19	11	30	15	15	30	4	11	15
Poison extract	3	27	30	6	24	30	2	13	15
Liquid paraffin control	4	26	30	4	26	30	3	12	15
	$\chi^2 = 26.2, P < 0.001$			$\chi^2 = 11.4, P < 0.01$			$\chi^2 = 0.8, P > 0.05$		

results, we would expect these invading queens always to be attacked by the target colony workers. In all of the 15 laboratory introductions of single *L. kutteri* queens to *L. acervorum* colonies we have performed to date, such attacks have proved fatal (Franks, unpublished observations). However, it is likely that the probability of successful colony foundation in nature is very low. When attacked by the target colony workers, the parasite could use its propaganda substances to confuse its aggressors, allowing it to escape immediate danger and to proceed to gain the acceptance of its hosts by means which are at present unknown. It could seek to enter a cavity in the host nest where it could remain undetected, in order to pick up the colony odor from the nest material. Or it may deliberately court attack in order to acquire the colony odor by cuticular contact, as does the myrmecophilous beetle *Myrmecaphodius excavaticollis* (Vander Meer and Wojcik, 1982), deploying the propaganda substance only if the attack becomes too severe. There is evidence that *L. acervorum* workers who have not met the parasite can pick up the propaganda substance from nestmates which have. This would speed up the spread of the substances through the defending force, potentially vital for a single parasite pitting itself against an entire colony.

The use of Dufour's gland secretions as chemical weapons in the penetration of host colonies may also occur in the inquiline *Doronomyrmex pacis*. Buschinger (1974b) discovered the Dufour's gland to be hypertrophied in this species as well. Intriguingly, it is also a close relative of *L. kutteri*, and parasitizes the same host species, *L. acervorum*. However, another related parasite of *L. acervorum*, *L. goesswaldi*, appears to have a Dufour's gland only slightly larger relative to its body than that of *L. acervorum* (Buschinger, 1974b).

In the slave-maker *H. sublaevis*, our results confirm the hypothesis (Buschinger, 1974a) that this ant possesses a chemical weapon produced in quantity by the Dufour's gland. In this species there is no difficulty in seeing how substances which provoke internecine battles in host workers serve the needs of the parasite, since both colony-founding *H. sublaevis* queens and workers on slave raids aim to drive all adult host ants from the brood of their target colonies. However, an explanation remains to be found for the strong similarity both in the behavioral effects of the propaganda substances of *L. kutteri* and *H. sublaevis* and also in their glandular origin. This similarity may be due to a convergent evolution, especially since the parasites share the same host. Alternatively, the close phylogenetic relatedness of these two parasites, and of both to their host (Buschinger, 1981), argues for common ancestry as the explanation. Chemical analysis of Dufour's gland secretions could be used to discriminate between these alternatives. Since these secretions appear to override nestmate recognition, the analysis of these substances may also provide special insights into the chemical basis of colony-specific odor.

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PREDATOR DETERRENCE BY MANDIBULAR GLAND SECRETIONS OF BEES (HYMENOPTERA: APOIDEA)

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Abstract—Volatile lipids from the mandibular gland secretions of bees (Hymenoptera: Apoidea) are potent olfactory repellents of foraging ants (*Formica, Crematogaster*) in biologically relevant contexts and quantities. In contrast, differential success in capture of bee and fly prey by predatory asilid flies (*Efferia*), reduviid bugs (*Apiomerus*), and arachnids (*Agelenopsis, Argiope*) is better explained by prey size than by chemical repellence, aposematism, or possession of a sting. Supernormal doses of some allomones, applied to worker honeybees (*Apis mellifera*) that were fed to *Argiope aurantia* spiders, elicted more frequent preenvenomation pauses following ensnarement but did not significantly increase other prey-handling times. These pauses merely delayed the bee's demise. Mandibular gland secretions of solitary bees augment their other secondary defenses in at least two contexts: (1) during intranest encounters when repelling intruding ants, and (2) retaliation delivered to their arthropodan predators which, if the bee is nearly too large for the predator to handle, may allow the bee to escape.

Key Words—Defense, allomone, bees, Hymenoptera, Apoidea, repellent, *Argiope, Apiomerus*, Asilidae, Diptera Formicidae.

INTRODUCTION

The 15,000+ species of nonparasitic solitary bees (Apoidea, excluding eusocial Apidae) are relatively k-selected "prudent" species sensu Hutchinson (1978) in that females generally produce fewer than 30 eggs during their 3- to 6-week adult life-spans (Michener, 1974). Concomitantly, their protective adaptations effectively lessen reproductive losses. Mass provisions of pollen and nectar/oil are both isolated from the elements and visually concealed from predators (anachoresis sensu Edmunds, 1974) within subterranean, pebble, resin, wood, or stem nests. Earthen nest cells are typically varnished with hydrophobic mem-

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branes (Cane, 1981) that protect moisture-sensitive larvae and provisions; females may also fumigate these cells using mandibular gland volatiles prior to provisioning (Cane et al., 1983). Among the adult females' protective adaptations are two secondary defenses which may enhance their chances to repel or escape their arthropodan predators. These defenses are the sting and the mandibular gland secretion.

The mandibular glands, apparently possessed by both sexes of all Apoidea (Nedel, 1960), are the sole known sources of volatile repellents from solitary bees. This multifunctional secretion may also serve as a premating pheromone (Tengö, 1979; Cane and Tengö, 1981; Hefetz, 1983; Vinson et al., 1982) and nest cell disinfectant (Cane et al., 1983). To our noses, the bouquet of a bee's mandibular gland can resemble essences of citrus, garlic, banana, butter, melting styrene, etc. Both sexes release these volatiles during handling (e.g., Linsley and Zavortink, 1977). Their known molecular structures include various low-weight (<300 amu) aliphatic, isoprenoid, and occasionally aromatic hydrocarbons, alcohols, aldehydes, ketones, ketals, and acids. Quantification of a bee's potential glandular volume is available for *Colletes cunicularius* (10 μg linalool/bee) (Bergström and Tengö, 1978) and three species of *Exoneura* (40–80 μg total lipids/bee, 0.1–3.0 μg salicylaldehyde) (Cane and Michener, 1984).

Solitary bees of all life stages succumb to diverse predators and parasites. A few are as follows: kleptoparasitic "cuckoo" bee larvae (e.g., *Sphecodes*, Nomadinae, Melectini) consume the host bee's eggs, larvae, and provisions (Bohart, 1970). Some meloid beetles are parasites of larval bees (Erickson et al., 1976). Detrivorous or carnivorous ants may raid solitary bee nests (Schultz, 1977). The *Nomada* kleptoparasites rely upon kairomones for host nest assessment and selection (Cane, 1983). Foraging ants, of course, also respond to semiochemicals.

Adult solitary bees can be victims of arthropodan predators and parasitoids during mating, foraging, and nesting activities. Predatory arthropods include spiders (especially Araneidae and Thomisidae) (Bilsing, 1920; Bromley, 1948), robber flies (Asilidae) (Linsley, 1960; Dennis and Lavigne, 1975), assassin bugs (Reduviidae) (Usinger, 1958; Stephen et al., 1969; Weaver et al., 1975), ambush bugs (Phymatidae) (Caron, 1978), preying mantids (Mantidae) (personal observation), sphecoid wasps (especially Philanthini) (Evans and Lin, 1959), and occasional ants (Formicidae) (Schultz, 1977; Cane, personal observation). The kleptoparasites and ants contrast with the robber flies, spiders, and assassin bugs in that the latter groups are typically comprised of long-legged, swift-moving, sit-and-wait predators which seem to rely more upon visual or tactile rather than chemical information, as in the former group, for prey localization and recognition. The three latter taxa inject fast-acting toxins and/or digestive enzymes into their prey, thereby achieving rapid prey immobilization. Some of these arthropodan predators may be capable of associative learning for prey defenses and recognition (Gelperin, 1968).

Faced with sundry mortality factors and limited reproduction potential, solitary bees must bear adaptations that largely prevent or circumvent fitness losses. Other than the sting and mandibles, the mandibular gland secretion could serve a secondary defensive function in startling or repelling a predator or parasite. Given diverse predators, parasites, and contexts of encounter, the secretion is unlikely to confer effective defense against all adversaries at all times. This study characterizes some of these behavioral and ecological contexts for allomonal defenses of solitary bees.

METHODS AND MATERIALS

Reduviidae. Assassin bugs, *Apiomerus flaviventris*, were taken during 1980 and 1984 from flowering *Baccharis* shrubs growing about water tanks in the San Simon Valley near Rodeo, New Mexico. These bugs regularly feed upon bee, wasp, or fly prey. The bugs were housed in glass terraria in the insectory at the Southwest Research Station of the American Museum of Natural History (S.W.R.S.) near Portal, Arizona. Cut vegetation provided perches. Daily, an active, freshly caught bee was offered to each of five or six *A. flaviventris*, and the ensuing predation attempts recorded. Inconclusive experiments with particular prey species were repeated. Tested bees are ranked by body size and noted for observable aposematic colorations, sex (and so sting), and noticeable (by my nose) mandibular gland odors.

Asilidae. Both sexes of the abundant robber fly, *Efferia rapax*, prey upon bees and flies of the desert scrub and canyon meadows of the San Simon Valley. In these settings, live bees tethered with a long hair to the tip of a 2-m long stick were flown above perched robber flies. Lack of response (N), approach/inspection (A), and actual striking (S) responses were recorded for each *E. rapax* individual, following the criteria of Dennis et al. (1975).

Formicidae. Ants were tested in both the laboratory and field. At a nesting aggregation of *Colletes thoracicus* in Ithaca, New York, foraging *Formica glacialis* were baited to 10-cm^2 filter papers smeared with honey. This ant species preys upon nest contents and adults of *Andrena alleghaniensis* (Cane, personal observation). Foragers were then briefly presented, at antennal length, with a micropipet bearing either (1) dried extract of one, two, or four mandibular gland equivalents of *C. thoracicus*, (2) dried hexane solvent, or (3) a small aqueous sample of the bee's provisions. Hexane effectively extracts lipids like linalool from *Colletes* mandibular glands (Bergström and Tengö, 1978). Each ant's first response was classified as either feed (F), no response (N), jump back (J), retreat (R), or bite (B). The 440 responses of 87 foragers were compared using a G statistic for a 5×5 test of independence, followed by an a posteriori test by STP for homogeneity (Sokal and Rohlf, 1969).

In the laboratory, queenless colonies of *Formica subsericea* and *Crema-*

togaster cerasi were housed in 30 × 40 × 10-cm Plexiglas nesting boxes that connected, via short plastic tubes, with 50 × 75 × 10-cm Plexiglas foraging arenas. Although *Formica* will raid bee nests, *C. cerasi* is a small woodland species that likely tends aphids and coccids (M. DuBois, personal communication).

Colonies were fed for four consecutive days/week from 0900–1000 hr following 1 hr of illumination. Foragers were offered a microscope slide at the arena's center bearing 50-μl milk and honey droplets. As the first foragers returned to the nest, this bait was replaced by four cleaned slides (in two rows) placed equidistantly at the arena's end opposite the nest. Each slide bore a 4-cm^2 filter paper square saturated with two drops of milk and one of honey. Centered on each filter paper was a 1-cm^2 black velvet square, impregnated with 5 μl of test chemical, or hexane as a control. Thus, foragers were simultaneously presented both with the "allomone" (without physical contact) and attractive food, as might occur if confronted by a female bee while raiding her nest.

A weekly test consisted of three volatiles selected from the following: butyric acid, citral, citronellol, farnesol, geraniol, linalool, 2-octanone, salicylaldehyde, or skatole. Solids were dissolved in a minimum of hexane. Treatments were selected randomly each week, and distributed in a 4 × 4 Latin-square design, with days and feeding station positions randomized. These treatments represent a chemical diversity of known mandibular gland secretions of bees (see Discussion). This design controls for day and position effects, time of day, and order of experimentation. On the three nonexperimental days of the week, ants received water and dead cockroaches ad libitum in their nest boxes.

During the 1-hr experiments, ants at the feeding stations were counted every 3 min, resulting in 20 cumulative censuses. Actual totals of foraging individual ants were impossible without marking. Square-root transformations of these frequency data yielded normal distributions by rankits analysis. A 4 × 4 Latin-square ANOVA was applied to each week's tests for each ant species, using BMDP79 programs. Following a significant result, a posteriori Duncan's multiple-range tests were used to detect intraexperiment differences between the control and the three test lipids used during each week's trials.

Arachnida. Two species of spiders were tested—*Argiope aurantia* (Araneidae) and *Agelenopsis aperta* (Agelenidae). The latter were housed and fed similarily to the reduviids. Owing to their terrestrial habits, the funnelweb spiders (Agelenidae) are unlikely to encounter bees (Bilsing, 1920). In contrast, *A. aurantia* commonly ensnares flower-visiting insects such as bees (Bromley, 1948) in its orb webs. The diets of *Argiope* and *Araneus* (=*Epeira*) in Ohio typically consisted of 14–24% bees, particularly honeybees (Bilsing, 1920).

In 1982, three *A. aurantia* were individually housed in 1 × 1 × 0.1-m observation frames faced with removable glass and backed by window-screening to the outside. Freshly caught, live bees were tossed into the peripheral

halves of the spider's webs. The times until contact (A), duration of offensive silk wrapping (W), and time until feeding (F) were recorded, as was the anatomical point of envenomation (adopted from Robinson and Olazarri, 1971). Prey were offered sequentially until either a spider no longer responded or four prey had been consumed. Final offerings were dropped from analysis when the spider's refusal was due to satiation, which was tested by offering a muscid fly, being a known desirable prey item.

In 1984, six *A. aurantia* were individually caged outdoors in 30 × 30 × 60-cm screen enclosures. These were fed active *Apis mellifera* foragers which had been briefly (<5 min) chilled, perfumed with one synthetic mandibular gland lipid, and quickly (30–60 sec) revived in warm sunlight within their individual vials. Doses of 7–14 μg, 68–71 μg, 136–141 μg, or 860–1170 μg, diluted to 1 μl with hexane, were administered topically to the chilled bee's thorax. Control bees received either no chemical or merely 1 μl hexane. The treatments were butyric acid, citral, citronellol, 2-decanone, farnesol, geraniol, linalool, and salicylaldehyde. Butyric acid, 2-decanone, and linalool were only delivered at 7–14 μg owing to time constraints and interest in testing biologically relevant quantities (see Introduction). Each spider daily received one or two bees sequentially. Dosage, compound, and order of feeding were randomized across days and spiders. The six spiders were thus blocked as six replicates, each receiving each dose of each compound once. The spider's time until prey encounter following web contact, the duration of the spider's offensive silk wrap, and the duration if any of the spider's pause while astride the ensnared bee prior to envenomation were all recorded.

Statistical comparisons were necessarily nonparametric owing to excessive ties of 0- and 1-sec handling times that defied normalization. Runs tests (Sokal and Rohlf, 1969) were first performed for up or down trends for the two prey-handling times (median contact and wrap times) and the proportion of pausing behaviors for the experimental days ($N = 10$) to both assess the success of randomization and to check for either habituation or learning. Four Friedman tests (adjusted for ties) (Conover, 1980) were then applied, treating the six spiders as blocks and either the test volatiles or the dosages as treatments. Two tests compared median contact times, and two compared median wrap durations. Finally, a G statistic was applied to the frequencies of pausing to assess independence from the treatment dosage, followed by an STP a posteriori comparison of frequencies where justified (Sokal and Rohlf, 1969).

Standardization. Although the dosage of each compound applied to each bee prior to release was controlled, the initial quantity encountered by the spider upon contacting the captive bee is lessened during the bee's brief warming-up and flight into the web. The proportion of the original dosage remaining was assessed in the laboratory. Live, chilled bees were perfumed with 7, 68, 860, and 1720 μg of citral diluted to 2 μl with hexane. As in the field experiments, these bees were then warmed for 2 min and allowed to fly encaged for 1 min.

Each live bee was then captured and plunged into methylene chloride solvent containing an internal hydrocarbon standard. A dead bee with a matching dosage was handled identically. After 5-min of extraction, an 1 : 10,000 aliquot (1 μl) was drawn and injected onto a gas chromatograph (HP5710A GC with FID, 4.5 mm $\times$ 1 m, 10% UC-W98 packed column, 170°C isothermal, and HP3380 integrator). The extractable proportions of the original dosages on these live bees were compared with those from the dead bees using the integrated peak areas (adjusted to equilize the areas of the hydrocarbon standards).

RESULTS

Reduviidae. The assassin bug *Apiomerus flaviventris* handily takes offered prey representing all five apoid families as well as flies (Table 1). They are sit-and-wait predators, rearing up on their meso- and metathoracic legs with forelegs laterally outstretched. Bees are typically clutched mediodorsally across the thoracic dorsum. In this position, the bee's legs, sting, and mandibles are all incapable of contacting the reduviid. Prey were often initially pierced through their cervices or mediodorsally through other membranes. The reduviids' encounter times with prey in their glass terraria varied from 6 sec to over 30 min (N = 27 timed bouts, median = 5 min). Prey smaller than *A. argemonis* (intertegular width < 2.7 mm) were captured on the first lunge nearly 95% of the time. For prey larger than the honeybee (intertegular width >3.2 mm), only one of the 15 prey items offered was successfully captured despite 35 total lunges. Notably, the large black syrphid fly, *V. mexicans*, which lacks mandibles, sting, and any apparent defensive secretion, remained uncaught (Table 1). Conversely, honeybees, which possess these defenses, were victims of *A. flaviventris* 93% of the time (Table 1). The stalk and lunge of an *Apiomerus* was readily thwarted by resting bumblebees as they thrust it aside with a kick from the mesothoracic leg. The *Apiomerus* were unable to successfully grip many of these larger prey.

Asilidae. Perched *Efferia rapax* darted to meet 68% of the offered tethered insects (N = 76 bees and 5 flies) and attempted capture for 47% of these close approaches. Prey smaller than 2.5-mm intertegular width (*Melissodes* sp. 2) were invariably at least approached (N = 25 bouts) (Table 1). The brightly colored, strong-odored *A. argemonis* was frequently approached and struck, but in general, the flies responded less frequently to the larger (intertegular width >2.5 mm) potential prey.

Formicidae. Foraging *Formica glacialis* ants at the *Colletes thoracicus* nesting aggregation responded differentially to the extract presentations. The G test indicated significant differences among the treatments ($P < 0.001$). The a posteriori tests show significant differences ($P < 0.05$) between the ants' responses to provision extract, to hexane control, and to mandibular gland ex-

TABLE 1. RESPONSES OF REDUVIID, ASILID, AND AGELENID PREDATORS TO POTENTIAL BEE PREY

		Predator			
		Apiomerus flaviventris[c]		Efferia rapax[d]	Agelenopsis aperta[e]
Prey[a]	Intertegular width (mm)[b]	O, C	Success (%)	N, A, S	E, C
Volucella mexicans+	5.7	6, 0	0		
*Centris ruthannae**	5.3	2, 0	0		1, 0
*Bombus sonorus**+	5.1	4, 0	0		
*Protoxaea gloriosa**	4.7	1, 1	100	2, 0, 1	
Melissodes sp. 1*	3.3	1, 0	0		
Triepeolus spp.*+	3.3	1, 0	0	7, 3, 4	
Apis mellifera	3.2	15, 14	93	2, 0, 1	
*Andrena argemonis**+	2.8	10, 5	50	5, 4, 8	0, 2
Melissodes sp. 2*	2.5	1, 1	100	10, 5, 4	0, 1
Megachile spp.	2.5	2, 2	100	0, 1, 1	
Anthidium spp.+	2.3	3, 3	100		0, 1
Musca spp.	2.2			0, 3, 2	0, 1
Agapostemon tyleri	2.0			0, 2, 3	0, 1
Calliphora spp.	2.0	2, 2	100		
Exomalopsis solani	2.0	4, 3	75		
Agapostemon melliventris	1.9	1, 1	100	0, 5, 3	
Nomia nevadensis+	1.9	7, 7	100		
Diadasia olivaceae	1.9	1, 1	100		
Chalicodoma spp.+	1.7	2, 2	100		
Lasioglossum spp.	0.9	1, 1	100	0, 3, 2	1, 1

[a] The menu of insects includes both flies (*Volucella*, *Musca*, and *Calliphora*) and bees, the latter including social (*Bombus*, *Apis*), kleptoparasitic (*Triepeolus*), and solitary representatives drawn from 14 different tribes that bracket most of the available size range of bees (roughly *Drosophila*-sized to large bumblebees). Bees are subjectively classified as to having an odoriferous mandibular gland secretion (*), and exhibiting aposematic or mimicry patterns (+).

[b] Intertegular width, being the shortest distance across the thorax between the insect's wing tegulae (wing bases), is an excellent estimator of bee dry weight (Cane, unpublished).

[c] The number of offered (O) prey items captured (C) is reported as a capture success rate (success).

[d] The frequencies of no response (N), aerial approach to investigate within 10 cms (A), and actual striking (S) of the tethered bee or fly are reported.

[e] The number of prey items making web contact are classified as either escaped (E) or captured (C).

tracts. Responses to the three dosages of mandibular gland extract (1, 2, and 5 glandular equivalents) did not differ significantly from one another. The ants met provision extracts with either feeding responses with mouthparts extended (35%) or no observable response ($N = 74$ ants). Hexane generally (92%) elicited no behavioral responses ($N = 173$ ants). Mandibular gland extracts never elicited feeding responses ($N = 193$ ants), but, of the 94 tested ants, 69% gave

avoidance responses (jump or retreat) while the remaining 19 directed defensive sprays at the pipet bearing the volatilizing mandibular gland extract of the *Colletes* bees. This represents 90% of all of the retaliatory responses elicited. Provision extracts, then, generally elicit approach and feeding responses, the solvent alone elicits no response, and the mandibular gland extracts elicit avoidance, startle, or retaliatory responses from foraging ants in the field.

During the laboratory ant studies, the ranges of cumulative forager counts at any one feeding station for a day's experimentation ranged from 0 to 127, $\bar{X} = 23.5$ (*Formica subsericea*) and from 0 to 629, $\bar{X} = 105$ (*Crematogaster cerasi*). Neither day nor position affected forager distributions at the feeding stations ($P > 0.5$). In all experiments, the control attracted at least as many foragers as any bait with odor ($P < 0.05$). For both ant species, neither farnesol nor skatole were significantly repellent ($0.05 < P < 0.1$). Citral, citronellol, geraniol, and linalool were all effective repellents ($P < 0.01$) of both ant species. Salicylaldehyde was also strongly repellent (2% of foragers feeding) for *F. subsericea* ($P < 0.001$), but only mildly so for *C. cerasi* ($P < 0.05$). Butyric acid and 2-octanone were marginally repellent chemicals ($0.01 < P < 0.05$) for both ant species.

Arachnida. Agelenopsis aperta, the funnelweb spider, was reasonably successful in capturing bee prey (Table 1). Only one escape in eight bouts was seen. A large, chilled *Centris* bee was offered to the spider, which immediately began wrapping it in silk. After 1 min, however, the bee revived and ripped free from the webbing. The spider retreated to its funnel refugium. In contrast, the spider took *A. argemonis* females handily, despite these bees' mandibular gland secretions that were noticeably released from their spread mandibles when first contacted by the spider.

Among *Argiope aurantia*, the four largest bees (from three families) all successfully escaped from these spiders' webs, despite the spiders' attempts at prey immobilization with silk (Table 2). The *Megachile* lack any notable mandibular gland volatiles. Of the remaining 11 smaller prey bees, only one escaped. Median handling times for all 16 prey were 2 sec to contact, 10 sec spent silk wrapping, and 11 additional sec until steady feeding. The three flies were not wrapped in silk prior to envenomation.

During the 1984 experiments, none of the 135 perfumed honeybee workers escaped *A. aurantia* once contact was made. In neither this experiment, nor any of the above experiments, was a predator ever injured by either a bee's mandibles or sting. Median handling times (and 25–75% quantiles, range) were similar to the above experiments. Contact was made in 1 sec (1–6 sec, range 1–66 sec), and offensive silk wrapping persisted for a median of 8 sec (5–12 sec, range 1–47 sec). In 22% of all cases, the spider paused astride the wrapped bee for 1–300 sec following silk wrapping but prior to envenomation (median 10 sec). As for *Apiomerus*, venom was typically injected mediodorsally on the bee, but for *A. aurantia* it was more often in the thoracic dorsum.

TABLE 2. DURATION OF PREY-HANDLING SEQUENCES OF *Argiope aurantia* SPIDERS IN RESPONSE TO OFFERED ACTIVE BEE PREY TOSSED INTO WEB

Prey	Intertegular width (mm)	*Argiope aurantia* (A, W, F) sec[a]
*Bombus pennsylvanicus**+	5.3	5, 65, E
Megachile fortis	4.7	1, 35, E
*Svastra obliqua**	4.6	1, 25, E
Apis mellifera	3.2	3, 5, 8
		3, 15, 15
Melissodes spp.*	2.5	1, 20, 22
		1, 25, 26
		20, 20, E
*Andrena accepta**	2.5	2, 5, 7
		2, 8, 10
		1, 10, 11
		1, 13, 14
		270, 70, 430
bombyliid spp.	2.5	3, 0, 3
		5, 0, 3

[a] Spider approach time (A) following web contact, duration of offensive silk wrapping (W), and delay until actual feeding (F) are all reported in seconds. Some potential prey forcefully escaped (E) following attempted silk-wrapping by the spider. See Table 1 for explanation of * and +.

No significant habituation or learning trends across experimental days were detected with the runs tests. Comparing the nine compounds (including hexane controls) at the 7- to 14-μg dose ($N = 45$), neither median contact ($P > 0.25$) nor wrapping ($P > 0.25$) durations were significantly prolonged (one-tailed) using Friedman tests. Similarily, no significant increases were found for median silk-wrapping durations ($P > 0.25$) across the dosage gradient from 0 to 200 μl ($N = 125$). Only median contact time was significantly enhanced (from 1 to 3 sec) by increasing dosages of volatiles applied to the bees ($P < 0.025$) (neglecting momentarily the incrementing type I errors accrued by repeated tests on the same family of data).

The proportional frequency of post-wrap pausing behavior across dosages was significantly greater (18–27% of bouts) when compared with hexane or no-treatment controls (4%) ($G = 123.4$, $G_{P<0.005} = 14.86$), but no differences among dosages were detectable by STP analyses. There were no significant differences among the compounds for pausing frequencies.

The extractable dosages of citral remaining upon live bees following their brief warm-up and flight preceding web ensnarement varied considerably. From 39 to 100% of the original dosage was recovered from dead bee whole-body extracts. Relative to these figures, extractable citral remaining upon live bees

ranged from 1.3% to 80%, with proportionately more recovered from the larger initial dosages. Thus, dosages encountered by *Argiope* spiders upon first contact with their perfumed *Apis* prey ranged from aproximately 0.5 μg (7–14 μg initial dosage) to 950 μg (860–1170 μg initial dosage). This adequately brackets the known range of naturally occurring quantities of these volatiles from bees (see Introduction).

DISCUSSION

Insect exocrine lipid secretions can serve as allomones that deter attacks by their predators or parasitoids (Blum, 1981). To be an allomone, candidate exocrine lipids must repel known predators/parasitoids for biologically relevant contexts of encounter, all within the quantitative confines of the producing target-prey species. If synthetic allomones are used in the absence of the natural delivery system, then we must approximate the quantities encountered by the predator. If the secretion persists evolutionarily as a defensive adaptation, it must reduce the prey's probability of reproductive fitness losses (injury or death) despite the predator's counteroffensive adaptations. In short, an allomone is not merely a repellent per se, but a deterrent to predation as well.

The tested synthetics are present in a majority of known mandibular gland secretions of bees. Among 11 colletid species (*Hylaeus* and *Colletes*), citral is frequent, and linalool is common in the latter genus (Blum and Bohart, 1972; Bergström and Tengö, 1973, 1978; Hefetz et al., 1979b; Duffield et al., 1980). Citral is also known from andrenids, being reported from three species of *Calliopsis* and one *Nomadopsis* (Hefetz et al., 1982), *Panurginus potentillae* (Duffield et al., 1983) and *Andrena* (Bergström et al., 1982). Three species of *Andrena* also bear citronellol (Tengö and Bergström, 1976; Bergström et al., 1982) and ketones like methyl decanone (Tengö and Bergström, 1976, 1977). The oxaeid *Protoxaea gloriosa* secretes citral and geraniol (Cane and Buchmann, unpublished). So does the melittid *Dasypoda*, which also produces linalool and citronellol (Bergmark et al., 1984). A *Centris* bee produces geraniol and citral (Vinson et al., 1982). Among stem-nesting bees, *Ceratina* mandibular glands (three species) contain nerolic and geranoic acids and/or their acetates (Wheeler et al., 1977), *Pithitis* has the acetates plus salicylaldehyde (Hefetz et al., 1979a), and three species of *Exoneura* also produce salicylaldehyde (Cane and Michener, 1984). Butyric acid seems ubiquitous among worker *Bombus* (Cederberg, 1977), skatole and nerol have been isolated from *Melipona* (Smith and Roubik, 1983), and citral is common among both *Trigona* and *Lestrimellita* (Blum, 1966). Simple olfactory field evaluations suggest that these compounds will be found in more bee species.

Ants are effectively repelled by either authentic or synthetic mandibular gland components of bees, both in field and laboratory contexts. When present

in the airspace proximate to food, these volatiles render an otherwise attractive feeding station repellent or threatening. Prior experience with bees does not seem to influence repellence (comparing omnivorous *Formica* with aphid-tending *Crematogaster*). Repulsion is, to an extent, species-specific (comparative responses to salicylaldehyde), but some mandibular gland volatiles repel neither species. Mandibular gland equivalents are adequate to achieve significant repellence (experiments with linalool).

In what contexts do solitary bees encounter ants? Ants often share soil nesting aggregations with bees. Nesting tumuli of bees can contain odors of the provisions held within (Cane, 1981). Various ant genera opportunistically prey upon such ground nests (Cane, personal observation; Schultz, 1977). Ants raid stem-nesting bees both to consume the nest contents as well as to usurp the bee's hollow domicile. Michener (in Cane and Michener, 1984) notes that these nests are quickly taken over by ants if the resident bee is removed. A *Pithitis* or *Ceratina* female will smear her body with cephalic secretions upon encountering ants at her nest entrance. The mandibular gland secretions of a female bee, when released at her nest entrance, can repel marauding ants which might otherwise kill her or her progeny.

The arthropodan predators of flying insects (asilids, reduviids, arachnids) successfully counter the repellents and venoms of bees by virtue of combinations of hard exoskeletons, sit-and-wait tactics followed by rapid approaches, powerful long legs that facilitate prey handling and remotion of prey defenses, and rapid prey immobilization by injected venoms. Warning or mimicry patterns, volatile mandibular gland secretions, and/or stings are at best secondary to overall prey size and strength in determining outcomes of predator–prey conflicts for *Apiomerus flaviventris, Efferia rapax,* and *Argiope aurantia* (Tables 1 and 2).

Asilid prey records and the distribution of presumed allomones among prey taxa underscore these results. Linsley (1960) reported the hymenopteran prey from nine genera of southwestern U.S. asilids. Relatively large (his distinction) asilid species commonly take species of *Anthophora,* Apis, Bombus,* Centris,* Melissodes,* Protoxaea,** and *Xylocopa.** (The asterisk denotes my field observations of perceptable mandibular gland volatiles). Medium-sized asilids take various *Apis, Agapostemon, Colletes,* Exomalopsis, Halictus, Hesperapis,* Megachile, Melissodes,* Micranthophora,* Nomia,* Perdita,** and *Psaenythia.** Finally, the small asilids prey upon such bee taxa as *Colletes,* Exomalopsis, Lasioglossum,* and *Perdita.** Linsley (1960) noted distinct differences in prey selection for these asilids among the potentially available prey which he explained by the relative sizes of predator and prey. In Wyoming, Dennis and Lavigne (1975) found 10 species of robber flies to take prey averaging 40% of their own body lengths. Some taxa (*Mallophorina*) consumed up to 73% Hymenoptera. They captured most of the above genera plus *An-*

thidium, Dialictus, Dianthidium, Epeolus, Evylaeus, Nomada,* Pseudopanurgus,* Sphecodes,* and *Triepeolus.**

From the above lists and my own results (wherein the prey menu is controlled), it seems that bees emitting even copious amounts of supposed repellents do not escape the asilids' menus. Some asilid species even seem to prefer more colorful bees and wasps (Linsley, 1960). Lavigne and Holland (1969) and Dennis et al. (1975) demonstrated that asilids distinguish among artificial lures primarily by size. Size, then, outweighs olfactory repellence in these examples of asilid prey selection.

Less is known of the hymenopteran prey of reduviids, although some *Apiomerus* have earned the label "bee assassin" (Caron, 1978). Indeed, *A. pictipes* in Mexico seems to employ *Trigona* attractants to lure guard bees to their demise (Weaver et al., 1975). Like the asilids, *A. flaviventris* adults readily take bees regardless of the bee's scent, but fail to manage larger, although otherwise undefended insects. Their inability to capture the large syrphid fly *Volucella,* contrasted with their repeated success with smaller *Andrena argemonis* (which smells of burnt garlic) or the sting-endowed honeybee, illustrates this point. Contrasted with the asilids, the prey size assessment by *Apiomerus* seems to be accomplished only after repeated grappling attempts, rather than by close range inspection. Perhaps bees bearing repellent mandibular gland secretions may enjoy some enhanced predator deterrence when the bee is marginally large for a given reduviid (again, contrasting their lesser success with odoriferous *Andrena* vs. the somewhat larger *Apis*).

Bee predation by spiders presents a similar story. Spiders that might otherwise rarely encounter bees, owing to their web placement, such as *Lycosa, Agelina* (Bilsing, 1920), and *Agelenopsis* (Table 1), readily tackle offered bee prey. In Ohio, Bilsing (1920) found the webs of *Argiope, Neoscona,* and *Araneus* to contain enwrapped species of *Bombus,* Megachile, Melissodes,* Augochlora, Apis, Colletes,** and *Halictus* (again, * = scented). Bristowe (1941) reported that *Argiope* would occasionally be stung and temporarily paralyzed by a captive *Halictus,* and sometimes rejected *Nomada* bees. Neither response was ever observed in my experiments. In contrast, Suter (1978) noted that, among a diversity of offered prey, *Halictus* elicited the "least latency of first motion" and fewest pauses per approach, using thanatosis (playing dead) to minimize its web-borne vibrations. When Fabré (1912) fed large, odorous *Xylocopa* and *Bombus* to *Lycosa,* the spider often retreated. But, if it attacked, it never failed to immobilize the prey item. Again, the bee's mandibular gland secretion does not convey protection against spiders, even for arachnids whose diet rarely includes bees.

Although mandibular gland secretions of bees do not deter predation by spiders, they do alter aspects of the *Argiope* prey-handling sequence. Honeybees perfumed with various mandibular gland components did elicit signifi-

cantly more frequent preenvenomation pausing. Owing to prior "immobilization wrapping," however, the bee never escaped. Also, prior to envenomation, *Argiope* throws silk offensively from a distance for odoriferous *Trigona* bees (Robinson et al., 1969) and pentatomid bugs (Robinson and Olazarri, 1971). In their studies, these prey also elicited frequent "rest-on-prey" pauses, with intensive grooming, again suggesting that the spider finds these volatiles noxious but tolerable.

Clearly, mandibular gland lipids of bees are often olfactorily perceptable by both humans and the arthropodan predators of bees. While variably repellent to ants at the bee's nest entrance, these allomones are generally ineffective deterrents of the bee's arachnid, reduviid, and asilid predators.

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Book Review

Insect Communication. (edited by Trevor Lewis (ed.)). New York, Academic Press 1984, $55.00, 414 pp.

This book is a collection of papers delivered at the 12th symposium of the Royal Entomological Society of London, in honor of the society's 150th year. The topic, insect communication, was chosen because it spans the subject of insect biology and has enjoyed a rapid expansion in the past decade. The volume covers a variety of topics, ranging from discussions of specific communication channels to the evolution of communication and the practical aspects of disrupting communication as a control tactic.

Two chapters address acoustic communication in insects. The chapter by Bennet-Clark is a thorough, but straight-forward, description of the physical aspects of insect hearing. Behavioral aspects of this modality are taken up by Ewing, with specific reference to sexual behavior. Ewing amply demonstrates the richness of song diversity and its complexity in a wide range of organisms with respect to both calling and courtship sequences. One of the important problems addressed in this chapter, and common to a number of others in the book, is that too often songs are characterized as being involved in calling or courtship without critical analysis of the precise components of the song that are important for the behavior in question. In addition, while songs are often characterized as species specific, the essential or optimal stimulus rendering the specificity is mostly undetermined, and in many cases there is little evidence that females utilize song in discriminating between males, although this is almost always presumed.

Visual communication is also covered in two chapters. The first is a description of the insect eye, by Wehner and Srinivasan, with emphasis on visual acuity. The second, by Case, is a comprehensive and well written account of visual communication in fireflies. Emphasis is placed on the behavioral strategies used to maximize the capabilities of the visual system in overcoming the problem of detecting a point source of light against a varied and often shifting background. Again, the richness and complexity of these displays are well documented, despite the fact that for many groups very little is known about their mating behavior.

Chemical communication is given the greatest coverage in the book (nine of 16 chapters). In a chapter on the chemistry of insect communication, Silver-

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stein presents a discussion of the classification of compounds according to their functional group and three-dimensional structural properties (the chemist's view), and their behavioral activity as semiochemicals (the biologist's view). He correctly points out that, despite the pitfalls, the available technology and experience in chromatographic analysis, volatile collections, and behavioral assays allow (and demand) a greater precision in determinations of chemical structure and behavioral function of pheromonal compounds, and a greater degree of interaction between the chemist and behaviorist.

Chemical communication in bark beetles is one of the most complex systems known, and the chapter by Borden is an excellent critique of chemically mediated aggregation and dispersion in this group. He begins with a generalized discussion of the complex sequence of events in the aggregation of beetles on a host tree, emphasizing important relationships between host accessibility, beetle physiology, and the interactions between both that involve semiochemicals. This generalized scheme is then used to contrast three case histories, the mountain pine beetle, an aggressive attacker of trees; the elm bark beetle, which relies on a fungus to aid in weakening the host; and the ambrosia beetle that utilizes dead or dying hosts. A secondary theme of this chapter concerns the potential for disruption of the communication process as a method of controlling the beetles. Borden provides an extensive list of ways this might be accomplished, emphasizing the importance of basic biological information about the insect-host interaction in designing control strategies.

A number of chapters deal with chemical communication in the social Hymenoptera. Morgan reviews the variety of chemicals used by bees, wasps, and ants for recruitment and foraging. The theme of this chapter is that the richness of diversity in glands and their secretions (chemical "words") and the multiple use of these secretions in a variety of contexts (chemical "phrases") allows for greater complexity of chemical meaning. Ecological considerations are also central to the chapter by Howse on alarm and defense compounds in social insects, and in Koeniger's chapter on brood care and recognition of pupae in the honey bee and hornet. Howse shows how the tactics used in defense and alarm are intimately related to foraging strategies and to nest structure and colony size.

The electrophysiology of insect olfaction is covered by Boeckh. Whereas there is a great deal known about peripheral receptors and their activity, Boeckh points out that questions dealing with central processing are most pertinent to an understanding of two major problems in pheromone perception. The first concerns the sensitivity of males to the chemical stimulus and is partially answered by the tremendous degree of convergence of neural pathways that occurs in the macroglomerulus of the deutocerebrum. More perplexing, however, is the question of how insects discriminate odor quality or the precise ratio of

components in the multicomponent blends of most insects. The "labeled line" and "across-fiber pattern" hypotheses are both discussed as they relate to specific sex pheromones and food odors, respectively.

Boeckh is an acknowledged leader in the field of CNS studies of olfaction, and it is only in the past few years that extensive details of this area have become available. It is thus unfortunate that a major error of reference is made in this chapter. Boeckh refers to Roelofs and his collaborators (specifically the study by Baker et al., 1981) as proponents of the idea that individual chemical components play "different roles in the approach of a male (moth) to the female." In fact, this study as well as a number of others have shown clearly that in the Oriental fruit moth, the complete three-component blend acts as a unit to influence all stages of the behavioral sequence. Recent studies in our lab, involving identifications of more complete pheromone blends in the cabbage looper, red-banded leafroller, soybean looper, and European cornborer moths, support this idea as a general principle in pheromone perception (J. Chem. Ecol. *12*, 659). This correction is important because it relates directly to the question of how blend quality affects male behavior downwind of a source and how electrophysiological experiments can be designed to investigate the observed sensitivity of males to small amounts of minor components in the blend.

Olfactory sexual communication in the Lepidoptera is the subject of a chapter of Cardé and Charlton. Rather than a descriptive survey of the field, this chapter focuses on several challenging questions concerning the current debate over the functional role of multicomponent pheromone blends and the orientation of males flying in a windborne odor plume. The authors state in the introduction that the primary function of the chemical signal is the securing of a mate and that selection will favor factors that enhance the ability of males to rapidly detect and respond to the airborne signal. One important factor concerns the activity of males at the time females are releasing pheromone. The authors suggest that males are most probably in an active appetitive searching flight pattern rather than in a quiescent state. They then argue that wind-tunnel assays utilized to determine male sensitivity to the signal (a second important factor enhancing mate location) may not be valid because they involve male activation from a quiescent state rather than from appetitive flight and that the thresholds for these two steps (activation wind fanning vs. locking on to the odor plume) might be very different. As evidence, unpublished flight tunnel results with the gypsy moth are provided. The authors claim that whereas males are able to fly upwind in a plume of 2.6×10^4 molecules/cm^2/$\frac{1}{2}$ sec, activation from a quiescent state in a flux of 2.6×10^6 molecules/cm^2/sec takes an average of 16 sec. It is undoubtedly true that appetitive flight is an important aspect of male precopulatory behavior and should be a serious consideration for future studies. It should be noted, however, that the gypsy moth displays a much longer latency

for this behavior than several other moth species with which this reviewer is familiar, and thus the observations with this insect may not be of general application to other Lepidoptera.

The problems associated with male sensitivity lead directly to a discussion of discrimination of blend quality, and a shift from the gypsy moth to the Oriental fruit moth, which uses a three-component blend. The authors ask "are the thresholds for attraction (locking on the upwind flight) lowest for the entire blend, or do portions of the blend evoke these behaviors at the same concentration? [or]. . can there be more than one active space [for the pheromone]?" One of the presumptions leading to these questions is the idea that, since minor components are present in such low quantities relative to the major component, they cannot be detected at the same distance as the major component. Yet there are numerous examples showing that upwind flight is dependent on a precise ratio of isomers and that males are able to detect very slight changes in isomeric ratios. It has also recently been demonstrated in our lab for the Oriental fruit moth, red-banded leafroller, and cabbage looper moths, that addition of minor components results in significant levels of upwind flight to low dosages of the major component that alone elicit no locking on or upwind flight. Thus it can be argued that males do not perceive the blend as a sequence of individual components with different spheres of activity, but rather that the blend acts as a unit to effect male sensitivity and specificity. The fact that some males are able, at low concentrations of pheromone, to exhibit flight to partial blends or off ratios (at much slower flight speeds than to the same concentration of the optimal blend) may suggest that a portion of the population has a broad range of specificity and may not be representative of meaningful threshold behavior.

I would also take exception with the authors claim (based on their interpretation of the figure shown from Linn and Roelofs, 1983) that "discrimination of the ratio of components occurs during upwind flight, with specificity for the optimal blend most pronounced in later behaviors." In fact, the figure shows that specificity is most profoundly effected in the early orientation or locking on phase of the response. It is clear for this insect and a number of others that taking flight is not a discriminating behavior, but rather that this occurs in the locking on and initiation of upwind flight phases. In addition, since the Oriental fruit moth initiates a response very rapidly in the tunnel (flight and locking on taking less than 3 sec), it is unlikely that the thresholds for this behavior would be different for insects in flight.

The importance of addressing these arguments in some detail relates not only to the comparative data that allow an alternative view, but also to statements presented by the authors in the introduction concerning the functional role of the chemical signal. If it is true that the chemical signal is critical in mate location, then it can be argued that males would be selected to be tuned to the complete female released signal, a signal that enhances the sensitivity

and ability of males to discriminate odor quality in the earliest (downwind) stages of the response. The fact that certain components are present in smaller amounts should not preclude their importance at any stage in the male precopulatory response.

The remainder of the chapter by Cardé and Charlton deals with a discussion of orientation mechanisms. This area has enjoyed much attention over the past decade and a great deal is now understood about how flying insects orient to an odor source. The authors note, however, that recent studies have radically altered previous views about movement of a pheromone plume in a fluctuating wind flow and raised questions about how this relates to the movement of the insect. Future studies must concentrate on these problems as they relate to the field to determine if the principles discovered in the arena of the wind tunnel are correct.

As noted earlier, one of the unstated, but important, themes that occurs throughout this volume concerns the functional aspects of signals used in communication. The evolutionary aspects of this problem are dealt with in two chapters. In the first, West-Eberhard argues strongly that sexual selection is *the* driving force in the evolution of species-specific signaling and courtship displays, as opposed to these being primarily reproductive isolation mechanisms. She argues that many signals should be considered as competitive rather than cooperative, in that they lead to differential success in obtaining a resource. After discussing several properties of socially competitive traits (strength of selection, potential for change, diversity of sensory channels, and perpetuity), she shows that many sexual and nonsexual displays can be considered as competitive and thus influenced by sexual selection, including nonsexual communication in social insects, threat displays, territorial displays, courtship, male external genitalia, and possibly female calling. Sexual selection theory, however, involves both intraspecific competition (male–male) and intersexual selection (female choice). There is at present considerable controversy over the role of these two factors and, unfortunately, the problems associated with separating them in the analysis of a display are not dealt with in this chapter. Rather there is a more general emphasis on the potential for rapid signal divergence that can occur under social selection and its role in speciation.

In the other chapter dealing with evolutionary questions Hölldobler presents a more general view. He first notes that, to discuss the evolution of communication, one must decide what communication is. Ideas concerning a definition of communication vary from one extreme, that it must be mutually beneficial to both participants, to the other extreme, that communication is the manipulation of one member (usually the receiver) by another (the sender). Hölldobler adopts a central position and asserts further that the "comparative analysis of the mechanisms of animal communication provides the basis for understanding the adaptive significance and for reconstructing the evolutionary

history of communicative behavior." A discussion of sexual selection then follows that is somewhat more balanced and cautious than West-Eberhard's. While species-specific signals may mediate reproductive isolation, individual components of the signal promote mate assessment and mate choice. He points out that the available evidence from studies of geographic variation in mating signals suggests that species diversity occurs primarily in allopatry and that cases of character displacement are rare. This is in agreement with West-Eberhard, as is Hölldobler's analysis showing that significant variation can exist in the ability of males to display, thus allowing for sexual selection to be a strong selective force. He cautions, however, that separation of intrasexual and intersexual selections is very difficult in practice, as is determination of the genetic basis for female choice. These ideas are then developed in a comparative analysis of individual recognition, worker communication, division of labor, and recruitment in the social hymenoptera. This group certainly offers a vast store of material for testing the hypotheses proposed for the evolution of communication signals.

The only chapter, by Vinson, dealing with interspecific aspects of communication concerns parasitoid host location. As with several other chapters, this one is a discussion of the diversity of strategies utilized by parasitoids in locating a host. Emphasis is placed on the interaction of the host habitat and the use of multiple signals to locate the host. These include host habitat odors, host odors (or kairomones), and visual or tactile components.

The final chapter of this volume, by Wall, is a discussion of the exploitation of insect communication by man and is devoted entirely to chemical communication. Wall first discusses the imbalance in research and development in this area, with identification of compounds and use in detection far outstripping monitoring, mass trapping, or disruption. Neglected areas of research include population studies, behavioral studies in the field, formulations, and meteorological studies. It is clear that effective use of chemicals to monitor or disrupt insect communication demands greater attention to trap design, trap efficiency, the behavioral strategies of the insects in the field, and effective formulation of the appropriate compounds for monitoring and disruption.

The field of insect communication is indeed a broad one and would be difficult to cover adequately in one volume. In the present work, whereas the major modalities involved in communication are dealt with, the choice of some of the chapters seems puzzling. I question, for example, the need to discuss the physical or morphological aspects of an apparatus used in communication (chapters by Wehner and Srinivasan; Bennet-Clark) when there is no discussion of the relevance of the behaviors involved. These technical aspects could have been better handled by references to other reviews. In addition, the chapter by Boeckh is the only one that contains any discussion of electrophysiology as it relates to communication. There is also a lack of integration between chapters,

and the introductory chapter by the editor, Lewis, makes no attempt at a synthesis. Rather, he discusses insect communication as it might relate to several aspects of human language, and then proposes four areas of concern for future study. These include electromagnetic communication, recognition and perception of pheromone blends, applied exploitation, and additional modes of communication. I would agree with the second and third of these choices but would voice an opinion, shared by a number of the authors themselves, that one of the most pressing problems concerns the functional aspects of signaling as its relates to the ecology and evolution of mating and the social aspects of communication in the Hymenoptera. I also question whether the theories proposed by Callahan constitute a serious concern for future studies.

Finally, the volume is of high quality, well illustrated, and extensively referenced. While it is not complete in its coverage, and thus might not serve adequately as a text, many of the chapters provide excellent reviews for researchers in other disciplines, and perhaps a number of stimulating questions for those in a particular discipline, as indeed was the case with this reviewer.

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PLANT ALLELOCHEMICALS AND INSECT PARASITOIDS Effects of Nicotine on *Cotesia congregata* (Say) (Hymenoptera: Braconidae) and *Hyposoter annulipes* (Cresson) (Hymenoptera: Ichneumonidae)[1,2]

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Abstract—Parasitoids developing within tobacco hornworms or fall armyworms exhibit significant differences in development and survival depending on whether their hosts fed on nicotine-free or nicotine-containing diets. The effects of nicotine were more severe on the relatively less adapted parasitoid, *H. annulipes* than the specialist parasitoid, *Cotesia congregata*. Labeled alkaloid originally placed in herbivore diet was incorporated in several parasitoid tissues. These results suggest that interactions between plant allelochemicals and parasitoids should be considered in the development of theory on insect herbivory and plant defense.

Key Words—Plant allelochemical, parasitoids, three trophic level interactions, nicotine, *Manduca sexta*, Lepidoptera, Sphingidae, *Spodoptera frugiperda*, Noctuidae, *Cotesia congregata*, Hymenoptera, Braconidae, *Hyposoter annulipes*, Ichneumonidae.

INTRODUCTION

The role of plant nutrients and allelochemicals on the ecology of insect herbivores has been the subject of a great deal of research. Relatively little emphasis

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[2]Mention of a commercial product does not constitute an endorsement.

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has been placed on the role of plant allelochemicals, within the tissues of herbivores, on the survival and ecology of secondary consumers such as insect parasitoids. Several researchers have speculated on the nature of these three trophic level interactions. Their conclusions were generally based on correlations between low levels of parasitism and the consumption by herbivores of plants with allelochemicals known to be toxic, the demonstration of the presence of an allelochemical within a parasitoid without an evaluation of its effects, or some other form of indirect or incomplete evidence (Narayanan and Subba Rao, 1955; Smith, 1957; Jones et al., 1962; Reichstein et al., 1968; Altahtawy et al., 1976; Rothschild et al., 1977; Smith 1978; Benn et al., 1979). Although many of the conclusions and speculations made in these studies are intuitively appealing, they remain speculations and assumptions to be fully and critically tested.

Morgan (1910) and Gilmore (1938a,b) suggested that nicotine might influence levels of parasitism of *Manduca sexta* (L.) (the tobacco hornworm) parasitized by *Cotesia congregata* (Say) (= *Apanteles congregatus*). Thurston and Fox (1972) found a significantly lower larval parasitoid emergence from hosts treated with nicotine. Development time of parasitoid larvae from nicotine-treated hornworms was longer, but the difference was not significant. Campbell and Duffey (1979, 1981) provided the most detailed and extensive investigations of the role of an allelochemical, within host tissues, on parasitoid survival and fitness. Campbell and Duffey (1979) showed that tomatine, a major alkaloid of tomato, caused prolonged larval parasitoid development, reduced pupal eclosion, reduced adult size and longevity, and caused morphological abnormalities in *Hyposoter exiguae* (Viereck), a parasitoid of *Heliothis zea* (Boddie) (corn earworm). Campbell and Duffey (1981) suggested that the detrimental influences of tomatine resulted from the possible disruption of sterol metabolism.

Our study attempted to confirm and extend the results of Thurston and Fox (1972). In addition to documenting other biological effects of nicotine, the study was designed to determine if nicotine was present at all three trophic levels and thus confirm the role of nicotine as the causal agent. A comparison was made of the outcome of interactions among a plant allelochemical, an herbivore, and its parasitoid using two herbivores, *Manduca sexta* and *Spodoptera frugiperda* (Smith) (the fall armyworm). The parasitoids of the hornworm and fall armyworm which were evaluated were *Cotesia congregata* and *Hyposoter annulipes*, respectively.

METHODS AND MATERIALS

M. sexta and *S. frugiperda* were reared on synthetic diets (Bio-Serv. Inc., Frenchtown, N.J.) which were either nicotine free or had nicotine incorporated. The concentration of nicotine in the fall armyworm diet (0.025% wet weight)

was $\frac{1}{4}$ that of the tobacco hornworm diet (0.1% wet weight) due to the higher sensitivity of the fall armyworm to nicotine toxicity. These concentrations are at the low end of the range of concentrations normally found in tobacco (Sisson and Saunders, 1983) and cause no significant mortality of host herbivores. To follow the low levels of nitocine incorporated into insect tissues, both diets were supplemented with 100 μCi/liter of generally labeled tritiated nicotine-*d*-bitartrate with specific activity of 1.27 μCi/mmol (Amersham Corp Arlington Heights, Illinois).

Newly enclosed tobacco hornworm and fall armyworm larvae, because of their sensitivity to manipulation, were left on nicotine-free diet before being used in experiments. Seven-day-old hornworm larvae (usually second instars) were individually parasitized and placed in 227-cm^3 (8-oz.) paper cups. Larvae of this age are the host stage preferred by *C. congregata*. Half the parasitized larvae were placed on nicotine-free diet and the other half on nicotine-containing diet. For *S. frugiperda*, half of a cohort of 3-day-old larvae were transferred to nicotine-containing diet while half were placed on nicotine-free diet. All larvae were parasitized on day 4, which is the preferred host stage of *H. annulipes*. Larvae were placed in 10-cm-diam. plastic Petri dishes with diet in batches of 18/dish and exposed to parasitoid females.

C. congregata is gregarious, and larvae emerge from their hosts and spin cocoons in which they pupate and from which adults emerge. After larval parasitoid emergence, all host larvae were dissected and unemerged parasitoids counted. *H. annulipes*, a solitary parasitoid which consumes all host tissues except for its integument, emerges as a larva and spins a cocoon from which the adult emerges.

Samples of synthetic diet, parasitoid adults, cocoons, and meconia were taken to isolate, identify, and quantify the labeled alkaloid. Samples were frozen, weighed, lyophilized, reweighed, and ground in a mortar and pestle with 40% (v/v) methanol containing 0.1% (v/v)1 N HCl. The homogenate was centrifuged at 10,000 *g* for 10 min and the supernatant filtered through 0.45 μ nylon 66 membrane filters. The samples were applied to an HPLC chromatographic system with a Waters automatic injector and a UV absorbance monitor with a Waters Model 440 detector. The specific radioactivity of each of the peaks in the eluant was monitored with a Radiomatic Model Flo-one HS radioisotope detector using Flow Scin II cocktail. Nicotine was separated and quantitated on a 10-μ C_{18} reverse-phase column using a mobile phase at 0.6 ml/min 60% (v/v) methanol containing 0.1% (v/v) phosphoric acid buffered to pH 7.0 with triethylamine. Total radioactivity in each sample was checked by measuring the level of radioactivity in an aliquot of the extract with a Packard Tricarb scintillation counter as described previously (Saunders et al., 1977). Specific quench curves for each type of biological sample were used to quantitate the concentration of labeled compound in tissues.

RESULTS AND DISCUSSION

Nicotine in the tissues of an herbivore can effect the development and survival of its parasitoids. In tobacco hornworms reared on nicotine-containing diets, the proportion of parasitoid larvae that failed to emerge from their hosts was significantly greater than those failing to emerge from hornworms on nicotine-free diets (Table 1). These data confirm the results of Thurston and Fox (1972). Of the parasitoid larvae which emerged from their hosts, a greater proportion failed to form cocoons if they had emerged from nicotine-fed hornworms. No statistically significant differences were demonstrated in pupal mortality, average number of larvae emerging, or the average total number of adults resulting per host (Table 1). Larval and pupal development as well as the size of adult *C. congregata* were unaffected by nicotine (Table 2).

The effects of nicotine on *H. annulipes* were similar to those on *C. congregata* but somewhat more severe on survival and development (Table 3). Of the fall armyworms reared on nicotine-containing diet and exposed to parasitism, only about 46% produced parasitoid larvae compared to almost 76% of the hosts reared on nicotine-free diet. These results are, at least in part, comparable but in sharp contrast to the lack of significant differences in the total number of *C. congregata* produced. Clearly, some of the difference may have been due to fewer nicotine-fed armyworm being parasitized. The extremely small size of 4-day-old larvae and the large size of *H. annulipes* relative to its host made accurate determination of individual parasitization, rather than random contact, impossible. As with *C congregata*, the ability of *H. annulipes* to

TABLE 1. INFLUENCE OF NICOTINE ON SURVIVAL OF *Cotesia congregata* (=*Apanteles congregatus*) A PARASITOID OF *Manduca sexta*

	Survival[a]					
Treatment	Total No. larvae produced	Larvae failing to emerge (%)[b]	Larvae failing to form cocoons (%)[b]	Pupal Mortality (%)[b]	Total No. larvae emerging	Total No. adults emerging
Nicotine (0.1%)	93.0a	23.3a	19.9a	18.5a	67.7a	42.6a
Control	99.7a	8.1b	6.1b	15.5a	79.6a	54.6a

[a] All figures are least-square estimates of means of 54 nicotine-diet and 34 control-diet reared hornworms. Values in columns followed by different letters are significantly different at $P < 0.05$ (SAS, Proc GLM, Type III SS).

[b] All percentages were transformed to arcsin $\sqrt{\%}$ prior to analysis. The values reported are the back-transformed mean percentages.

TABLE 2. INFLUENCE OF NICTONE ON DEVELOPMENT OF *Cotesia congregata* (=*Apanteles congregatus*) A PARASITOID OF *Manduca sexta*

Treatment	Development			Size (dry wt, mg/individual)[a]
	Larval (days)[b]	Pupal (days)[c]	Total (days)	
Nictone (0.1%	11.9a	6.7a	17.7a	0.224a
Control	11.7a	6.8a	17.5a	0.244a

[a] All figures are least-squares estimates of mean of 54 nicotine-diet and 34 control-diet reared hornworms. Values in columns followed by the same letter are not significantly different (SAS, Proc GLM, Type III SS).
[b] Time period between oviposition and cocoon formation.
[c] Time period between cocoon formation and adult emergence.

form cocoons is influenced by nicotine. Another important difference between parasitoid species is that while larval development in *C. congregata* is unaffected by nicotine, nicotine exposure prolongs larval development in *H. annulipes*.

Perhaps most important were differences in adult size. *H. annulipes* adults from hosts reared on nicotine-containing diet were significantly smaller than those produced from hosts free of nicotine. These results are in marked contrast to those with *C. congregata* where the slight numerical differences between nicotine and nicotine-free adults were not statistically significant. The decline

TABLE 3. INFLUENCE OF NICOTINE ON SURVIVAL AND DEVELOPMENT OF *Hyposoter annulipes*, A PARASITOID OF *Spodoptera frugiperda*[a]

Treatment	Survival			Development			
	Parasitism (%)	Larval failing to form cocoons (%)	% Adults emerging (%)	Larval	Pupal	Size (dry wt, mg/individual)	Six ratio (% females)
Nicotine (0.025%)	46.4a	12.8a	74.2a	10.4a	6.3a	0.643a	48.4a
Control	75.8b	3.7b	81.3a	8.9b	6.2a	0.787b	41.3a

[a] Values in columns with different letters are significantly different at $P < 0.05$.

in adult parasitoid weight associated with the presence of nicotine in hosts may have important negative consequences for *H. annulipes* fecundity and overall fitness. Other developmental parameters were not affected by nicotine. Essentially identical results have been obtained in field experiments using low and high nicotine cultivars in which parallel observations were made (Thorpe and Barbosa, 1986, 1319–1328).

Determination of the presence and quantity of the labeled compound in parasitoid tissues confirms the transfer of a plant allelochemical from the diet of an herbivore to the herbivore and subsequently to its parasitoid (Tables 4 and 5). Campbell and Duffey's (1979) study was the first and this is the second to detect directly a plant allelochemical in the tissues of a parasitoid and simultaneously to demonstrate its deleterious effects.

Tables 4 and 5 show that the parasitoids of both the tobacco hornworm and the fall armyworm may deal with accumulated concentrations of a plant allelochemical by shunting the material to cocoon silk and meconium (waste

TABLE 4. AMOUNT OF LABELED ALKALOID IN TISSUES AND EXCRETORY PRODUCTS OF *Cotesia congregata* EMERGING FROM TOBACCO HORNWORMS (*Manduca sexta*) REARED ON 0.1% NICOTINE DIET[a]

	Adult	Cocoon	Meconium
No. of samples	37	37	35
Mean dry wt (mg)/sample	16.72	17.94	3.07
Mean μg labeled alkaloid/g dry wt	8.18a	126.5c	82.0b

[a] Values in rows followed by the same letter are not significantly different. Wilcoxon rank-sum test, $\alpha = 0.048$ (Bonferroni's adjustment for multiple a-posteriori pairwise tests).

TABLE 5. AMOUNT OF LABELED ALKALOID IN TISSUES AND EXCRETORY PRODUCTS OF *Hyposoter annulipes* EMERGING FROM ARMYWORMS (*Spodoptera frugiperda*) REARED ON 0.025% NICOTINE DIET[a]

	Adults	Cocoons	Meconia
No. of samples	6	6	7
Mean dry wt (mg)/sample	8.50	2.77	1.67
Mean μg labeled alkaloid/g dry wt	3.8a	43.5b	36.3b

[a] Values in row followed by the same letter are not significantly different. Wilcoxon rank-sum test, $\alpha = 0.048$ (Bonferroni's adjustment for multiple a-posteriori pairwise tests).

products remaining in the cocoon after adult emergence). The highest concentrations of radioactive alkaloid per gram dry weight were observed in the cocoon silk and meconium. Relatively little radioactivity remained in adult tissues. In *C. congregata*, a significantly greater concentration of alkaloid is shunted to the cocoon silk rather than into the meconium. This is an efficient mechanism since cocoon biomass is six times greater than that of meconia. No such efficiency is exhibited in *H. annulipes*. Nicotine may (1) be directly toxic to parasitoid larvae, (2) interfere with parasitoid nutrition, or (3) act indirectly by reducing host size beyond that caused only by parasitism. Host size and nutrition can also affect both parasitoid growth and development (Beckage and Riddiford, 1978, 1983). Regardless of the specific mechanism, the presence of nicotine in parasitoid tissues supports the role of nicotine as the causal factor in the biological changes observed.

In *C. congregata* the only aspects of development which are detrimentally affected are those changes occurring just prior to and just after larval parasitoid emergence. The fact that any detrimental changes were noted is somewhat unexpected since the parasitoid was exposed to very little of the ingested nicotine. Indeed the concentration of nicotine in the diet of the hornworm causes no significant hornworm mortality. Hemolymph is the principal food source of parasitoid larvae. Of the small amount of nicotine that remains in hornworm tissues, the lowest concentrations are found in the hemolymph. Self et al., (1964) demonstrated that the percent recovery of ^{14}C-labeled nicotine in hornworm larvae 4, 15, and 24 hr after ingestion was 5.2%, 4.1%, and 0%, respectively, while after 24 hr, 92.4% of ingested nicotine is recovered in the feces (Self et al., 1964). Both data sets suggest the rapid egestion of nicotine. Six hours after ingestion, the ratio of recovered nicotine in hemolymph, larva, and feces was 1:6:182. Although one might speculate that parasitoids may concentrate blood alkaloids, there is no evidence indicating that parasitoids possess such a capacity, and thus it is most reasonable to assume that the food consumed (blood) contains relatively little alkaloid. A second reason why, a priori, one would not expect any major effect due to nicotine is that the evolution of *C. congregata* is closely associated with that of *M. sexta* and thus closely associated with nicotine. Although *C. congregata* is recorded from other Sphingidae, the relative abundance of other hosts is scarcely comparable to that of *M. sexta*. In addition, whether or not the parasitoids emerging from these other sphingid hosts are actually *C. congregata* is a subject of debate (Krombein et al., 1979).

Hyposoter annulipes, on the other hand, is not routinely exposed to nicotine. It attacks a variety of noctuids which feed on a large number of plant families (i.e., a wide variety of plant allelochemicals). Tobacco (and thus nicotine) is not a prime or frequent host plant of any *H. annulipes* hosts. In the Maryland area, *S. frugiperda* attacks tobacco only when its populations reach relatively high densities on other crops like corn. These factors may, at least in

part, explain the more severe effects of nicotine on this parasitoid compared to those on *C. congregata*. However, since little is known of the metabolism of nicotine by the fall armyworm, intrinsic changes (i.e., metabolic alteration of nicotine) may be responsible for the modification of survival and development in *H. annulipes*.

Finally, data like these may have a significant effect on theories describing insect–plant interactions. Although the role of plant allelochemicals in host-habitat and host-finding has been extensively reviewed (Vinson 1975, 1976, 1977, 1981; Price, 1981; etc.), there has been less emphasis on the effects of plant allelochemicals within the tissues of herbivores on their natural enemies. This study, Campbell and Duffy's (1979, 1981), and others (see Introduction) suggest that the development of ecological theories on plant–insect interactions should consider the role of natural enemies. Issues that might be affected by such consideration include the role of insect herbivory in the evolution of certain forms of plant defense, the relationship between type of defense and growth forms of plants, the protection of "valuable" tissues at the potential expense of less important tissues, etc. We suggest that parasitoids (and other natural enemies) are critical participants in three trophic level interactions and may affect the evolution of plant and herbivore. Thus, theoretical predictions that include parasitoids (or other third-trophic-level organisms) may be quite distinct from those that exclude them from consideration and may be more parsimonious with ecological and evolutionary patterns (see Barbosa and Saunders, 1985; Schultz, 1983).

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EFFECTS OF COMSUMPTION OF HIGH AND LOW NICOTINE TOBACCO BY *Manduca sexta* (LEPIDOPTERA: SPHINGIDAE) ON SURVIVAL OF GREGARIOUS ENDOPARASITOID *Cotesia congregata* (HYMENOPTERA: BRACONIDAE)[1]

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Abstract—The significance of nicotine in the three trophic level interaction involving tobacco (*Nicotiana tabacum*), the tobacco hornworm (*Manduca sexta*), and the parasitoid *Cotesia congregata* was investigated in field plots of two varieties of tobacco which had about a 10-fold difference in their nicotine content. While *M. sexta* mortality, rates of parasitism by *C. congregata*, and the total number of *C. congregata* larvae produced per host were similar on each of the two varieties, the number of parasitoids reaching adulthood on the low nicotine treatment was nearly twice that on the high nicotine treatment. This difference was due to the significantly greater proportion of parasitoid larvae which failed to emerge from the host or that died prior to pupation after emerging from hosts which fed on the high nicotine variety. A greater proportion of larvae from hosts which fed on the low nicotine tobacco died as pupae. No treatment differences occurred for either sex of the parasitoid in individual dry weight, longevity, or pupal development time, except that female pupal duration was prolonged in the high nicotine treatment. These results support the suggestion that plant allelochemicals, which may function to provide plant resistance against pest herbivores, can be detrimental to natural enemies of the pest.

Key Words—Plant allelochemical, parasitoid, three trophic level interaction, antibiosis, nicotine, tobacco, *Manduca sexta*, Lepidoptera, Sphingidae, *Cotesia congregata*, Hymenoptera, Braconidae.

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INTRODUCTION

An extensive body of theory has been developed to explain the role of plant allelochemicals in plant–insect herbivore interactions (Feeny, 1975 1976; Rhoades and Cates, 1976; Rosenthal and Janzen, 1979). While early attention focused primarily on the effects of plant chemicals within a two trophic level system, emphasis has more recently expanded to include interactions with natural enemies, which occupy the third trophic level (Lawton and McNeill, 1979; Price et al., 1980; Schultz, 1983). Natural enemies consuming hosts which in turn are feeding on plants being defended by allelochemicals can be affected in a variety of ways. These compounds can increase a herbivore's susceptibility to natural enemies by prolonging development time (Feeny, 1976; Blau et al., 1978; Lincoln et al., 1982), increasing consumption (Aizawa, 1963; Doane and McManus, 1981), or increasing the duration of such noncryptic activities as feeding and intra- and interplant movement (Erikson and Feeny, 1974; Schultz, 1983). However, adverse effects of allelochemicals on natural enemies have also been demonstrated (Eisner, 1970; Campbell and Duffy, 1979; Price et al., 1980; Barbosa and Saunders, 1985).

Nicotine, an alkaloid found in tobacco (*Nicotiana tabacum* L.), is an allelochemical which has been shown to be highly toxic to insects (Self et al., 1964; Yang and Guthrie, 1969; Parr and Thurston, 1972). Thurston and Fox (1972) reported reduced emergence of larval *Cotesia congregata* (=*Apanteles congregatus*) (Say) from their *Manduca sexta* (L.) hosts after adding nicotine to the host's synthetic diet. Campbell and Duffey (1979, 1981) demonstrated that tomatine [a major alkaloid of tomato (*Lycopersicon esculentum* Mill.)] can cause prolonged development, reduced pupal eclosion, smaller size, and shortened adult longevity in *Hyposoter exiguae* (Viereck) when incorporated into its host's synthetic diet. Laboratory studies by Barbosa et al. (1986) showed that *C. congregata* developing in *M. sexta* reared on artificial diet containing 0.1% nicotine suffered higher larval mortality than those reared on hosts fed diet without nicotine. The nicotine treatment significantly reduced the number of *C. congregata* larvae that emerged from the host and those that did emerge spun fewer cocoons.

The effects of nicotine on *C. congregata* have never been experimentally tested in the field. Morgan (1910) and Gilmore (1938) observed that *M. sexta* parasitism appeared to occur at much lower levels on tobacco than on other nearby solanaceous plants and attributed this difference to the toxic effects of nicotine. Thurston and Fox (1972) sampled *M. sexta* larvae from field plots of dark and burley tobacco varieties (presumed to have high and low levels of nicotine, respectively), but found no difference between the varieties in percent emergence of *C. congregata* larvae. The objectives of this study were to test the effects of a high and a low nicotine variety on the survival of *M. sexta*, rates

of parasitism by an endemic population of *C. congregata*, and to evaluate the effects of the different host diets on parasitoid survival and development.

METHODS AND MATERIALS

A 0.14-hectare field of tobacco at the University of Maryland Tobacco Experiment Station, Upper Marlboro, Maryland, was divided into 25 blocks of 40 plants each. Row and interplant spacing were 1 and 0.6 m, respectively. Each block was subdivided into two treatment plots, each containing 20 plants of either NC-95, a flue-cured tobacco variety widely used in North Carolina which has an alkaloid level of 2.65% on a cured-leaf basis (Chaplin and Weeks, 1976), or LAFC-95, an experimental line created by back-crossing a low alkaloid line to NC-95 (Chaplin, 1975). This variety has an alkaloid level of 0.18%. Each block was separated from adjacent blocks by two rows of MD-201, a typical commercial variety used in Maryland. All treatment plants were started from seed in a greenhouse and then transplanted by hand on June 27, 1983. Metalaxyl and pendimethalin were incorporated into the soil prior to planting for disease and weed control, but no additional pesticides were used. Subsequent weed control was by mechanical cultivation.

The *M. sexta* used in this study were obtained as eggs from stock cultures (North Carolina State University). Larvae were transferred upon hatching to 2-liter plastic crispers containing strips of Bioserv® hornworm diet. When the treatment tobacco plants were ca. 50 cm tall, a population of *M. sexta* was established by transferring 1- to 3-day-old laboratory-reared larvae from diet to tobacco leaves at a mean density of one larva per treatment plant. The actual dispersion of the larvae was determined by sampling from the negative binomial distribution with $k = 4$ (Southwood, 1978). Borth and Harrison (1984) found that this distribution approximates that of endemic *M. sexta* populations in Maryland tobacco fields. Interplant migration does not occur at these densities (McFadden, 1968). Six hundred larvae were introduced on July 25 (cohort 1) and 400 on August 9 (cohort 2). All individuals were subsequently counted twice weekly. Missing larvae were assumed lost to predation (Lawson, 1959). Since the development of individual larvae was carefully monitored, any *M. sexta* found to be at an inappropriate growth stage were assumed to have originated from wild populations and were removed.

Rates of *C. congregata* parasitism over time were determined by sampling three or four larvae from each treatment in each of five blocks (cohort 1) or three blocks (cohort 2) twice weekly. (The actual number of larvae sampled is shown in Table 2). Sampled larvae were subsequently reared for two weeks on excised tobacco foliage and then dissected to determine parasitism. On August 10 and 25, all remaining larvae from cohorts 1 and 2, respectively, were collected and reared individually in the laboratory in 177-ml plastic cups on ex-

cised leaves of the same tobacco variety from which they were collected. All larvae were examined daily for parasitoid emergence. The number of *C. congregata* larvae that emerged and spun cocoons was recorded for each host. Three days after parasitoid emergence, all *M. sexta* larvae were frozen and later dissected to determine the number of *C. congregata* larvae which failed to emerge. All adult *C. congregata* were counted, sexed, and oven-dried at 60°C to a constant weight. *C. congregata* adult longevity and pupal development time were determined from samples of 10 cocoons from one host out of each treatment from each of 10 blocks (cohort 2 only). Each cocoon was isolated in a 27-ml plastic cup with a streak of honey and placed in a growth chamber under a regime of constant light and 27 ± 2°C. All cocoons were checked daily for adult emergence and death.

Unless otherwise indicated, all data were analyzed by analysis of variance, using the mean square error for the block × treatment interaction to test for differences between the two tobacco varieties. All data expressed as percentages were arcsine $\sqrt{\%}$ transformed prior to analysis.

Leaf samples were prepared for nicotine quantitation by cutting two veinless 0.5-g sections from the centers of randomly sampled leaves. One of these sections was dried at 50°C for 24 hr to determine percent moisture. The other was ground in 10 ml of 40% (v/v) methanol containing 0.1% (v/v) 1 N HCl with a Ten Broeck homogenizer. The homogenate was then centrifuged at 11,000 *g* for 15 min, and the supernatant filtered through a 0.45-μm Millipore filter prior to injection. The samples were processed and nicotine quantified using high-performance liquid chromatography. A description of the equipment and procedures used is given by Saunders and Blume (1981).

RESULTS

Table 1 shows the concentrations of nicotine found in samples of leaf tissue from each of the tobacco varieties on three dates. There was at least a 10-fold difference in nicotine concentration between the varieties on each sampling date. Chaplin and Weeks (1976) found a similar difference in alkaloid level between these two varieties.

The cumulative mortality of the *M. sexta* larvae placed in the field on the two varieties was not significantly different ($P = 0.66$; *t* test). The cumulative average number of larvae missing out of the 20 larvae initially established per plot increased linearly from 1.8 per plot on the third day after introduction to 9.3 per plot on the 14th day after introduction.

Rates of *C. congregata* parasitism (Table 2) were higher for the first cohort of *M. sexta* (July 25–August 8) than for the second (August 9–August 24), (60–100% and 18.2–88.9%, respectively). Rates of parasitism did not differ signif-

TABLE 1. SEASONAL VARIATION IN LEAF NICOTINE CONCENTRATION IN TWO VARIETIES OF TOBACCO GROWN IN MARYLAND, 1983[a]

Variety	Sample date		
	Aug. 8	Aug. 25	Oct. 5
NC-95 (high nicotine)	17.8 ± 2.5	50.2 ± 7.6	35.5 ± 4.6
LAFC-53 (low nicotine)	0.8 ± 0.4	1.3 ± 0.5	3.6 ± 0.3

[a] $\bar{X}$ ± SE mg nicotine/g dry weight. $N = 3$ for all samples.

TABLE 2. TEMPORAL DISTRIBUTION OF PARASITISM OF *M. sexta* LARVAE BY *C. congregata* ON TWO TOBACCO VARIABLES

Cohort[a]	Tobacco variety	Parasitism (%) on day[b]			
		4	7	11	14
1	NC-95	60(15)	95(20)	100(18)	100(16)
	LAFC-53	73.3(15)	94.7(19)	100(19)	100(19)
2	NC-95	18.2(11)	54.5(11)	83.3(12)	80(5)
	LAFC-53	30(10)	83.3(12)	88.9(9)	80(5)

[a] Cohorts 1 and 2 were introduced on July 25 and Aug. 9, respectively.
[b] Number of days since larvae were introduced into the field. Values are % parasitism of (N) *M. sexta* larvae averaged across five (cohort 1) or three (cohort 2) blocks.

icantly between varieties ($P = 0.16$) (date and cohort effects were not partitioned or tested). Sixty-seven percent of the larvae in cohort 1 were parasitized by the fourth day after exposure in the field, and by the 11th day, 100% of the larvae were parasitized. The apparent drop in the percentage of larvae parasitized in cohort 2 from day 11 to 14 may be a sampling artifact, or it may indicate selective elimination of parasitized individuals from the population.

The effect of high and low nicotine varieties on *C. congregata* survival and development is shown in Tables 3 and 4. While the total number of *C. congregata* larvae which developed in *M. sexta* feeding on each of the two varieties was not significantly different, significantly more male and female *C. congregata* adults were produced from hosts feeding on the low nicotine variety (Table 3). This was because a significantly greater proportion of larvae failed to emerge from hosts on high nicotine tobacco, and a significantly higher proportion of those that did emerge died prior to spinning cocoons. A significantly greater proportion of the *C. congregata* which developed in hosts which fed on

TABLE 3. SURVIVAL AND DEVELOPMENT OF *Cotesia congregata* PARASITIZING *Manduca sexta* LARVAE REARED ON HIGH AND LOW NICOTINE TOBACCO VARIETIES[a]

Tobacco variety	Total No. larvae produced	Larvae[b] failing to emerge (%)	Larvae[b] failing to form cocoons (%)	Pupal[b] mortality (%)	No. adults emerging	
					Male	Female
NC-95 (high nicotine)	139.8	55.3	5.8	1.4	17.3	20.2
LAFC-53 (low nicotine)	125.8	34.6	2.6	3.3	29.5	35.0
$P =$	0.27	0.02	0.03	0.02	0.02	0.0007

[a] All values are least-squares estimates of means of 37 (NC-95) and 41 (LAFC-53) *M. sexta* larvae.
[b] All percentages were transformed to arcsin $\sqrt{\%}$ prior to analysis. The values reported are the back-transformed mean percentages.

TABLE 4. PUPAL DEVELOPMENT TIME, ADULT LONGEVITY, AND DRY WEIGHT OF *Cotesia congregata* PARASITIZING *Manduca sexta* LARVAE REARED ON HIGH AND LOW NICOTINE TOBACCO VARIETIES

Tobacco variety	Pupal[a,b] develop. time (No. of indiv.)		Longevity[a] (No. of indiv.)		Dry weight (mg)/indiv.[c] (No. of indiv.)	
	Male	Female	Male	Female	Male	Female
NC-95	5.76	5.64	13.94	14.50	0.22	0.27
(high nicotine)	(41)	(44)	(49)	(44)	(34)	(36)
LAFC-53	5.54	5.30	13.03	13.78	0.20	0.27
(low nicotine)	(35)	(54)	(35)	(54)	(41)	(38)
$P =$	0.14	0.002	0.28	0.48	0.48	0.76

[a] Values are least-squares estimates of means of (*N*) *C. congregata*.
[b] Time between cocoon formation and adult eclosion in days.
[c] Values are least-squares estimates of means of mean *C. congregata* dry weight/*M. sexta* individual. No. of *M. sexta* larvae examined is given in parentheses.

the low nicotine tobacco died as pupae, but this source of mortality was low (<4%) in both treatments. Since the exact time of parasitization was not known, larval development time could not be measured. However, pupal development time was significantly greater for females under the high nicotine treatment (Table 4). There were no significant differences between treatments in longevity or dry weight of *C. congregata* adults.

DISCUSSION

Wilson and Huffaker (1976) state that plant resistance and biological control should form the core of integrated pest management programs. Campbell and Duffey (1979) and Barbosa et al. (1982) suggest that these tactics may be incompatible or even antagonistic if the mechanism of host plant resistance interferes with the biology of natural enemies. Some incompatibility appears to have occurred in this study since two tobacco varieties which differed in their levels of the allelochemical nicotine were found to affect the survival of *C. congregata* differentially.

The two tobacco varieties in this study differed significantly not only in their alkaloid level, but also in their levels of reducing sugars and total nitrogen (Chaplin and Weeks, 1976). Some of the difference in total nitrogen between the two varieties may be due to the difference in the levels of nicotine, which contains nitrogen; however, the reduction in the alkaloid fraction of the low nicotine variety cannot account for all of the reduction in total nitrogen (Chaplin and Weeks, 1976). While it is possible that some portion of the detrimental effects of the high nicotine tobacco variety on *C. congregata* were attributable to nutritional factors rather than toxicity, total nitrogen, which is considered to be a good index of nutritional quality (Mattson, 1980), is higher in the high than in the low nicotine variety, suggesting that the high nicotine variety is nutritionally superior. The close agreement between the results of this study and those of Barbosa et al. (1986), in which nutritional quality was controlled by using artificial diet, further supports the role of nicotine as the factor responsible for the reduction in *C. congregata* survivorship on hosts which fed on the high nicotine tobacco.

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LASER MICROBEAM STUDIES OF ROLE OF AMPHID RECEPTORS IN CHEMOSENSORY BEHAVIOR OF NEMATODE *Caenorhabditis elegans*

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Abstract—Amphid sensilla, historically considered the primary chemosensory structures of nematodes, were found to be necessary for the detection of only one of the six chemical stimuli that were tested. Only the attraction to cAMP was eliminated by damaging the two lateral lips, which bear the amphid sensilla. The inner labial sensilla, one of which occurs on each of the six lips, are probably the primary receptor structures for the other chemical stimuli. Damaging all six lips, which should destroy all anterior chemosensory input, not only eliminted the attraction to sodium and chloride ions, but reversed the nematodes' response to them. Nematodes with all six lips destroyed showed reversal behavior when exposed to these attractants. Nematodes with damage to all six lips appeared to recover much of their normal chemosensory function within 24 hr after treatment.

Key Words—*Caenorhabditis elegans*, nematode, chemotaxis, chemoreception, amphid, laser microbeam.

INTRODUCTION

For many years, the amphids have been thought to be the major sense organs for reception of chemical stimuli by nematodes (Steiner, 1925). Evidence for this reasonable supposition is their location near the anterior end and their structure, which consists of nerve cell endings within a cavity that opens to the exterior (Coomans, 1979; Wright, 1980). Until recently, there was no experi-

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mental evidence to support this presumed function. We have approached the question of amphid chemosensory function by using a laser microbeam to destroy the amphids in *Caenorhabditis elegans* and measuring their response to a variety of known stimulus chemicals.

Laser microbeams have previously been used to study the function of sensory structures (Samoiloff et al., 1973; Chalfie and Sulston, 1981). Since treatment with the laser microbeam requires the handling of individual specimens, a behavioral assay that works well with individual nematodes is desirable. The tail-tether technique (Dusenbery, 1980) appears to be the best available in this regard. In this technique, a nematode is held by the tail with a suction pipet, while a stream of water carrying chemical stimuli flows past. Its behavior is recorded by projecting its shadow on an array of photodetectors connected to a polygraph. Reversal bouts and other responses to adverse stimulation are readily determined. The distribution of these activities during repeated cycles of stimulation is used to determine the response score.

Detailed studies of the anterior end of *C. elegans* have been made by serial-section electron microscopy (Ward et al.,1975; Ware et al., 1975). It was demonstrated that the only anterior sensilla that open to the exterior are the amphids and the inner labial sensilla. The only other sensilla that are known to open to the exterior are the pair of phasmids located on the tail (Sulston et al., 1980). The amphids are relatively complex sensilla. Each amphid contains the endings of 12 neurons, eight of which appear to be exposed to the outside environment. Each inner labial sensillum contains the endings of two neurons, one of which is exposed to the exterior. Each phasmid contains the endings of two neurons, both of which are exposed to the exterior. The sensilla of other nematodes are basically similar, except that the number of neurons in each type of sensillum varies (Coomans, 1979; Wright, 1980).

The six inner labial sensilla are distributed radially around the mouth. One inner labial sensillum is associated with each of the six lips. Additionally, an amphid sensillum is located on each of the two lateral lips. Since each lip is only about 1 μm across, it is not practical to destroy a single type of sensillum on the lateral lips. However, one can exploit the fact that destruction of the lateral lips should inactivate both amphids but only one third of the inner labial sensilla. This pattern of damage allows the detection of chemosensory responses that are mediated by the amphid receptors.

METHODS AND MATERIALS

Unless otherwise specified, young adult nematodes were taken from standard cultures (Brenner, 1974). For microbeam treatment of the lateral lips, the nematodes were anesthetized with either 0.1% tricaine and 0.01% tetramisole (Kimble et al., 1982) in N buffer (100 mM NaCl, 25 mM potassium phosphate,

pH 6.0) or with sodium azide (Nelson, personal communication) at a final concentration of 100 μg/ml in N buffer. Anesthetized nematodes were transferred to an agar pad (Sulston et al., 1980) and oriented with their lateral lips in the same focal plane by placing a length of 6-O surgical silk across the nematode prior to addition of the cover glass. For treating all six lips, the nematodes were anesthetized in either tricaine/tetramisole or cold N buffer saturated with CO_2, or with sodium azide as described above. The tricaine/tetramisole-treated nematodes were allowed to recover from anesthesia overnight, whereas the CO_2- and sodium azide-treated nematodes recovered within 30–90 min. Unfortunately, the CO_2 treatment did not immobilize the nematodes sufficiently to use the orientation technique for treating the lateral lips.

The laser microbeam system was patterned after that of J.G. White (Sulston and White, 1980) and employed a Candella, model SLL-66 flashlamp-pumped dye laser focused through a Zeiss Universial microscope equipped with differential interference contrast optics. Using coumarin-2 dye, the microbeam system focuses on the order of 0.1 megawatt of 450 nm photons through an area about 1 μm in diameter for about 0.1 μsec. This extreme intensity leads to nonlinear, multiphoton processes, and no absorbing pigment is required. The laser intensity was sufficient to produce visible damage to target tissues as evidenced by coagulation which was visible with the differential interference contrast system. To ensure complete destruction of dendritic endings, target lips were subjected to multiple hits until the entire interior lip tissue was visibly coagulated. From those specimens which showed no alteration in chemotaxis behavior as a result of the laser treatment, representatives were heat killed and reexamined by differential interference contrast microscopy to confirm the extent of lip damage.

After recovery from anesthesia, treated nematodes were assayed for altered responses to sodium, chloride, carbon dioxide, D-tryptophan, high osmolarity, and cAMP. Responses were quantitated by the tethered worm technique (Dusenbery, 1980) which gives a graphic recording of the nematode's movement patterns. This assay technique allows for repeated, cyclic exposure of each nematode to alternate stimulus and control solutions. Both experimental and control solutions were prepared using one of three standard buffers. MOPS (morpholinopropane sulfonic acid) buffer contained 10 mM MOPS, 5 mM KCl, 1 mM $CaCl_2$, and 1 mM $MgSO_4$ adjusted to pH 7.0. MES [2(*N*-morpholino)ethane sulfonic acid] buffer was the same as the MOPS buffer with MES substituted for the MOPS and adjusted to pH 6.0. Finally, a phosphate buffer contained 5 mM Na_2HPO_4, 5 mM KH_2PO_4, and 50 mM Na_2SO_4 adjusted to pH 7.0.

For each assay, the test and control solutions were prepared as shown in Table 1. Nematodes were alternately exposed for 1-min intervals to test and control solutions. Both experimental nematodes and anesthetized controls were

TABLE 1. COMPOSITION OF BEHAVIORAL ASSAY SOLUTIONS

Assay	Test solution	Control solution
Na^+	100 mM NaCl in MOPS buffer	50 mM $CaCl_2$ in MOPS buffer
Cl^-	100 mM NaCl in MOPS buffer	50 mM Na_2SO_4 in MOPS buffer
D-Tryptophan	1 mM D-tryptophan and 100 mM NaCl in MOPS buffer	100 mM NaCl in MOPS buffer
Osmotic	200 mM NaCl in MOPS buffer	100 mM NaCl in MOPS buffer
CO_2	1 mM $NaHCO_3$ and 100 mM NaCl in MES buffer	100 mM NaCl in MES buffer
cAMP[a]	2 mM cAMP in phosphate buffer	Phosphate buffer alone

[a] Since Ward (1973) found that Cl^- inhibits the response to cAMP, no chloride was included in the test or control solutions.

observed for at least 10 cycles of stimulation. Controls were generally anesthetized with the experimentals and allowed to recover without microbeam exposure. However, controls for the sodium assay had one subdorsal and one subventral lip targeted in order to verify that the observed responses were not simply a result of the microbeam procedure.

For quantitation of each cycle, the solution in which the greater reversal activity occurred was determined. Each cycle of test and control solutions was designated as positive, negative, or even. A positive cycle was one in which reversal behavior was greater in the control solution. If reversal activity was greater in the stimulus solution, the cycle was scored as negative. Response values (R) were calculated by the formula:

$$R = \frac{N_+ - N_-}{N_+ + N_- + N_0} \times 100$$

where N_+ = positive cycles, N_- = negative cycles, and N_0 = even cycles in which reversal activity was either absent or equal in both solutions. This procedure produces a scale on which +100 corresponds to complete attraction, −100 to complete avoidance, and zero corresponds to no response. Mean response (R) values and standard deviations were computed for each experimental and control group. Rank-sum statistical analyses were performed on the mean R values of different experimental and control groups. In any comparison, if the probability that the two means were from the same population was less than 5% ($P < 0.05$), the two means were considered to be significantly different.

In experiments to evaluate the nematode's capacity to recover from laser damage, both young (3–4 days old) and old (6–7 days old) nematodes were

assayed twice for their responses to sodium. The first assay was performed immediately (2 hr) after recovery from anesthesia. Nematodes were then removed from the tail-tether apparatus and held overnight on separate, fresh bacterial plates. After 24 hr the same nematodes were assayed again for response to sodium to detect any recovery of chemosensory function.

RESULTS

If the amphids were the only receptors for sodium ions, damaging the lateral lips should eliminate the response to sodium. However, as seen in Table 2, nematodes treated in this way responded nearly 80% as strongly as untreated controls. Although lateral lip damage significantly decreased the response from that of untreated controls (Table 2 and Table 3, column L-0), the results of this procedure did not differ significantly from those obtained by destruction of a subdorsal/subventral lip pair (Table 2 and Table 3, column L-SD/SV). Bilateral lip damage eliminates all amphid-mediated sensory input while leaving four of the six inner labial sensilla unaltered and presumably functional. Thus, the data indicate that the inner labial sensilla, rather than the amphids, are of primary importance for the detection of sodium.

In order to test the involvement of inner labial sensilla, all six lips were targeted, and nematodes were tested for response to sodium and chloride. Interestingly, destruction of all six lips did not eliminate the responses as expected. Instead, this treatment reversed the responses so that avoidance behavior was greater in the sodium or chloride solutions (Table 2). Nematodes that were anesthetized with tricaine/tetramisole did not show the characteristic re-

TABLE 2. RESPONSES TO Na^+ AND Cl^-[a]

		Lips targeted			
		Twa			
Stimulus	Anesthetic	Lateral	SD/SV	Six	None
Na^+	Na azide	78 ± 18(6)	93 ± 11(6)	−70 ± 19(5)	100 ± 0(5)
Na^+	T/T[b]	82 ± 5(5)	89 ± 16(5)	66 ± 29(8)	99 ± 4(8)
Na^+	CO_2			−76 ± 17(5)	94 ± 5(7)
Cl^-	CO_2			−60 ± 35(5)	88 ± 16(6)

[a] Tabulated above is the mean response (*R* value) ± standard deviation and (*N*) for the following groups: lateral lips ablated (Lateral); one subdorsal and one subventral ablated (SD/SV); all six lips ablated (Six); and anesthetic controls with no lips targeted (None).

[b] With this anesthetic, a 24-hr delay occurred between microbeaming and assay to allow for recovery from anesthesia.

TABLE 3. STATISTICAL ANALYSIS

Stimulus	Anesthetic	*P* value of groups compared				
		L-SD/SV	L-6	L-0	6-0	SD/SV-0
Na^+	Na azide	0.066	0.002	0.015	0.002	0.094
Na^+	T/T	0.050	0.724	0.030	0.020	0.434
Na^+	CO_2				0.001	
Cl^-	CO_2				0.002	

[a] Rank-sum statistical tests were performed on data from the groups reported in Table 2. The probability that the groups compared were from the same population is tabulated above. Worms with lateral lip ablation (L) were compared with worms having all six lips ablated (6) and with control groups in which either two lips other than the laterals were ablated (SD/SV) or in which the worms were anesthetized and not ablated (0).

versal of response to sodium when all six lips were damaged. However, with this anesthetic, 24 hr were required for recovery. During this extended time, functional recovery from the laser damage is a possibility. Such recovery could return their responses toward normal by the time of the assay.

In order to test the possibility of recovery from laser damage, a separate battery of experiments was conducted in which nematodes were anesthetized with sodium azide. Since recovery from azide anesthesia was rapid, nematodes could be assayed for sodium detection within 2 hr of treatment. Each individual nematode could then be allowed to recover overnight and could be assayed a second time after 24 hr. Table 4 shows the results of these experiments. Young nematodes (3–4 days old) showed statistically significant recovery of chemosensory function over 24 hr. Interestingly, older nematodes (6–7 days old) showed no significant recovery over the same time period. Statistically, even the younger nematodes did not recover sufficiently to equal the ability of control nematodes in detecting sodium.

TABLE 4. AGE DEPENDENCE OF 6-LIP RECOVERY

Treatment group	Age (days)	2 hr postop[a]	24 hr postop[a]	Significance[b] (2 hr vs. 24 hr)
6-lip	3–4	$-70 \pm 19(5)$	$60 \pm 32(5)$	$P = 0.002$
control	3–4	$100 \pm 0(5)$	$100 \pm 0(5)$	
6-lip	6–7	$2 \pm 16(5)$	$26 \pm 40(5)$	$P = 0.09$
control	6–7	$98 \pm 5(5)$	$96 \pm 9(5)$	

[a] Mean (R) value $\pm$ SD(N).
[b] Experimental groups of both ages at 2 and 24 hr after ablation were significantly different from control ($P = < 0.01$).

TABLE 5. RESPONSES OF LATERAL LIP-ABLATED WORMS TO KNOWN CHEMICAL STIMULI[a]

Stimulus	Lips targeted		Statistical significance (P)	
	Lateral lips (experimental)	No lips (control)	Exp. < control	Exp. vs. no stimulus
None		0 ± 7(5)		
CO_2	−82 ± 17(9)	−92 ± 14(8)	>0.05	0.01
D-Tryptophan	−67 ± 11(6)	−81 ± 14(5)	>0.05	0.04
Osmotic	−76 ± 25(8)	−81 ± 10(6)	>0.05	0.02
cAMP	26 ± 37(8)	74 ± 13(8)	0.003	>0.05

[a] Mean response (R) value ± SD(N).

Since the experiments suggested that the inner labial sensilla, and not the amphids, were responsible for sodium detection, other known stimuli were tested to determine the role of amphid sensilla in their detection. The responses of young, lateral lip treated nematodes to CO_2, D-tryptophan, high osmolarity, and cAMP are shown in Table 5. Of the various stimuli tested, only the response to cAMP was impaired by damaging the lateral lips. The response of amphid-damaged nematodes to cAMP was significantly weaker than that of control nematodes and was similar to the behavior of nematodes receiving no stimulation.

DISCUSSION

The present findings indicate that the amphids are not essential for the response to most stimuli tested, but they may still play a minor role. The various experiments involving the response to Na^+ suggest that they might. Damage to the lateral lips had a greater impact on the response than damage to two of the other lips (Table 2), although, the difference is of marginal significance (Table 3). It may be that all anterior chemosensory sensilla participate to some extent in sodium detection. Thus, damage to the lateral lips would affect both amphids as well as two inner labial sensilla, while damage to any other pair would affect only two inner labials.

Since *C. elegans* responds to sodium as strongly and reproducibly as to any known stimulus, this finding is not a simple case of eliminating a weak or minor response. The only other experiments bearing on this surprising result are some previous genetic dissection studies on *C. elegans*. Lewis and Hodgkin (1977) described a strain of *C. elegans* that was defective in attraction to Na^+ and Cl^- and had anatomical defects in the inner labial sensilla but not the amphids. This result is entirely consistent with our microbeam studies. More recently, Albert et al. (1981) have described two strains as defective in attraction

to Na^+ and Cl^- but having anatomical defects in the amphids and not in the inner labial sensilla. This apparent contradiction can be explained by assuming that these strains have defects in the inner labial sensilla that were not observed by electron microscopy. However, when taken together, the studies of mutant strains are contradictory. The microbeam experiments described here are more convincing, and we conclude that several chemosensory responses of *C. elegans* are not dependent upon the amphids.

The reversal of response to soduim and chloride that occurred with young nematodes in which all six lips were damaged and tested within a few hours is difficult to explain. This phenomenon is further complicated by the fact that it rarely occurs with older nematodes. The reversed response could result from a mechanism by which the nematode normally compares stimulation at the head and tail and responds to the difference between them. Sensilla resembling simple amphids, the phasmids, are known to be located in the tail of most nematodes (Wright, 1980), including *C. elegans* (Sulston et al., 1980). Damage to all six lips eliminates chemosensory input from the anterior end of the nematode. If the nematodes compare anterior and posterior chemosensory input, destruction of the anterior sensilla could shift the balance of sensory input toward the tail receptors and lead to a reversed response.

The tail-tether apparatus is designed so that the tail of the nematode being tested is held in a pipet by vacuum and is probably not exposed to stimulus solutions. Younger nematodes are smaller in diameter and fit more loosely in the pipet. It is possible that stimulus solutions can "leak" around the younger nematodes and come into contact with tail receptors. Although highly speculative, this might explain the reversal of response to sodium that occurs in young nematodes. Further experiments will be necessary to answer this question.

Anatomy of the amphid sensilla has historically resulted in their being assigned the function of primary chemosensory structures of nematodes. Yet, of the six chemical stimuli tested, only cAMP detection appears to be significantly impaired by lateral lip damage. Possibly, the role of the amphid as a chemoreceptor has been overestimated. However, it is more likely that the stimuli that are normally detected by the amphids have not yet been tested or even discovered.

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EFFECTS OF CUTICULAR DUVANE DITERPENES FROM GREEN TOBACCO LEAVES ON TOBACCO BUDWORM (LEPIDOPTERA: NOCTUIDAE) OVIPOSITION[1]

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Abstract—Five cuticular chemical components isolated from the green leaves of tobacco introductions (TIs) and a commercial tobacco cultivar were tested for their effects on tobacco budworm, *Heliothis virescens* (F), oviposition in cage bioassays, and field experiments. These chemicals were sprayed onto budworm-resistant TI 1112 tobacco which produces low levels of most cuticular components. Individual duvane diterpenes (α- and β-4,8,13-duvatrien-1-ols and α- and β-4,8,13-duvatriene-1,3-diols) increased tobacco budworm egg laying on sprayed TI 1112 plants. *cis*-Abienol, docosanol, and docosanyl myristate were inactive.

Key Words—Tobacco budworms, *Heliothis virescens* (F), Lepidoptera, Noctuidae, tobacco, *Nicotiana tabacum* L., diterpenes, duvanes, host plant resistance, oviposition.

INTRODUCTION

The leaves of commercially grown tobaccos, *Nicotiana tabacum* L., are covered by sticky exudates that are excreted onto the leaf surface from glanded

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trichomes (Michie and Reid, 1968; Severson et al., 1984). The major cuticular components consist primarily of diterpenes (labdanes and duvanes), hydrocarbons, and sucrose esters (Enzell et al., 1977, Severson et al., 1984). *N. tabacum* varieties and tobacco introduction (TIs) produce primarily labdanes (some oriental and cigar tobaccos), primarily duvanes (many flue-cured and burley tobaccos), or a combination of both types of diterpenes (Reid, 1979, 1980; Sato et al., 1982; Severson et al., 1984). Tobaccos with simple (nonglanded) trichomes, such as TI 1112, produce only trace amounts of diterpenes and sucrose esters, and these tobaccos are resistant in the field to green peach aphids, *Myzus persicae* (Sulzer) (Johnson and Severson, 1982), and tobacco budworms, *Heliothis virescens* (F) (Elsey and Chaplin, 1978; Severson et al., 1983; Johnson and Severson, 1984), and tobacco hornworms, *Manduca sexta* (L.) (Chaplin et al., 1976, Johnson, 1978).

In controlled cage studies with tobacco budworm moths, Jackson et al. (1983) demonstrated that one mechanism of host plant resistance in TI 1112 is ovipositional nonpreference. The whole leaf wash (WLW) extracted from the budworm-susceptible commercial flue-cured variety, NC 2326, stimulated tobacco budworm oviposition when sprayed onto TI 1112 potted plants in cage and field experiments (Jackson et al., 1984). NC 2326 WLW was fractionated into a methanol–water-soluble (MWS) fraction, containing predominantly α- and β-4,8,13-duvatriene-1,3-diols (α- and β-diols), and a hexane-soluble (HS) fraction consisting of hydrocarbons, fatty alcohols, and wax esters. Only the MWS fraction stimulated tobacco budworm oviposition when sprayed onto TI 1112 plants (Jackson et al., 1984).

Other tobaccos, TI 1223 and TI 1341, with surface chemical compositions different from NC 2326, are also susceptible to tobacco budworm damage in the field (Johnson and Severson, 1984). TI 1223 produces the duvane diterpenes, α- and β-4,8,13-duvatrien-1-ols (α- and β-ols) and the labdane diterpene, *cis*-abienol. A mixture of duvanes, α- and β-ols and α- and β-diols, is present in the cuticular extractions of TI 1341 (Severson et al., 1984). In controlled cage studies, high levels of budworm ovipositional activity are observed on both TI 1223 and TI 1341 (unpublished data).

The research described herein was done to determine if duvane and labdane diterpenes stimulate tobacco budworm ovipositon when sprayed onto the budworm-resistant TI 1112. Docosanol and docosanyl myristate were also tested. Data from cage and field tests will be reported.

METHODS AND MATERIALS

The solvents used for plant extraction, component isolation, and spray application were Burdick and Jackson distilled-in-glass grade and were used as received. Docosanol [99+% by glass capillary gas chromatography (GC-2)]

and myristic acid (99+% by GC-2) were obtained from Sigma Chemical Company (St. Louis, Missouri).

Whole leaf cuticular components were extracted from tobacco grown under flue-cured conditions at Clemson University Pee Dee Research and Education Center, Florence, South Carolina; the Tobacco Research Station, Oxford, North Carolina; and the University of Georgia Coastal Plain Experiment Station, Tifton, Georgia, as described by Jackson et al. (1984). the NC 2326 WLW and MWS fractions were prepared from materials obtained in late May and early June at Tifton for that test year. The other diterpene components and mixtures were isolated from WLW extracts from various tobaccos obtained at all locations the previous crop year. All components and fractions were analyzed by GC-2 as described by Severson et al. (1984).

α- and β-Diol Mixture. NC 2326 MWS (2.5–3.0 g in $CHCl_3$) was placed into a 10-ml injection loop and was pumped onto a 2.5 × 58-cm liquid chromatography column (Laboratory Data Control) containing a 44-cm bed of Sephadex LH-20. Chloroform was pumped through the system at 2 ml/min, and 5-ml gel fractions (GF) were collected. After the collection of GF 45, the solvent was changed to 10% MeOH in $CHCl_3$ and pumped through the system for 3 hr. The column was reconditioned overnight using a 0.5 ml/min flow of $CHCl_3$. The fractions rich in diols (GF 35–42) were combined, and the solvent was removed from the sample on a rotary evaporator at 40°C under reduced pressure (100–150 mm Hg). The residue was placed in a desiccator under vacuum (~1 mm Hg) for 4 hr to yield 1.8–2.0 g of α- and β-diol mixture (71% α, 23% β by GC-2). The major impurities were oxidized diols (oxydihydroxy and trihydroxy duvanes).

α- and β-Ols. About 3 g of TI 1341 WLW in 10 ml of $CHCl_3$ was placed onto a Sephadex LH-20 column and eluted as above. Gel fractions rich in α- and β-ols (GF 25–32) were combined and the solvent removed. The residue was dissolved in hexane and placed on a 50-g basic alumina column (activity grade 1, slurry packed in hexane) and eluted with 0.5 liters of 1:3 CH_2Cl_2–hexane. The α- and β-ols were then eluted with 1.5 liters of 1:1 CH_2Cl_2–hexane. Solvent was removed as described above to yield about 650 mg of a colorless oil (98% by CG-2, α- to β-ol ratio 9:1).

α-Diol, β-Diol, cis*-Abienol, and Docosanyl Myristate.* α-Diol (mp 65–66°C, 99+% by GC-2) (mp 65–66°C, Roberts and Rowland, 1962), β-diol (mp 122–123°C, 99+% by GC-2) (mp 126°C, Roberts and Rowland, 1962), and *cis*-abienol, as a monohydrate (mp 63–65°C, with softening, 99+% by GC-2) (mp 65%C, with prior softening, Gray and Mills, 1964) were isolated as described by Severson et al. (1982). Docosanyl myristate was prepared from docosanol and myristic acid (mp 55.5–56°C; *m/e* 536, $C_{36}H_{52}O_2$; 98+% by GC-2).

Formulation of Spray Materials. A three-year average yield of about 55 mg of α- plus β-diols (α- to β-diol ratio approx 3:1) per plant was obtained

from 6-week-old NC 2326 plants. Therefore, the materials for spray applications were formulated at the following one-plant equivalent levels: NC 2326 WLW, NC 2326 MWS, and α- plus β-diol mixture at 55 mg of α- plus β-diol (as determined by GC-2 analyses); α-diol, α- and β-ol mixture, and *cis*-abienol at 41.2 mg; and β-diol at 13.8 mg. Docosanol and docosanyl myristate were formulated at 2 mg/plant which was 10–20 times their average levels on NC 2326 in the field. The above were dissolved in hexane–CH_2Cl_2 (3:1) at a concentration of one plant equivalent per 0.5 ml. The solutions, including a solvent blank (SB) of hexane–CH_2Cl_2 (3:1), were divided into 2-ml lots into vials and stored at $-17.8°C$ in the dark until needed. The vials were brought to ambient temperature before spraying and quantitatively transferred with carrier solution (3:1 acetone–H_2O) to yield 40 ml of spray solution. One plant equivalent of material (10 ml of mixture) was sprayed onto each test plant.

Ovipositional Cage Bioassays. A screen cage bioassay for tobacco budworm oviposition on potted tobacco plants was used for tests during 1980–1983 at the Tobacco Research Station, Oxford, North Carolina. Ten 3-day-old female moths were released shortly before dark into each 2.4 × 2.4 × 2.0-m screened cage located outside. Four test plants were placed in the corner opposite four control plants. The morning following each test all eggs were counted. Details of plant production, insect rearing, cage design, egg monitoring procedure, and spray application of cuticular isolates were described by Jackson et al. (1983, 1984). Spray treatments are given in Table 1.

Before individual analyses of variance for each experiment, data were transformed to a percentage of the total eggs counted per replication. Preference for egg laying on a particular treatment versus the control was evaluated by a paired *t* test.

To determine the efficiency of the spraying procedure, cuticular leaf chemical samples were taken from sprayed and unsprayed tobaccos. One 2-diam. leaf plug was taken from each plant as soon as they dried after spraying (day 0) and one and two days later. These leaf plugs were dipped eight times into 10 ml of CH_2Cl_2 in 20-ml scintillation vials. These samples were frozen immediately, shipped on Dry Ice to Athens, Georgia, and stored at $-17.8°C$. The cuticular chemical samples were analyzed by GC-2 as described by Severson et al. (1984).

Field Experiments. The activities of the cuticular diterpenes from tobacco on budworm oviposition were tested in the field at Oxford, North Carolina, and Florence, South Carolina, during 1983. Five replications of six treatments of 6-week-old tobacco plants were arranged in a cross pattern in fields isolated from other tobaccos. The six treatments were: TI 1112 with SB; TI 1112 with NC 2326 MWS; TI 1112 with α-diol; TI 1112 with β-diol; TI 1112 with α + β-ols; and NC 2326 with SB. The centers of adjacent replications were 10 m apart. Within a replication, the treatments were positioned at the points of an

TABLE 1. OVIPOSITION OF TOBACCO BUDWORM MOTHS IN SCREEN CAGES ONTO POTTED TOBACCO PLANTS SPRAYED WITH CUTICULAR COMPONENTS EXTRACTED FROM GREEN TOBACCO LEAVES, OXFORD, NORTH CAROLINA, 1980–1983

Experiment number[a]	Entry A			Entry B			Percent of eggs on entry A
	Tobacco type	Spray treatment[b]	Amount applied (mg/plant)	Tobacco type	Spray treatment[b]	Amount applied (mg/plant)	
1	NC 2326	None		TI 1112	None		75.2[d]
2	TI 1112	NC 2326 WLW	55.0[c]	TI 1112	SB		76.5[d]
3	TI 1112	NC 2326 MWS	55.0[c]	TI 1112	SB		74.8[d]
4	TI 1112	α-Diol	41.2	TI 1112	SB		64.2[d]
5	TI 1112	β-Diol	13.8	TI 1112	SB		63.3[d]
6	TI 1112	α + β-Diols	55.0	TI 1112	SB		72.2[e]
7	TI 1112	α + β-ols	41.2	TI 1112	SB		65.6[d]
8	TI 1112	α-Diol	41.2	TI 1112	NC 2326 WLW	55.0[c]	55.5
9	TI 1112	β-Diol	13.8	TI 1112	NC 2326 WLW	55.0[c]	59.8
10	TI 1112	α + β-Diols	55.0	TI 1112	NC 2326 WLW	55.0[c]	64.9[e]
11	TI 1112	α + β-ols	41.2	TI 1112	NC 2326 WLW	55.0[e]	50.7
12	TI 1112	α-Diol	41.2	TI 1112	NC 2326 MWS	55.0[e]	50.4
13	TI 1112	β-Diol	13.8	TI 1112	NC 2326 MWS	55.0[c]	46.0
14	TI 1112	α + β-Diols	55.0	TI 1112	NC 2326 MWS	55.0[c]	48.7
15	TI 1112	docosanol	2.0	TI 1112	SB		50.5
16	TI 1112	*cis*-abienol	41.2	TI 1112	SB		45.2
17	TI 1112	docosanyl myristate	2.0	TI 1112	SB		45.2

[a] Experiment 1 had 109 replications over a four-year period, experiments 2–17 had 8–27 replications, which averaged 20.5–55.1 eggs per plant.
[b] WLW = whole leaf wash; MWS = methanol–water-soluble fraction of WLW; α-diol = α-4,8,13-duvatriene-1,3-diol; β-diol = β-4,8,13-duvatriene-1,3-diol; α + β-ol = combinations of α- and β-4,8,13-duvatrien-1-ol; SB = solvent blank of 0.5 ml hexane–methylene chloride (3:1) in 9.5 ml acetone–water (3:1)
[c] Amount of α- + β-diol in the mixture.
[d] Significantly different, paired *t* test ($P < 0.01$).
[e] Significantly different, paired *t* test ($P < 0.05$).

equilateral hexagon with the nearest plants of different treatments being ca. 2.5 m apart. Four plants per treatment per replication were arranged in a square with 0.5 m between plants. The Oxford and Florence tests had similar designs, except that 6-week-old potted plants (two weeks in greenhouse and four weeks in shade outdoors) were used at Oxford and 6-week-old field-grown plants were used at Florence. One spraying per test was made by the same technique as described above for cage bioassays. The Oxford plants were sprayed starting at 1100 hr on August 23. After they dried, the plants were moved to a recently mowed stubble field of harvested wheat and arranged in the pattern described above. This field was over 500 m from the nearest tobacco field. Two hundred mated female budworm moths (40 per replication) were released per night for four successive nights. Eggs were counted and removed on four successive days.

The design at Florence was similar. Spraying of this test was begun at 1500 hr on July 7. However, after completion of these chemical applications, a large thunderstorm appeared. To prevent loss of this experiment, the plants were covered. A stake was driven into the ground in the center of each group of plants and they were covered with a tent of clear polyethylene. The rain prevented the release of moths that evening. The following morning the polyethylene was removed and a shade of a 1.2 × 1.2-m sheet of plywood was placed over each group of plants. These were removed just prior to moth release that evening. Therefore, day 0 for the Florence test was 24 hr after spray application. The field spray-back tests were sampled for chemical analyses as described above for cage tests.

Since the experimental designs were the same at the two locations, data from the Oxford and Florence tests were transformed to log $(x + 1.0)$ and combined prior to analysis of variance. Treatment means were separated by Duncan's new multiple-range test.

RESULTS

Ovipositional Cage Bioassays. As previously reported (Jackson et al., 1983, 1984), ca. 75% of the budworm eggs were deposited on unsprayed NC 2326 plants when moths were given a free choice between them and unsprayed TI 1112 plants (experiment 1, Table 1). Also, as previously shown (Jackson et al., 1984), both NC 2326 WLW and NC 2326 MWS were active in stimulating tobacco budworm oviposition onto TI 1112 plants (experiments 2 and 3, Table 1).

Tobacco budworm oviposition was significantly higher on TI 1112 plants sprayed with any of the individual duvanes or combinations of duvane isomers than it was on control TI 1112 plants sprayed only with the solvent blank (experiments 4–7, Table 1). This activity also persisted in experiments where du-

vane-treated TI 1112 were tested against control TI 1112 plants treated with NC 2326 WLW or NC 2326 MWS (experiments 8–14, Table 1). Neither docosanol, *cis*-abienol, nor docosanyl myristate stimulated budworm oviposition in the cage bioassays.

The levels of cuticular chemicals remaining on treated TI 1112 plants used in the cage bioassays declined over time after spraying. The α + β-4,8,13-duvatrien-1-ols and *cis*-abienol broke down most rapidly and significantly less (ca. 10%) of these compounds remained on the plants by two days after spraying (Table 2). Both the α- and β-4,8,13-duvatriene-1,3-diols applied individually or in NC 2326 MWS were more stable, with ca. 21–30% remaining after two days. Nearly 50% of the docosanol remained on the TI-1112 plants after two days (Table 2).

Field Experiments. All TI 1112 plants sprayed with duvane diterpenes or NC 2326 MWS had significantly ($P = 0.05$) more eggs deposited on them than TI 1112 plants sprayed only with solvent blank (Table 3). This trend continued for three days after spraying. Only the TI 1112 with NC 2326 MWS treatment had similar numbers of eggs as the NC 2326 control plants the first night after spraying. All of the treatments had significantly fewer eggs than NC 2326 by the second night after spraying.

However, as shown in Figure 1, all the diterpenes degraded rapidly in the field. This fast degradation explains the decrease in eggs found after the second night on these spray treatments relative to NC 2326. No changes were observed over time in the cuticular component profiles of NC 2326 and TI 1112 sprayed only with SB. The α- and β-ols disappeared at the fastest rate and were nearly absent two days after applications. The α- and β-diols were somewhat more stable, and they approached TI 1112 levels only after three days in the field.

DISCUSSION

Each of the duvanes tested in cage bioassays and field experiments stimulated tobacco budworm oviposition onto TI 1112 plants sprayed with these materials. None of the nonduvane cuticular components from tobacco increased egg laying. These data are evidence that the observed ovipositional nonpreference resistance of TI 1112 by *H. virescens* is due in part to the absence of duvane diterpenes which are major components of the leaf surface chemical profiles of commercial American tobaccos.

Our data indicate that β-diol may have a higher activity than the α isomer. The α:β ratio for NC 2326 MWS was ca. 3:1, and this ratio changed little over time (Table 2). Severson et al. (1984) reported an α:β ratio of 2.9:1.0 for NC 2326, and this ratio approximated 3:1 for 11 varieties of flue-cured, burley, Maryland, dark-fired, cigar wrapper, cigar binder, and Turkish tobaccos. Roberts and Rowland (1962) showed that α-diol is more heat and light labile than

TABLE 2. STABILITY OF CUTICULAR COMPONENTS SPRAYED ONTO TI 1112 TOBACCO PLANTS USED IN OVIPOSITION CAGE BIOASSAYS AT OXFORD, NORTH CAROLINA, 1983

Plant type	Spray treatment[a]	Components measured	Level ($\mu g/cm^2$) at days after application		
			0	1	2
TI 1112	NC 2326 MWS	$\alpha + \beta$-ols	0.3	0.1	Trace
		α-Diol	40.9	24.8	16.0
		β-Diol	15.3	9.4	6.1
TI 1112	$\alpha + \beta$-ols	α-ol	60.7	25.7	7.5
		β-ol	6.6	2.9	0.9
TI 1112	α-Diol	α-Diol	50.5	29.4	17.9
		β-Diol	0.2	0.2	0.2
TI 1112	β-Diol	α-Diol	0.2	0.2	0.4
		β-Diol	15.0	7.9	3.2
TI 1112	*cis*-Abienol	*cis*-Abienol	66.4	11.3	6.1
TI 1112	Docosanol	1-Docosanol	2.7	1.9	1.2
NC 2326	None (check)	α-ol	0.1	0.1	0.1
		β-ol	0.1	0.1	0.1
		α-Diol	11.1	9.7	6.8
		β-Diol	4.5	3.8	3.1
		cis-Abienol	0.0	0.0	0.0
		1-Docosanol	0.2	0.2	0.1
TI 1112	None (check)	α-ol	0.0	0.0	0.0
		β-ol	0.0	0.0	0.0
		α-Diol	0.1	0.1	0.1
		β-Diol	0.1	0.1	0.1
		cis-Abienol	0.0	0.0	0.0
		1-Docosanol	0.0	0.0	0.0

[a]Materials applied in 0.5 ml of spray treatment per plant in 9.5 ml acetone–H_2O (3:1). MWS = methanol–water-soluble fraction of whole leaf wash; $\alpha + \beta$-ols = $\alpha + \beta$-4,8,13-duvatrien-1-ols; α-diol = α-4,8,13-duvatriene-1,3-diol; β-diol = β-4,8,13-duvatriene-1,3-diol.

TABLE 3. TOBACCO BUDWORM OVIPOSITION IN 1983 FIELD TESTS ON 6-WEEK-OLD TI 1112 OR NC 2326 PLANTS SPRAYED WITH DUVANE DITERPENES, METHANOL–WATER-SOLUBLE FRACTION OF NC 2326 WHOLE LEAF CUTICULAR WASH, OR SOLVENT BLANK

Treatment[a]	Average number of eggs/plant on indicated day after spraying[b]		
	1	2	3
TI 1112 with SB	4.9a	7.0a	7.7a
TI 1112 with β-Diol	7.7b	12.4b	11.8b
TI 1112 with α + β-ols	9.7bc	10.4b	19.4b
TI 1112 with NC 2326 MWS	15.9d	12.7b	12.3b
TI 1112 with α-Diol	12.4c	16.1b	21.3b
NC 2326 with SB	20.0d	25.4c	25.4c

[a] SB = solvent blank; α-diol = α-4,8,13-duvatriene-1,3-diol; β-diol = β-4,8,13-duvatriene-1,3,-diol; α + β-ols = mixture of α- and β-4,8,13-duvatrien-1-ols; NC 2326 MWS = methanol–water-soluble fraction from NC 2326 whole leaf wash.

[b] Data combined from Oxford, North Carolina and Florence, South Carolina; means in the same column followed by the same letter are not significantly different ($P = 0.05$); Duncan's multiple-range test.

β-diol. For our field tests we also saw a slightly more rapid decline of α-diol than β-diol when these materials were applied individually (Figure 1). When applied as part of the NC 2326 MWS these chemicals did not disappear as rapidly, but α-diol still declined more rapidly than β-diol. The α- and β-ols broke down very rapidly and were nearly gone by the second day after spraying (Figure 1).

The data indicate that the diterpenes are continuously synthesized and secreted onto the surface of the rapidly growing leaves, and then decompose (Table 2). They are believed to be converted to higher oxidation states by phytoxidation (Reid, 1975) which then further decompose to numerous volatile terpenes that may be important flavor and aroma components (Colledge et al. 1975; Enzell, 1977; Kawashima and Gamou, 1979; Wahlberg et al., 1977; Demole and Dietrich, 1977; Reid, 1979). Mass spectral analysis indicated that the oxidized duvanes are hydroxyepoxy, hydroxyoxy, and trihydroxy degradation products of the parent α- and β-ols and diols reported by Demole and Dietrich (1977), Enzell (1977), and Enzell and Walhberg (1980). A slight increase in the levels of the oxidized duvanes after three days in the field does not appear to account for all of the decreases in the parent duvanes. Thus, it would indicate the losses occur due to the formation of volatile components and/or degradation products that are not soluble in CH_2Cl_2 or are not GC volatile. The data presented here showed that the presence of duvane diterpenes is important in stimulating budworm oviposition. However, more studies are needed to determine

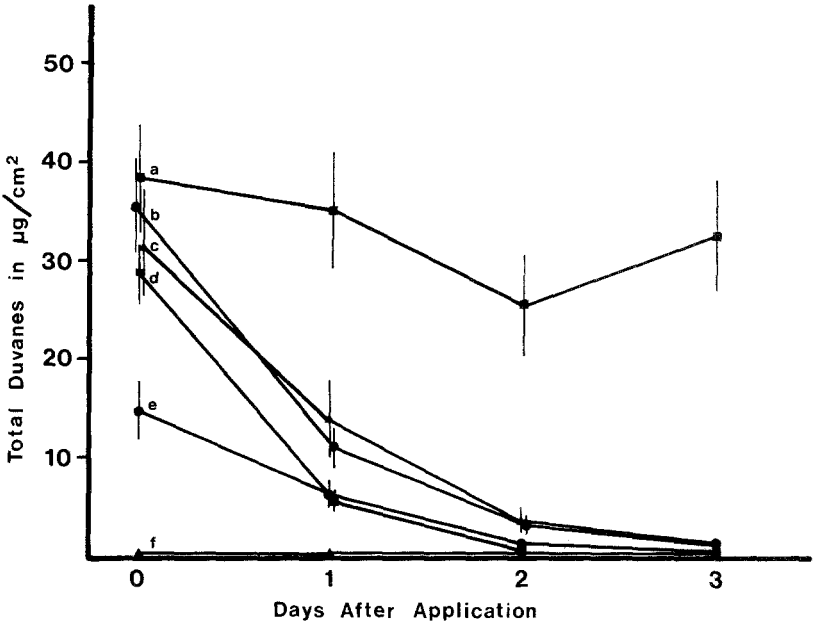

FIG. 1. Total duvane diterpenes on NC 2326 (a) and on TI 1112 plants sprayed with a solvent blank (f), the methanol–water-soluble fraction of NC 2326 whole leaf wash (c), α-4,8,13-duvatriene-1,3-diol (b), β-4,8,13-duvatriene-1,3-diol (e), or α- plus β-4,8,13-duvatrien-1-ols (d) in field tests with potted plants at Florence, South Carolina, and Oxford, North Carolina, 1983. Vertical lines indicate plus or minus standard error of the means; there were 10 samples per mean.

if oxidized duvanes or their volatile degradation products are also active in inducing budworm oviposition.

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SEX PHEROMONE OF *Grapholita funebrana* Occurrence of *Z*-8- and *Z*-10-Tetradecenyl Acetate as Secondary Components

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Abstract—*Z*-8-Dodecenyl acetate (Z8–12 : Ac), *E*-8-dodecenyl acetate (*E*8–12 : Ac), *Z*-8-tetradecenyl acetate (Z8–14 : Ac), *Z*-10-tetradecenyl acetate (Z10–14 : Ac), and *Z*-8-dodecen-1-ol (Z8–12 : OH) were identified in the proportions 100 : 1 : 30 : 5 : 2 in female sex gland extracts of *Grapholita funebrana*, accompanied by saturated acetates from 12 to 20 carbons with tetradecyl acetate predominating. Z10–14 : Ac has not previously been described as a lepidopteran sex pheromone component. Best attraction of males is obtained with Z8–12 : Ac in the presence of a higher proportion of *E*8–12 : Ac than in the female. Inclusion of the 14-carbon acetates did not augment *G. funebrana* catches but inhibited *G. molesta*. On the other hand, addition of Z8–12 : OH at the level optimal for *G. molesta* reduced attraction of *G. funebrana*.

Key Words—*Grapholita funebrana*, plum fruit moth, *Grapholita molesta*, Lepidoptera, Tortricidae, *Z*-8-dodecenyl acetate, *Z*-8-tetradecenyl acetate, *Z*-10-tetradecenyl acetate, sex pheromone, delta-10-unsaturation.

INTRODUCTION

Grapholita funebrana Tr. (Lepidoptera, Tortricidae) is recorded as a pest on a wide variety of *Prunus* spp., especially plums across Eurasia (Bovey, 1966; Carter, 1984). After the identification of *Z*-8-dodecenyl acetate (Z8–12 : Ac; for

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other short forms, see Table 1) as a sex pheromone component of *G. molesta* (Roelofs et al., 1969), this compound was also found to attract *G. funebrana* in the field (Granges and Baggiolini, 1971). Mating disruption in *G. funebrana* with this chemical has met with variable success (Arn et al., 1976; Mani et al., 1978; Charmillot et al., 1982); this is the first report on a chemical analysis of the sex pheromone of *G. funebrana*.

METHODS AND MATERIALS

Insects. Since *G. funebrana* is difficult to rear, we collected first-generation larvae from infected fruit in June and the diapausing generation in corrugated paper bands wound around tree trunks in September. The latter were held over winter in an insectarium. Adults were segregated according to sex on emergence at 24°C and 56% relative humidity in a 16:8 hr light–dark cycle (6000:1 lux) and held in polystyrene boxes supplied with sucrose solution.

Sex gland extracts of 150 2 to 4-day-old females were made midway through the scotophase by removing the everted ovipositor tips and placing them in hexane (ca. 1 μl/gland, Merck "for residue analysis").

Chemical Analysis. Gas chromatography (GC) with electroantennographic detection (EAD) (Arn et al., 1975) was made on a 25-m Silar 10c high-resolution glass capillary column (3 min at 40°C, 10°/min to 60°C, and 4°/min to 200°C). The detector species (Guerin et al., 1985), *Pammene rhediella* and *Spilonota ocellana*, were obtained from field traps baited with their respective attractants *Z*-8,*E*-10-dodecadien-1-ol and Z8–14:Ac.

Mass spectrometric (MS) analysis of 50 female equivalents (FE) was made with a Finnigan 4015 instrument using the Silar column. Dimethyl disulfide (DMDS) adducts from 20 FE were derived as described by Buser et al. (1983) and analyzed on a 50-m SP-1000 column (2 min at 80°C, 20°/min to 120°C and 5°/min to 240°C).

Field Trials. These were done in 6–10 replicates using rubber caps and traps as in Arn et al. (1979) in plum orchards in Switzerland and in peach orchards bordered by plums in Hungary. Separation of *G. molesta*, also caught at the latter location, from *G. funebrana* was done on the basis of genitalia.

RESULTS

Chemical Analysis. GC-EAD analysis of the female sex gland extract using the male *G. funebrana* antenna indicated biologically active components at the retention times of 12:Ac (weaker signal) and Z8–12:Ac. A systematic search with the key ions *m/z* 61 ($CH_3COOH_2^+$ and M^+-CH_3COOH indicated the presence of two dodecenyl acetates (*m/z* 166) in the gland extract, one at the retention time of Z8–12:Ac, the other, at a much smaller amount, matching *E*8–

TABLE 1. COMPONENTS IDENTIFIED IN *G. funebrana* SEX GLANDS[a]

Compound	Short form	Identified by	Amount (ng/FE)
Dodecyl acetate	12:Ac	GC-MS	0.08
E-8-Dodecenyl acetate	*E*8-12:Ac	GC-MS	0.005
Z-8-Dodecenyl acetate	*Z*8-12:Ac	GC-MS, DMDS, GC-EAD	0.5
Z-8-Dodecen-1-ol	*Z*8-12:OH	GC-EAD	0.01
Tetradecyl acetate	14:Ac	GC-MS	1
Z-8-Tetradecenyl acetate	*Z*8-14:Ac	GC-MS, DMDS, GC-EAD	0.15
Z-10-Tetradecenyl acetate	*Z*10-14:Ac	GC-MS, DMDS	0.03
Hexadecyl acetate	16:Ac	GC-MS	0.15
Octadecyl acetate	18:Ac	GC-MS	0.03
Eicosyl acetate	20:Ac	GC-MS	0.2

[a]In order of elution on Silar 10c and SP-1000.

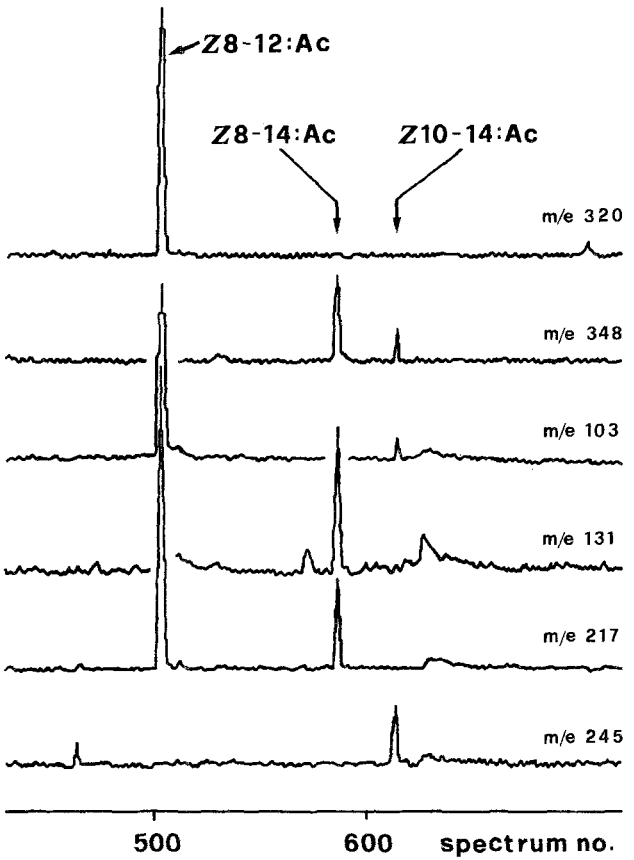

FIG. 1 Mass chromatograms of DMDS-derivatized *Grapholita funebrana* female extract on SP-1000 using the respective molecular ions 320 and 348 for dodecenyl and tetradecenyl acetates, the typical fragment ions 103 and 131 for omega-4 and omega-6 unsaturation, and those of 217 and 245 for delta-8 and delta-10 monoenic acetates.

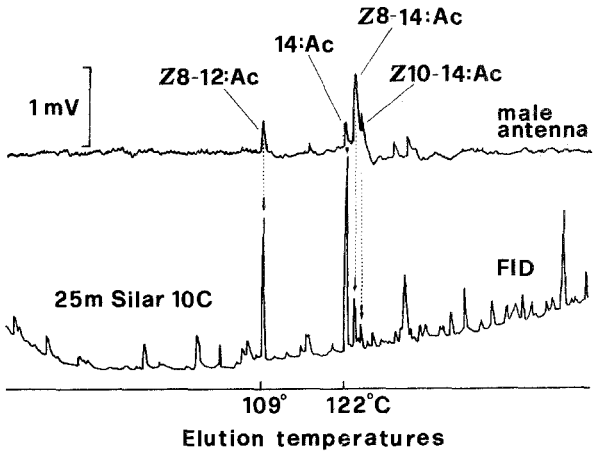

FIG. 2. GC-EAD analysis of *Grapholita funebrana* sex gland extract (10 FE) on 25-m Silar 10c using as detector a *Spilonota ocellana* male antenna most sensitive to Z8–14:Ac.

12:Ac. In addition, we found two tetradecenyl acetates (*m/z* 194) and saturated acetates from 12 to 20 carbons (Table 1).

Assignments of double-bond positions were made from DMDS adducts. The recorded mass spectra revealed one major peak at *m/z* 320 for dodecenyl acetate adducts with the diagnostic ions *m/z* 103, 217, and 157, for delta-8-unsaturation (Figure 1). The level of the *E*8–12:Ac derivative was below the detection limit. At *m/z* 348 for tetradecenyl acetates, two peaks with dissimilar mass spectra were obtained at the ratio seen in the underivatized extract, indicating positional isomers. Based on the fragment ions *m/z* 131, 217, and 157 for the first and 103, 245, and 185 for the second, they were assigned delta-8 and delta-10.

In GC-EAD (Figure 2) the antenna of *S. ocellana*, specifically sensitive to Z8–14:Ac (Arn et al., 1974), gave the strongest response to the early eluting tetradecenyl acetate with an intensity matching that of a synthetic Z8–14:Ac; the *E*8 isomer was comparatively inactive. Isomer assignment of the second tetradecenyl acetate was based on retention times. Although we did not detect Z8–12:OH in the extract by GC-MS, analysis with the antenna of *P. rhediella* indicated the presence of ca. 2.5% of this product (relative to Z8–12:Ac), matching both retention time and activity of the synthetic standard. *P. rhediella* is attracted to Z8,*E*10–12:OH but also is selectively sensitive to Z8–12:OH (Guerin et al., 1983); the closely eluting isomers on Silar 10c, Z7, *E*8, *E*9, and Z9 were relatively inactive.

Field Trials. In two tests in Switzerland, we varied the amounts of *E*8–12:Ac and the 14-carbon compounds identified (Table 2, A and B). Despite

TABLE 2. *Grapholita funebrana* FIELD TESTS

Blend composition[a]							
Z8–12:Ac	*E*8–12:Ac	Z8–14:Ac	Z10–14:Ac	14:Ac	Z8–12:OH	*G. funebrana*	*G. molesta*
A: Switzerland, May 17–July 15, 1983 (10 replicates)							
100	1	25	5			69 a	
100	1					74 a	
100	1	5				92 a	
100	1	25				68 a	
100	1	100				81 a	
100	1		1			89 a	
100	1		5			84 a	
100	1		25			88 a	
B: Switzerland, May 15–August 16, 1983 (10 replicates)							
100	1	25	5			130 c	
100	1	25	5	50		99 c	
100	1	25	5	200		125 c	
100	1	25	5	1000		145 c	
100	2	25	5			315 b	
100	2	25	5	200		300 b	
100	4	25	5			636 a	
100	4	25	5	200		760 a	
C: Hungary, August 1–22, 1984 (9 replicates)							
100	4					193 a	16 b
100	4	25	5	200		232 a	0 c
100	4				3	73 b	79 a
100	8				3	69 b	80 a

[a] Amounts in micrograms per rubber cap.
[b] Figures in the same test followed by the same letter are not significantly different at $P = 0.05$ as indicated by log $(x + 1)$ transformation, two-way analysis of variance, and Duncan's mutliple-range test.

our detecting only 1% *E*8–12:Ac in the female, better catch was obtained with 4%, confirming earlier findings (Arn et al., 1976; Biwer and Descoins, 1978). None of the tetradecenyl acetates added singly or in pairs, nor 14:Ac added to the four-component blend augmented catch.

Selectivity tests on *G. funebrana* and *G. molesta* were made in Hungary where both species flew simultaneously (Table 2, C). Blends containing Z8–12:Ac with 4 or 8% *E*8–12:Ac attracted both species. Addition of the three 14-carbon acetates in the proportions found in the females failed to improve attraction of *G. funebrana*. (In Switzerland these two treatments caught 2303 and 2244 males, respectively, in five replicates from May 17, to September 5,

1984). Furthermore, direct field observations in Hungary indicated that of those males orienting to the pheromone source, neither the percentage contacting the rubber cap nor those subsequently displaying hairpencils was augmented by inclusion of the 14-carbon acetates (in all 66 males were observed). However, *G. molesta* was inhibited in the presence of the 14-carbon compounds. On the other hand, addition of Z8–12 : OH to the binary blend at the level found in the *G. funebrana* sex gland reduced captures of the latter by a factor of three while enhancing those of *G. molesta*.

DISCUSSION

The identification of Z8–12 : Ac in the *G. funebrana* sex pheromone gland is not surprising considering its field activity on this and other *Grapholita* species. On the other hand, Z8–14 : Ac and Z10–14 : Ac, identified here, are not common as components of sex attractants. To our knowledge, Z10–14 : Ac has not been previously identified in Lepidoptera, although field trials have established it as an attractant component for moth species in the tribes Archipini and Olethreutini and the family Gracillariidae (Roelofs and Brown, 1982; Arn et al., 1974; Ando et al., 1977; Booij and Voerman, 1984). Reference to the occurrence of Z8–14 : Ac is only made for the tortricids *Ctenopseustis obliquana* (Young et al., 1985) and *Planotortrix excessana* (Galbreath et al., 1985) in New Zealand, and for *S. ocellana* based on GC-EAD retention time by Arn et al. (1975). Z8–14 : Ac is also attractive to *Spilonota laricana* (Priesner, 1977) and another Eucosmini species, *Pseudexentera spoliana* (Roelofs and Brown, 1982).

While the pathways leading to pheromone components with double bonds in odd-numbered positions are well established, less is known about the biosynthesis of unsaturated compounds of the type found in *G. funebrana*. A delta-10-desaturase, as claimed by Roelofs and Bjostad (1984), acting on both 14 and 16 carbon chains, in conjunction with chain-shortening, could account for the production of Z10–14 : Ac and Z8–/E8–12 : Ac on one hand, and Z8–14 : Ac on the other.

The occurrence of 14-carbon acetates in the Grapholitini is in itself novel (cf. Roelofs and Brown, 1982). Although not involved in attraction, these compounds may contribute to specificity of the *G. funebrana* attractant blend. Inhibition of *G. molesta* by 12 : Ac, another component of the *G. funebrana* sex gland, has already been established (Rothschild, 1974). On the other hand, Z8–12 : OH reduces *G. funebrana* catch at the optimum required for *G. molesta*. However, since *G. molesta* is sexually active in the afternoon and *G. funebrana* in the early morning, isolation by pheromone blend is less critical here than, for example, between *G. molesta* and *G. prunivora* (Baker and Cardé, 1979).

Further optimizations of the *G. funebrana* atttractant blend could include testing effects of lower amounts of Z8–12 : OH and the role of each of the 14-carbon acetates in inhibition of *G. molesta*.

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AGGREGATION OF LARVAE OF *Blattella germanica* (L.) BY LACTIC ACID PRESENT IN EXCRETA

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Abstract—Lactic acid has been identified in extracts of filter paper "conditioned" by the German cockroach, *Blattella germanica* (L.). One- to two-week old larvae aggregated on filter paper wetted with solutions of lactic acid.

Key Words—Aggregation, lactic acid, German cockroach, *Blattella germanica*, excreta, Dictyoptera.

INTRODUCTION

Ishii and Kuwahara (1967) first demonstrated the aggregation of larvae of the German cockroach, *Blattella germanica* (L.), on filter paper which had been "conditioned" by being placed in a culture of the cockroach. Similarly, Watler (1979) showed that conditioned substrates aggregated larvae of *Acheta domesticus* (L.). McFarlane et al. (1983) found volatile fatty acids on the papers conditioned by *A. domesticus* and showed that one of them, propionic acid, aggregated the larvae. In similar work with *B. germanica*, McFarlane (1984) found propionic, isovaleric, and valeric acids to be repellent; isobutyric acid to be slightly repellent; and acetic and butyric acids to be without effect.

In analyses of frass for volatile fatty acids by gas–liquid chromatography, lactic acid was frequently observed to be present. The demonstration of lactic acid on conditioned filter paper, and the effect of lactic acid on aggregation in *B. germanica*, are the subjects of the present article.

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METHODS AND MATERIALS

German cockroaches were maintained on Ralston Purina dog chow at 30±1°C and 55±5% relative humidity and at a photoperiodic regime of 14:10 light–dark. Tests were carried out with DL-lactic acid, obtained from the Fisher Scientific Company, Montreal.

The method of testing was the same as used previously (McFarlane, 1984). The experiments were conducted at room temperature. The larvae were 2–12 days old; newly hatched larvae and older larvae tended not to settle under the conditions of the experiment. Two 14 × 3.8-cm strips of Whatman No. 1 filter paper folded twice (to form a W) were placed on edge and spaced evenly in a culture dish (diam. 19.2 cm). Lactic acid was tested by making serial dilutions and treating a filter paper strip with 0.68 ml of the solution. The control paper was wetted with 0.68 ml distilled water. Twenty larvae were introduced into the culture dish, and the dish was immediately covered with fine-mesh Tergal® which was secured with a rubber band. Each culture dish was then covered with a small cardboard box which maintained darkness for the test. If this was not done, the larvae would not settle on the papers. In fact, in nearly all trials, the great majority of the larvae came to rest on the papers, and these were counted. Larvae not on the papers were not considered in the analysis of results, as the normal response to the experimental situation was to come to rest on a paper; wandering larvae could not therefore be said to have been attracted or repelled. The few trials in which less than half the larvae came to rest on the papers were also not considered, for the same reason.

Where the solutions were tested immediately, i.e., while still wet, larvae were counted 1.5 hr after the experiment was set up, and thereafter at 0.5-hr intervals for 6 hr. Where the solutions were applied to the filter paper the previous day, and allowed to dry before testing, counts were made at 0.5-hr intervals after the experiment was set up for a total of 2 hr. Response to the dried papers was found to be quicker than to the wet papers in preliminary trials.

In the analysis of results, the previously found bias towards the left-hand paper (McFarlane, 1984) was taken into account, and statistical treatment was carried out independently for left and right papers. Student's *t* test was used to test for the significance of differences between mean numbers on treated and control papers.

Lactic acid has a pK of 3.08. Tests were also carried out with various organic and inorganic acids with similar pK values. Fumaric acid (pKs of 3.03 and 4.44), citric acid (pKs of 3.14, 4.77, and 639) and phosphoric acid (pKs of 2.12, 7.21, and 12.67) were tested.

Methods for Lactic Acid Determination in Conditioned Filter Papers. Two methods were used to analyze for lactic acid by gas–liquid chromatography; the identity of lactic acid was confirmed by mass spectrometry.

Method I. Filter paper strips (14 × 3.8 cm) were conditioned by placing two strips in a culture jar containing 40 adult and 40 larval cockroaches and leaving them in the jar for 1–25 days. On removal from the culture jar, the filter papers were tested (against untreated paper) for their ability to aggregate larvae.

The conditioned filter papers (6) were cut into small pieces (approximately 0.5 cm^2) and placed in a beaker (100 ml). A quantity (15 ml) of distilled water was added to the beaker, and the mixture was allowed to stand for 30 min with intermittent mixing. The liquid was squeezed from the paper and collected; an additional 5 ml of distilled water was then added to the beaker. The mixture was agitated and the liquid was once more squeezed from the paper and collected. A total of 10 ml of extract was collected; this extract was used for analysis of lactic acid.

The same procedure was followed for a sample of untreated filter paper.

For lactic acid determination by gas chromatographic analysis, the lactic acid was converted to benzyl lactate; clean-up and derivatization procedures for benzyl lactate were performed according to the method of Jones and Kay (1976). Conditions of gas chromatographic analysis were as follows: Varian gas chromatograph (model 3700) equipped with a flame-ionization detector; glass column (1.8 m long × 2 mm internal diameter); column packing material was 1% butane-1,4-diol succinate polyester on Chromosorb W, 80–100 mesh; nitrogen carrier gas (flow rate = 20 cc/min); temperature programming: initial temperature = 70°C, initial hold = 8 min, program rate = 4°C/min, final temperature = 150°C, final hold = 1 min. The benzyl lactate in the sample was identified by reference to a standard benzyl lactate preparation. Peak areas were calculated by means of a HP 3390A Reporting Integrator (Hewlett-Packard Co., Avondale, Pennsylvania).

Method II. Filter papers were conditioned by placing four strips (14 × 3.8 cm) in each of two jars with 40 adult males each, and four in each of two jars with 40 adult females each. The papers were removed after 21 days and tested immediately for attraction to larvae. As the male papers gave a quicker aggregation response than the female papers, only the male papers were used in the analysis for lactic acid (Table 1).

Whole papers were extracted as in method I. This does not give an accurate idea of the uniformity of lactic acid and the concentration on the papers, as excreta is spotted on the papers and not uniformly distributed. Hence, spotted areas were cut out with a cork borer (5 mm diameter) and independently analyzed, along with similar nonspotted areas of the same papers.

For lactic acid determination by gas chromatographic analysis, the method of Jones and Kay (1976) was used for preparation of the benzyl lactate derivatives. Conditions of gas chromatographic analysis were as follows: Varian gas chromatograph (model 3700) equipped with a flame-ionization detector; DB 17 (Chromatographic Specialties Ltd., Brockville, Ontario, Canada) capillary col-

Table 1. Rate of Aggregation of Larvae to Filter Papers Conditioned by Male or Female Adult Insects (Eight replicates/test. Mean ± SD)

Time after start (hr)	Males, number on		Females, number on	
	Treated	Control	Treated	Control
0.5	19.1 ± 1.0	0.3 ± 0.5	11.9 ± 4.0	2.0 ± 1.2
1.0	19.4 ± 0.5	0.0	16.3 ± 3.7[a]	1.6 ± 1.4
1.5	20.0	0.0	17.8 ± 2.2	1.0 ± 1.4
2.0	19.8 ± 0.5	0.0	18.1 ± 1.6	1.3 ± 1.8

[a] Significantly greater than 0.5-hr number at $P = 0.025$.

umn (30 mm × 0.25 mm ID); helium carrier gas (flow rate = 1 cc/min; makeup gas flow rate = 30 cc/min); temperature programming: initial temperature = 70°C, initial hold = 12 min, program rate = 4°C/min, final temperature = 180°C, final hold = 1 min. Identification and quantitation of the lactate was performed using the procedures described in method I.

The identity of the benzyl lactate peak was confirmed by mass spectrometry using a Kratos MS 50 TCTA mass spectrometer coupled to a Kratos DS 55 data system (Kratos, Manchester, England); conditions of mass spectrometry were: electron impact source, run at 79 eV; source temperature = 200°C.

Analysis of Frass and Conditioned Filter Paper for D- *and* L-*Lactic Acid.* Extracts of whole conditioned filter paper (eight papers conditioned for 17 days) were prepared using the procedure described previously. The extracts were analyzed for D-lactic and for L-lactic acids using the enzymatic assay method of Sigma Chemical Company, 1984, (Sigma Diagnostics, pyruvate/lactate; quantitative enzymatic determination in whole blood at 340 μm; procedure No. 820-Uv, St. Louis, Missouri).

RESULTS

Analyses of conditioned filter papers for lactic acid by method I, with six papers used for each analysis, gave the following results: conditioned for 1 day, 0.0072 mg lactic acid/filter paper; for 5 days, 0.0077; for 10 days, 0.0114; for 15 days, 0.0155; and for 25 days, 0.0280. The amount of lactic acid found increased with the duration of conditioning. All papers used in the analyses were first tested for aggregation of larvae and all gave positive results. No lactic acid was detected in extracts of untreated filter papers.

Analyses for lactic acid of conditioned filter papers from a culture of male adult cockroaches by method II showed 0.0329 mg lactic acid/filter paper, and 0.2098 mg/filter paper in the contaminated spots of filter paper. Lactic acid was not detected in uncontaminated areas of filter paper. It may be seen that the

TABLE 2. RESPONSE OF LARVAE TO WET PAPERS TREATED WITH 0.68 ml OF LACTIC ACID (Mean ± SD)

Lactic acid concentration (M)	Number on left paper				Number on right paper			
	Trials	Treated	Trials	Control	Trials	Treated	Trials	Control
10^{-1}	18	5.8 ± 7.6[d]	17	15.2 ± 6.5	17	3.3 ± 5.9[d]	18	13.1 ± 7.2
10^{-2}	18	17.7 ± 4.5[a]	18	8.1 ± 7.6	18	10.3 ± 7.1[a]	18	1.6 ± 4.5
10^{-3}	18	16.5 ± 3.2[b]	18	11.1 ± 7.9	18	7.8 ± 7.6[c]	18	2.1 ± 2.8
10^{-4}	18	11.5 ± 6.1[e]	18	15.2 ± 4.1	18	3.7 ± 3.4[e]	18	7.6 ± 6.3

[a] Significantly greater than control at $P = 0.001$.
[b] Significantly greater than control at $P = 0.02$.
[c] Significantly greater than control at $P = 0.01$.
[d] Significantly less than control at $P = 0.001$.
[e] Significantly less than control at $P = 0.05$.

amount of lactic acid/filter paper after conditioning for 21 days is similar to that for papers conditioned for 25 days in the analyses given in the preceding paragraph. It may also be seen that when contaminated spots were analyzed, the concentration of lactic acid was more than six times that of whole filter paper. Confirmation of the presence of lactic acid in these extracts was obtained from the mass spectrometric analysis. The results of the analysis for the isomers of lactic acid was as follows: 0.0563 mg D-lactic acid per conditioned filter paper, and 0.0623 mg L-lactic acid per conditioned filter paper.

The results of "wet" trials using lactic acid solutions are given in Table 2: 0.68 ml of 10^{-2} and 10^{-3} M lactic acid led to significant aggregation, whereas 10^{-1} and 10^{-4} M were repellent. Therefore, there is an optimal concentration for aggregation. The amounts of lactic acid on the filter papers ranged from 0.612 mg/paper at 10^{-2} M to 0.0612 mg/paper at 10^{-3} M. The amount of lactic acid actually found in contaminated spots is therefore within this range. The rate at which larvae responded to the filter papers in the experiments with 10^{-2} M lactic acid is given in Table 3.

When previously dried papers were tested, 10^{-2} M lactic acid was repellent, and lower concentrations had no effect.

Tests using fumaric acid showed repellency at 10^{-1} M and 10^{-2} M and no effect at 10^{-3} M, no effect with citric acid, and repellency at 10^{-1} M phosphoric acid and no effect at 10^{-2} M (Table 4).

DISCUSSION

Lactic acid aggregates larvae of *B. germanica* at concentrations of 10^{-2} to 10^{-3} M, when the larvae are exposed to filter paper wetted with 0.68 ml of the solution. This amount is greater than the amount found on whole con-

Table 3. Rate of Response of German Cockroach in Experiment with 10^{-2} M Lactic Acid [a]

Time after initiation of experiment (hr)	No. of trials in which there was	
	Response	No response
1.5	24	12
2.0	30	6
2.5	30	6
4.0	34	2
6.0	36	0

[a] "Response" indicates that 50% or more of 20 larvae had come to rest on the test papers; "no response" indicates that 50% had reacted to the test papers.

ditioned filter papers. However, the lactic acid on conditioned filter paper is concentrated in many small spots of excreta, and when these spots are analyzed, the concentration sensed by the larva is in the range 10^{-2}–10^{-3} M.

However, when the larvae are exposed to the dried paper, similar concentrations are either repellent or ineffective. This is the same kind of result which McFarlane et al. (1983) obtained with *A. domesticus* and propionic acid, where wet papers aggregated the larvae but dried papers repelled. The repellent effect

Table 4. Response of Larvae to Wet Papers Treated with 0.68 ml of Fumaric, Citric, and Phosphoric Acids

	Number on left paper				Number of right paper			
Acid conc. (M)	Trials	Treated	Trials	Control	Trials	Treated	Trials	Control
Fumaric acid								
10^{-1}	9	7.8 ± 7.9[a]	9	15.0 ± 4.9	9	3.1 ± 3.3[b]	9	10.8 ± 7.7
10^{-2}	6	8.7 ± 5.5[c]	6	19.0 ± 1.3	6	0.3 ± 0.8[c]	6	10.5 ± 5.8
10^{-3}	6	10.2 ± 5.5	6	10.8 ± 4.7	6	7.3 ± 4.5	6	7.2 ± 5.2
Citric acid								
10^{-1}	6	15.8 ± 2.9	6	18.0 ± 2.4	6	1.3 ± 1.8	6	2.5 ± 2.9
10^{-2}	6	16.5 ± 2.1	6	17.7 ± 2.7	6	1.2 ± 1.8	6	2.2 ± 2.3
Phosphoric acid								
10^{-1}	6	1.7 ± 1.5[d]	6	18.3 ± 1.2	6	0.2 ± 0.4[d]	6	17.6 ± 2.0[d]
10^{-2}	9	11.2 ± 7.7	9	12.9 ± 6.1	9	4.8 ± 7.1	9	6.3 ± 8.1

[a] Significantly less than control at $P = 0.025$.
[b] Significantly less than control at $P = 0.01$.
[c] Significantly less than control at $P = 0.0025$.
[d] Significantly less than control at $P = 0.0005$.

of 10^{-2} M lactic acid presented dry may be explained if drying of the filter paper leads to local increases in concentration which are repellent. Lower concentrations of lactic acid on dried paper do not seem to be detected by the larvae.

With wet papers, arrest of movement of the larvae begins while the paper is still wet, and aggregation follows. In fact, there seem to be two distinct aspects to aggregation. First there is the arrest of movement when conditions are favorable. Then there is the attraction of larvae for each other.

The fact that there is an optimal concentration range for lactic acid using wet papers may explain why a dry paper with an arrestant concentration was not found. For if, as was suggested, increases in concentration accompany drying, then there is no control of the distribution of lactic acid on the dried papers.

Inasmuch as no aggregation was obtained with fumaric, citric, and phosphoric acid, it is concluded that the attraction of lactic acid lies in the nature of the molecule itself, not its acidity.

Wileyto and Boush (1983), in a study of the attractancy of various food components for the German cockroach, state that "the dregs in a beer bottle have more attractive power than anything tested in the laboratory [unpublished data]. It is of interest that the lactic acid concentration of beer ranges from 65–450 mg/liter (Charalambous, 1981), which is similar to the concentrations we have found to aggregate the German cockroach.

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THE FEMALE SEX PHEROMONE OF SUGARCANE STALK BORER, *Chilo auricilius* Identification of Four Components and Field Tests

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Abstract—Four pheromonal components have been detected in ovipositor washings and volatiles from female sugarcane stalk borers, *Chilo auricilius* Dudgeon (Lepidoptera: Pyralidae), using combined gas chromatography–electroantennography. The components have been identified as (I) (*Z*)-7-dodecenyl acetate, (II) (*Z*)-8-tridecenyl acetate, (III) (*Z*)-9-tetradecenyl acetate, and (IV) (*Z*)-10-pentadecenyl acetate by comparison of their gas chromatographic behavior with that of synthetic standards. In field tests carried out in northern India during 1982–1984, a combination of II, III, and IV in their naturally occurring ratio (8:4:1) was shown to provide a highly attractive synthetic source for trap use. (*Z*)-7-Dodecenyl acetate was found to reduce catches of male *C. auricilius*, both when dispensed with the other three components and when released from dispensers surrounding a trap baited with the other three components.

Key Words—Sugarcane stalk borer, *Chilo auricilius*, Lepidoptera, Pyralidae, sex pheromone, sex attractant; (*Z*)-7-dodecenyl acetate, (*Z*)-8-tridecenyl acetate, (*Z*)-9-tetradecenyl acetate, (*Z*)-10-pentadecenyl acetate.

INTRODUCTION

Chilo auricilius Dudgeon (Lepidoptera: Pyralidae) is an important pest of sugarcane found in India, Southeastern Asia, Indonesia, and northern Australia. The larvae damage the cane by attacking shoots and by internode boring, which impairs growth and results in losses of weight of cane and sugar recovery; yield

losses of up to 30% have been recorded in India. Sorghum and rice are also attacked by this species. Its life history and factors affecting its incidence are described by Gupta et al. (1981), who also reported that no suitable and effective control measures were available. The presence of a female sex pheromone was demonstrated in northern India by trapping of male *C. auricilius* in virgin female baited traps (Kalra and David, 1971). Identification and synthesis of the female sex pheromone was undertaken with the aim of providing a control agent compatible with existing cultural practices in India.

METHODS AND MATERIALS

Insect Material. Pupae were field collected in northern India and sent by air freight to London. They were sexed and the sexes segregated and maintained in an environmental cabinet until adult emergence (conditions: reversed light–dark cycle, 12 hr:12 hr; temperature 26°C photophase, 24°C scotophase; relative humidity 85%). Adult moths were maintained under the same conditions with a supply of 10% sucrose solution.

Pheromone Collection. Ovipositor washings in carbon disulfide or heptane were usually prepared from virgin female moths 6–8 hr into the first dark period after emergence. Some extracts were prepared after $1\frac{1}{2}$, 3, 4, 5, and $10\frac{1}{2}$ hr and from 2- to 3-day-old females in attempts to obtain higher pheromone yields. Volatiles were collected from individual virgin female moths as described previously (Nesbitt et al., 1979).

Electroantennography (EAG). Recording of male moth antennal responses to GC column effluent (GC-EAG) was carried out essentially as described previously (Moorhouse et al., 1969). "Puff" tests were carried out as described by Nesbitt and et al. (1977), with a 7-min recovery period between stimulations.

Gas Chromatography (GC). For analyses on packed columns, with and without simultaneous recording of EAG responses to part of the column effluent, columns and conditions were as follows: glass columns (1.8 m × 2 mm ID) packed with (A) 2.5% SE30 and 0.5% Carbowax 20 M on Chromosorb G AW DMCS, programmed from 120 to 225°C at 4°C/min; and (B) 1.5% Carbowax 20 M on Chromosorb G AW DMCS programmed from 120 to 200°C at 4°C/min. Carrier gas for A and B was 25 ml/min nitrogen. For analyses on fused silica capillary columns, columns were as follows (except where otherwise stated): 50 m × 0.32 mm ID Chrompack columns coated with (C) CP Sil 5CB (chemically bonded methyl silicone), and (D) CP wax 57CB (chemically bonded, cross-linked polyethylene glycol). Carrier gas for C and D was helium 0.8 kg/cm^2. A Grob splitless injector was used. Columns were maintained at 70°C for 2 min at the start of each analysis (except for analyses of pentadecenyl isomers); subsequent temperature profiles were as follows: For dodecenyl isomers: C and D, 70–120°C at 20°C/min, then isothermal; for tridecenyl iso-

mers: C, 70–135°C at 20°C/min, then isothermal; D, 70–100°C at 20°C/min, then isothermal (25 m × 0.24 mm ID column). For tetradecenyl isomers: C, 70–145°C at 20°C/min, then isothermal; D, 70–140°C at 20°C/min, then isothermal. Conditions for pentadecenyl isomers were as follows: C, 80°C for 1 min, 80–120°C at 40°C/min, 120–200°C at 1°C/min; D 70°C for 2 min, 70–110°C at 20°C/min, isothermal for 34 min, 110–200°C at 2°C/min.

Synthetic Chemicals. (*Z*)- and (*E*)-6-tridecenyl acetate, (*Z*)- and (*E*)-7-tridecenyl acetate, and (*Z*)- and (*E*)-10-tridecenyl acetate were purchased from the Institute for Pesticide Research, Wageningen, The Netherlands. The other monounsaturated acetates were synthesized by standard Wittig or acetylenic routes. The pheromone components used in field tests were purified by liquid chromatography on silica gel impregnated with 20% silver nitrate so that they contained less than 0.1% of the opposite geometric isomer.

Field Tests. These were carried out at two centers in Uttar Pradesh, N. India, during 1982–1984. The traps used were large water traps (60 cm × 60 cm) on 75 cm legs, similar to those described by Campion et al. (1974) but without lids; pheromone dispensers were suspended centrally just above the water from a cross rod. Dispensers for the synthetic compounds were polythene vials (35 mm × 8 mm × 1.5 mm thick); an equal weight of 2,6-di-*tert*-butyl-4-methylphenol (BHT) was added to the synthetic materials as antioxidant. Synthetic pheromone sources were not renewed during experiments. Virgin female moths used to bait traps were caged singly in small mesh containers positioned in the same way as the polythene vials. The female moths were renewed every three days.

For attractancy experiments involving a small number of treatments (Tables 1 and 2), traps were set out randomly in a row, crosswind, approximately 30 m apart. For the experiment detailed in Table 3, treatments were placed randomly in an 8 × 5 grid with approximately 30 m between traps. For the experiment detailed in Table 4, a 6 × 6 lattice design was used with trap spacing within blocks 30 m and blocks at least 100 m apart. For communication disruption experiments, six vials containing the test chemical were placed evenly in a circle around a trap baited with synthetic attractant at 1 m distance and at the same height above the ground as the attractant vial. In the experiment detailed in Table 5, the "surrounded" traps and control traps were set out randomly in two parallel rows, with approximately 30 m between traps in each row and 60 m between rows. In the two-trap experiments (Tables 6 and 7) traps were approximately 30 m apart and replicates were at least 100 m apart. In the experiments summarized in Tables 1, 2, 3, and 5, treatments were randomized at the beginning of the experiment only.

Statistical analyses were based on a factorial random block design with treatments and nights as the two factors. Catches were converted to $(x + 0.5)$ and subjected to analysis of variance. Differences between treatment means were tested for significance by least significant differences (LSD) tests. The

experiment described in Table 4 was a Latin square design and treatments were rerandomized each night. In the disruption experiments (Tables 6 and 7), the positions of treatment and control traps were exchanged each night.

RESULTS AND DISCUSSION

Structure Determination

GC-EAG analyses of ovipositor washings and female volatiles on nonpolar and polar packed columns (A and B) showed four EAG responses. Equivalent chain lengths of the EAG-active compounds based on the retention times of *n*-alkyl acetates (ECLs) suggested that these compounds were C_{12}, C_{13}, C_{14}, and C_{15} monounsaturated acetates or C_{14}, C_{15}, C_{16}, and C_{17} monounsaturated aldehydes (column A ECLs 11.9, 12.9, 13.9, 14.9; column B ECLs 12.4, 13.3., 14.3, 15.4). The EAG-active compounds, assumed to be pheromonal components, were designated I–IV in order of elution; the largest EAG response was to component II. In ovipositor washings, the average ratio of the GC peak heights for components I, II, III, and IV was 4:8:4:1. Entrained female volatiles were enhanced in the more volatile components I and II, and the ratio of the four components was very variable. The best yield of pheromone was 6 ng/female from ovipositor washings; usually much lower yields were obtained. When male moth EAG responses to tridecyl acetate and pentadecanal were compared (GC-EAG link, 10 ng injected), a 1.0-mV response was recorded to the acetate and no response to the aldehyde, suggesting that component II at least, was an acetate rather than an aldehyde. When GC retention times of components I–IV were compared with those of the positional and geometric isomers of dodecenyl, tridecenyl, tetradecenyl, and pentadecenyl acetates, respectively, on fused silica capillary columns C and D, the results described below were obtained:

Component I. All dodecenyl isomers were available for comparison, and the only one with retention times consistent with those of component I on both columns was (Z)-7-dodecenyl acetate, (Z7–12:Ac) (Figure 1).

Component II. *Z*- and *E*6, 7, 8, 9, 10-tridecenyl acetates were available for comparison. Component II had retention times consistent only with the Z8 isomer, (Z8–13:Ac) (Figure 2). By analogy with the retention times of the full series of dodecenyl and tetradecenyl acetates, which both follow a similar pattern, the (Z)-8-tridecenyl acetate retention times are thought to be unambiguous.

Component III. All tetradecenyl acetate isomers were available for comparison. The retention times for component III fitted only with those of (Z)-9-tetradecenyl acetate (Z9–14:Ac) on both columns (Figure 3).

Component IV. The structural assignment for this component is more tentative than for I–III; it was the least abundant component in ovipositor washings and yields in entrained female volatiles were further reduced by its relative

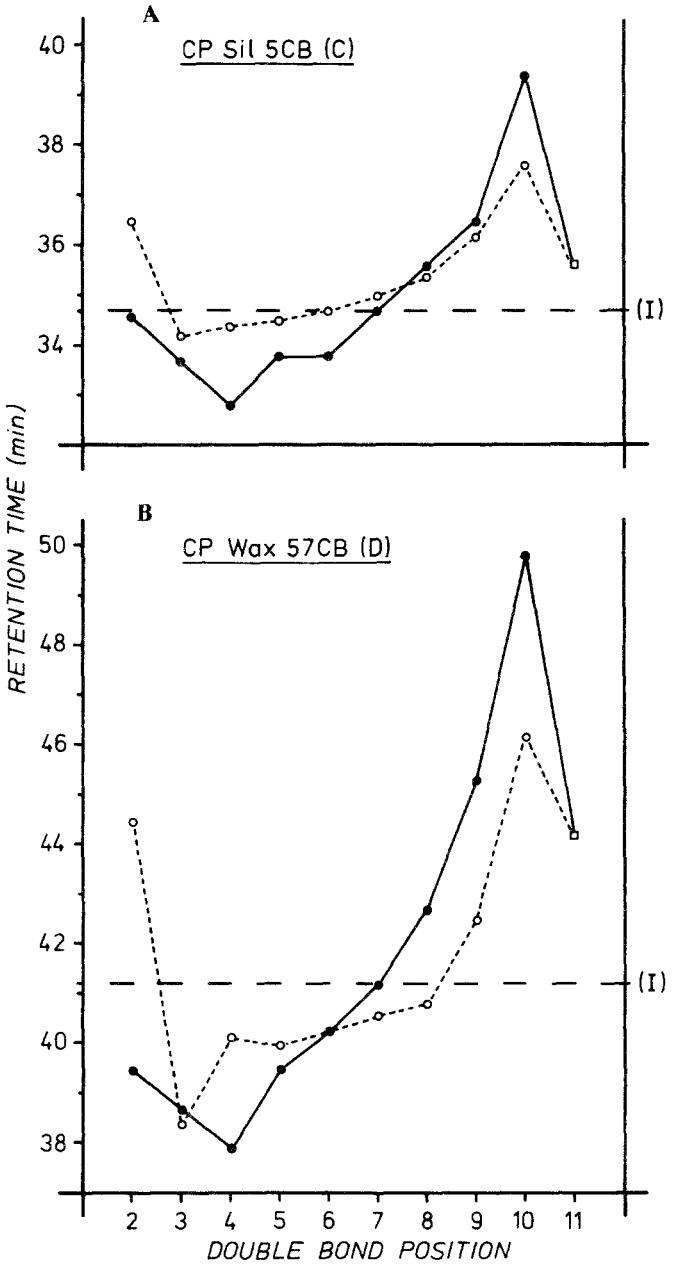

FIG. 1. Retention times of dodecenyl acetate isomers and pheromone component (I) on fused silica capillary GC columns coated with (A) CP Sil 5CB (column C) and (B) CP Wax 57CB (column D): ●—● *Z* isomers; ○- - -○ *E* isomers; □ no configuration. Retention times of dodecyl acetate on column C 37.37 min; column D 35.91 min.

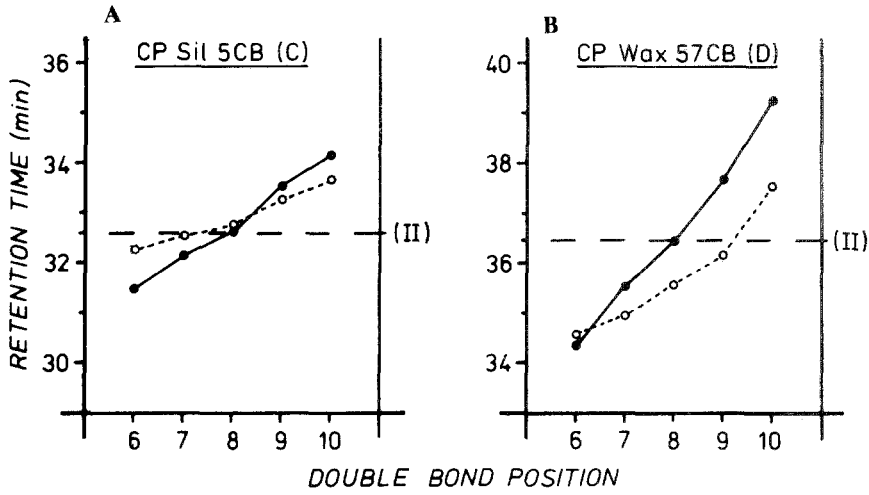

FIG. 2. Retention times of tridecenyl acetate isomers and pheromone component (II) on fused silica capillary GC columns coated with (A) CP Sil 5CB (column C) and (B) CP Wax 57CB (column D): ●—● *Z* isomers; ○- - -○ *E* isomers. Retention times of tridecyl acetate on column C 34.78 min, column D 31.69 min.

involatility. At the time when GC-EAG analyses were carried out on the natural pheromone only (*Z*)-9 and (*Z*)-10-pentadecenyl acetate were available for comparison with component IV. The latter was found to cochromatograph with the *Z*10 isomer (Z10–15 : Ac) on both polar and nonpolar columns. Later, when the *E*9, *Z*8, and *Z*7 isomers were available, these were shown to be fully resolved from the *Z*10 isomer under GC conditions similar to those used originally on column D.

When EAG responses to *Z*7–12 : Ac, *Z*8–13 : Ac, *Z*9–14 : Ac and Z10–15 : Ac were compared by puff-testing at the 2-ng level, the following results were obtained: *Z*7–12 : Ac, 1.4 mV; *Z*8–13 : Ac, 1.9 mV; *Z*9–14 : Ac, 1.2 mV; Z10–15 : Ac, 0.9 mV (mean of duplicate tests). Using the same antennal preparation, 0.25 female equivalent (ovipositor washing) gave 1.1 mV (mean of single tests on four different samples).

Field Tests

In 1982 the four synthetic acetates, *Z*7–12 : Ac, *Z*8–13 : Ac, *Z*9–14 : Ac, and Z10–15 : Ac dispensed from polythene vials were tested in traps using two ratios, 1 : 1 : 1 : 1 and 4 : 8 : 4 : 1, respectively, and two loadings, 0.1 and 1.0 mg. The results indicated that the naturally occurring 4 : 8 : 4 : 1 ratio at the higher loading was significantly more attractive to male *C. auricilius* than the other treatments (Table 1). Early in 1983 the 4 : 8 : 4 : 1 mixture, 1 mg loading, was compared with traps baited with virgin female moths. Catches with the synthetic

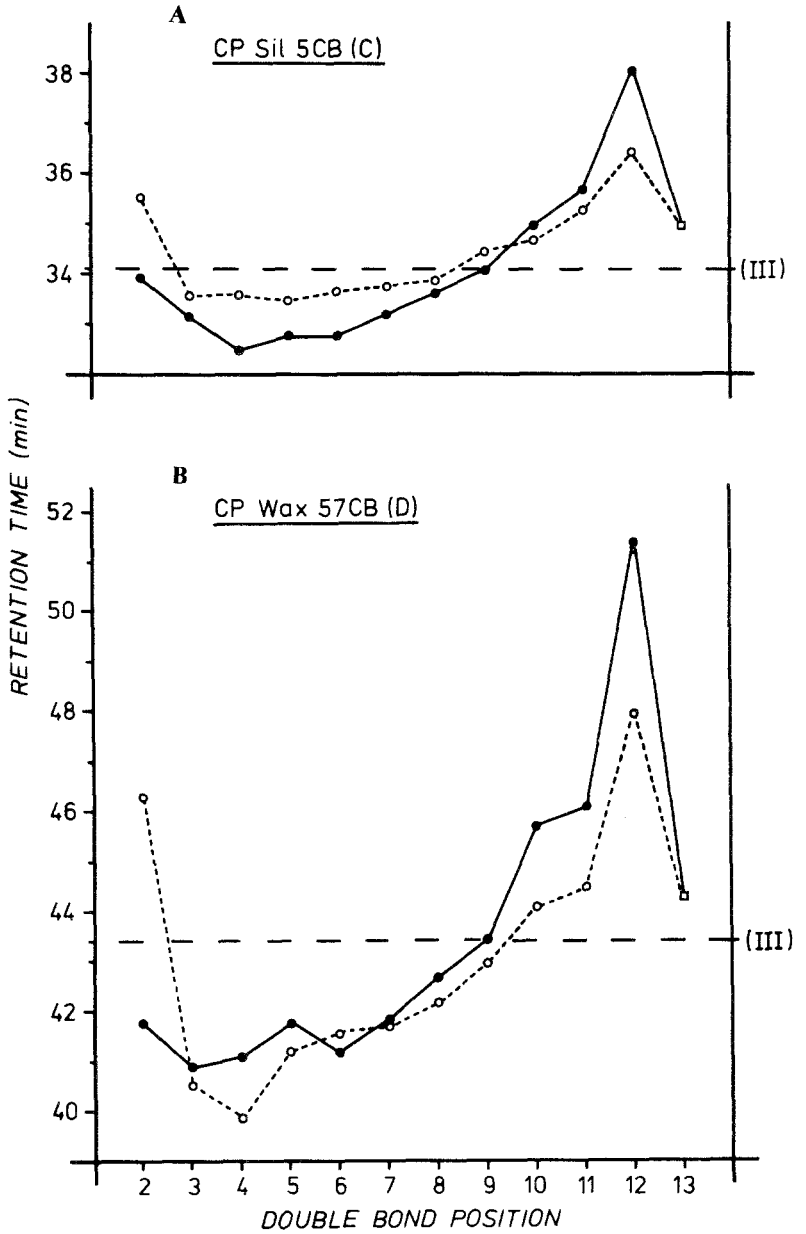

FIG. 3. Retention times of tetradecenyl acetate isomers and pheromone component (III) on fused silica capillary GC columns coated with (A) CP Sil 5CB (column C) and (B) CP Wax 57CB (column D): ●—● *Z* isomers; ○- - -○ *E* isomers; □ no configuration. Retention times of tetradecyl acetate on column C 36.28 min, column D 38.43 min.

TABLE 1. CATCHES OF MALE *C. auricilius* MOTHS IN TRAPS BAITED WITH DIFFERENT RATIOS AND LOADINGS OF FOUR PHEROMONE COMPONENTS (5 replicates, 63 nights)

Ratio of components				Total loading (mg)	Moth catch/ trap/night[a]
Z7–12 : Ac	Z8–13 : Ac	Z9–14 : Ac	Z10–15 : Ac		
4	8	4	1	1.0	2.36 a
4	8	4	1	0.1	0.17 b
1	1	1	1	1.0	0.10 b
1	1	1	1	0.1	0.08 b

[a] Means followed by the same letter are not significantly different at the 1% level by LSD test.

mixture were very much higher than with the virgin female moth (Table 2); it is not known why the female moths performed so poorly. To determine whether all four acetates were necessary for attraction, an experiment was carried out in which each acetate was tested on its own, and Z8–13 : Ac, the most EAG-active and abundant component, was tested in binary combination with each of the three other components (in their naturally occurring proportions). The 4 : 8 : 4 : 1 four-component mixture was included for comparison. The results showed that the individual acetates were only weakly attractive, but the Z8–13 : Ac/Z9–14 : Ac and Z8–13 : Ac/Z10–15 : Ac binary mixtures were significantly more attractive than the Z8–13 : Ac/Z7–12 : Ac mixture or the four-component mixture (Table 3). The latter results suggested that Z7–12 : Ac might be reducing trap catches and, in 1984, a three-component mixture without this component (Z8–13 : Ac, Z9–14 : Ac, and Z10–15 : Ac, 8 : 4 : 1) was compared with the three binary mixtures tested previously and the four-component mixture. The results showed that the three-component mixture was significantly more attractive than the other treatments; the Z8–13 : Ac/Z9–14 : Ac mixture emerged as the best binary mixture in this experiment (Table 4).

Having defined an attractive mixture for trap use, experiments were carried out to determine whether any of the individual components was a potentially

TABLE 2. CATCHES OF MALE *C. auricilius* MOTHS IN TRAPS BAITED WITH FOUR-COMPONENT MIXTURE AND WITH VIRGIN FEMALE MOTH (43 nights)

Attractant source	Replicates	Total moth catch	Moth catch/ trap/night
Z7–12 : Ac, Z8–13 : Ac, Z9–14 : Ac, Z10–15 : Ac (4 : 8 : 4 : 1, total, 1 mg)	20	3104	3.6
Virgin female moth[a]	5	33	0.15

[a] Virgin female renewed every three nights.

TABLE 3. CATCHES OF MALE *C. auricilius* MOTHS IN TRAPS BAITED WITH FOUR PHEROMONE COMPONENTS INDIVIDUALLY AND IN MIXTURES (5 replicates, 56 nights)

Ratio of components[a]				Total moth catch	Mean catch/ trap/night[b]
Z7-12 : Ac	Z8-13 : Ac	Z9-14 : Ac	Z10-15 : Ac		
1	—	—	—	21	0.08 d
—	1	—	—	38	0.14 d
—	—	1	—	39	0.14 d
—	—	—	1	275	0.98 c
1	2	—	—	889	3.18 b
—	2	1	—	1593	5.69 a
—	8	—	1	1524	5.44 a
4	8	4	1	756	2.70 bc

[a] Total loading, 1 mg.
[b] Means followed by same letter are not significantly different at the 5% level by LSD test.

useful mating disruption agent. This was done by testing the effect of surrounding a trap baited with synthetic attractant with six vials containing the individual components. In the first experiment, 1-mg vials containing the individual components were used, and the central traps and control traps were baited with the four component, 4 : 8 : 4 : 1, mixture (the most attractive source known at the time). "Surrounding" vials containing this mixture were included for comparison, although it was realized that such a complex mixture was unlikely to be an economic proposition for mating disruption. The results showed that only the Z7-12 : Ac vials and those containing the four-component mixture were effective in reducing trap catches. The traps surrounded by Z9-14 : Ac and Z10-15 : Ac vials caught significantly more male moths than the control traps (Table

TABLE 4. CATCHES OF MALE *C. auricilius* MOTHS IN TRAPS BAITED WITH DIFFERENT MIXTURES OF FOUR PHEROMONE COMPONENTS (6 replicates, 36 nights)

Ratio of components				Total loading (mg)	Mean catch/ trap/night[a]
Z7-12 : Ac	Z8-13 : Ac	Z9-14 : Ac	Z10-15 : Ac		
4	8	4	1	1.0	5.63 c
4	8	4	1	0.1	1.34 e
1	2	—	—	1.0	2.81 de
—	2	1	—	1.0	8.42 b
—	8	—	1	1.0	4.50 cd
—	8	4	1	1.0	11.97 a

[a] Means followed by the same letter are not significantly different at the 1% level by LSD test.

TABLE 5. CATCHES OF MALE *C. auricilius* IN TRAPS BAITED WITH FOUR-COMPONENT MIXTURE[a] SURROUNDED BY VIALS CONTAINING THIS MIXTURE OR INDIVIDUAL COMPONENTS (2 replicates, 21 nights)

Ratio of components in surround vials[b]				Mean catch/ trap/night[c]
Z7-12:Ac	Z8-13:Ac	Z9-14:Ac	Z10-15:Ac	
1	—	—	—	1.10 c
—	1	—	—	7.43 b
—	—	1	—	16.72 a
—	—	—	1	13.53 a
4	8	4	1	3.36 c
None (control)				7.62 b

[a]Z7-12:Ac + Z8-13:Ac + Z9-14:Ac + Z10-15:Ac 4:8:4:1, 1 mg.
[b]Six vials at 1 m from trap, 1 mg each vial.
[c]Means followed by the same letter are not significantly different at the 5% level by LSD test.

TABLE 6. CATCHES OF MALE *C. auricilius* MOTHS IN TRAPS BAITED WITH FOUR-COMPONENT MIXTURE,[a] SURROUNDED BY SIX VIALS CONTAINING 1 mg Z7-12:Ac (4 replicates, 36 nights)

Surround vials	Total moth catch	Trap catch reduction (%)
1 mg Z7-12:Ac	373	76
None	1557	

[a]Z7-12:Ac + Z8-13:Ac + Z9-14:Ac + Z10-15:Ac, 4:8:1:1 ratio, total 1 mg.

TABLE 7. CATCHES OF MALE *C. auricilius* MOTHS IN TRAPS BAITED WITH THREE-COMPONENT MIXTURE,[a] SURROUNDED BY SIX VIALS CONTAINING 2 mg Z7-12:Ac (2 replicates, 36 nights)

Surround vials	Total moth catch	Trap catch reduction (%)
2 mg Z7-12:Ac	188	95
None	3733	

[a]Z8-13:Ac + Z9-14:Ac + Z10-15:Ac, 8:4:1 ratio, total 1 mg.

5). The effect of the Z7–12 : Ac component on reducing trap catches was tested further in a replicated, two-trap experiment in which the surrounding vials were moved from one trap to the other each night. The results indicated a significant reduction (74%) in catch in the surrounded traps (Table 6). A similar experiment in which the loading of the Z7–12 : Ac vials was increased to 2 mg and the more attractive 8 : 4 : 1, Z8–13 : Ac, Z9–14 : Ac, Z10–15 : Ac mixture was used in the traps, resulted in a 95% reduction of catch in the surrounded traps (Table 7).

CONCLUSIONS

The analytical and field results reported indicate that Z7–12 : Ac, Z8–13 : Ac, Z9–14 : Ac, and Z10–15 : Ac are components of the female sex pheromone of *Chilo auricilius*. Moreover, a three-component mixture of Z8–13 : Ac, Z9–14 : Ac, and Z10–15 : Ac in an 8 : 4 : 1 ratio provides an effective synthetic attractant for trap use. The role of the Z7–12 : Ac component in the moth's reproductive behavior is obscure. Although a mixture of this component and the major component Z8–13 : Ac is more attractive than either component alone, Z7–12 : Ac added to a mixture of Z8–13 : Ac, Z9–14 : Ac, and Z10–15 : Ac in their natural ratio reduces trap catches. Moreover, Z7–12 : Ac released from vials surrounding a trap baited with the other three components also causes a reduction in trap catch. Pheromonal components which reduce trap catches have been reported in other lepidopterous species (Beevor and Campion, 1979; Mustaparta, 1984). In practical terms, Z7–12 : Ac is a potentially useful mating disruption agent for *C. auricilius* and disruption trials are in progress with a sprayable, microcapsule formulation of this chemical.

Although monounsaturated acetates are very common pheromonal components in Lepidoptera, odd-numbered chain lengths are rare, and a sequence of four acetates each differing in chain length by only one carbon atom is unique among pheromones identified to date.

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VITAMIN A DEFICIENCY MODIFIES RESPONSE OF PREDATORY MITE *Amblyseius potentillae* TO VOLATILE KAIROMONE OF TWO-SPOTTED SPIDER MITE, *Tetranychus urticae*

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Abstract—A volatile kairomone of the two-spotted spider mite, *Tetranychus urticae*, elicits a searching response of the phytoseiid predator *Amblyseius potentillae*, only when the predator is reared on a carotenoid-free diet. However, after addition of crystalline β-carotene or vitamin A acetate to the predator's rearing diet this searching response was absent. Because vitamin A and β-carotene are indispensable nutrients for diapause induction, the carotenoid-deficient predators increase their fitness by searching for two-spotted spider mites, when other spider mites are unavailable. Two-spotted spider mites, among others, contain the carotenoids required for diapause induction, but are an inferior prey due to the dense webbing they produce. When the predators have carotenoids at their disposal, they do better by searching for other spider mites that are more profitable in terms of reproductive success. Such a prey is the European red spide mite, *Panonychus ulmi*. The volatile kairomone of this prey elicits a searching response of the predator whether it has a carotenoid deficiency or not.

Key Words—*Amblyseius potentillae*, *Tetranychus urticae*, *Panonychus ulmi*, Acarina, Phytoseiidae, Tetranychidae, volatile kairomones, vitamin A, β-carotene, diapause induction.

INTRODUCTION

Amblyseius potentillae (Garman) (Acarina: Phytoseiidae) is a polyphagous predatory mite that can feed and reproduce on phytophagous spider mites (Acar-

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ina: Tetranychidae) (Kropczynska-Linkiewicz, 1971; Overmeer, 1981) and pollen of several plant species (Overmeer, 1981). The predator is known to respond to volatile kairomones. In Y-tube olfactometer experiments it was shown that starved *A. potentillae,* when reared on two-spotted spider mites (*Tetranychus urticae* Koch), respond to a volatile kairomone of the European red spider mite [*Panonychus ulmi* (Koch)] but not to a kairomone emitted from leaves infested by the two-spotted spider mite, to which other predatory mites respond (Sabelis and Van de Baan, 1983). *Tetranychus urticae* is an unprofitable prey species for *A. potentillae*, as the predator is hindered by the dense webbing produced by this spider mite (Sabelis, 1981). The European red spider mite that also produces silk but not a dense webbing is a more profitable prey. These differences in values of these prey species for *A. potentillae* also are reflected in the ability of the predator to control populations of these mite species. The predator is able to control populations of *P. ulmi* Van de Vrie, 1973; McMurtry and Van de Vrie, 1973; Rabbinge, 1976), but it is unable to control those of *T. urticae* (Sabeis, 1981).

When animals search for food, they have to make decisions about where to search, how long to search at a specific site, and which type of food to eat. In optimal foraging theory, it is assumed that predators make decisions so as to maximize their genetic contributions to future generations. It is therefore assumed that animals maximize their net rate of food intake (Pyke et al., 1977; Krebs, 1978). Although it has received little emphasis in the literature, nutrient composition also is important and should be optimized. Examples are available for spiders (Greenstone, 1979) and moose (Belovsky, 1978). General dietary components like nitrogen, amino acids, and proteins are usually thought to be important (Greenstone, 1979; Slansky and Feeny, 1977; Moss et al., 1972). In this paper, a specific nutrient is taken into account: vitamin A, which is an indispensable nutrient for diapause induction in the eyeless mite *Amblyseius potentillae* (Veerman et al., 1983). Our work shows that a deficiency of vitamin A or its precursor β-carotene in the diet of *A. potentillae* affects its response to a kairomone of one of its prey species, the two-spotted spider mite. To our knowledge this is the first time that any single nutrient has been shown to be of crucial importance in prey selection.

METHODS AND MATERIALS

Predators. Amblyseius potentillae was reared on plastic plates (McMurtry and Scriven, 1965) in a climate room at 25 $\pm$ 1°C, 60 $\pm$ 10% relative humidity, under continuous fluorescent light on four different diets. Two-spotted spider mites have been used as prey for many years. The mites were brushed off the host-plant leaves onto the plastic plates to circumvent the adverse effects of the dense webbing of this prey. For about five years, predators from this culture

have been reared on broad bean (*Vicia faba* L.) pollen, a carotenoid-free diet for *A. potentillae* (Overmeer and Van Zon, 1983). For some experiments we added crystalline β-carotene or vitamin A acetate (Sigma Co., St. Louis, Missouri) to the pollen (1 mg β-carotene or vitamin A acetate per 5 mg pollen). Overmeer and Van Zon (1983) and Veerman et al. (1983) showed that *A potentillae* take up these crystalline carotenoids when mixed with pollen.

Prey. The two-spotted spider mite was reared in our laboratory for many years on Lima beans (*Phaseolus lunatus* L.) at 20–30°C under continuous fluorescent light that was added to the sunlight regime.

The European red spider mites were collected from an apple orchard. The leaves, on which 20–40 active *P. ulmi* stages were present, were used in the bioassay on the day collected.

Bioassay. We used an olfactometer that consisted of a glass tube with an iron wire in the center, both of which are Y-shaped. Odor-emitting objects may be placed in PVC cages at the ends of both arms of the Y-tube olfactometer. Air was sucked out at the base of the tube and led to the outside of the climate room in which the olfactometer was positioned. The airstream speeds in both arms of the olfactometer were measured with a hot wire anemometer and standardized at 0.7 ± 0.1 m/sec by inserting dry cotton wool in the inlet of the airstream and/or changing the total air speed with a valve. Female predators that had been deprived of food and water for 20 hr in a plastic tube were placed individually on the wire at the base of the basal tube and observed. Starved predators were used as these showed a response to the volatile kairomone of *P. ulmi*, unlike well-fed predators (Sabelis and Van de Baan, 1983). When the predator walked upwind and reached the far end of one of the arms, the experiment was terminated. The maximum experimental time was 10 min. Predators that did not walk to the far end of one of the arms were left out of the statistical analysis.

The results have been analyzed using a sign test. The null hypothesis is that the probabilities of the predators to walk to the far end of either arm are equal to 50%. For a more detailed description of the olfactometer, the experimental procedure and statistical analysis, see Sabelis and Van de Baan (1983). The experiments were performed at 26 ± 1°C and 60 ± 10% relative humidity.

RESULTS AND DISCUSSION

Results of experiments using the Y-tube olfactometer show that when *A. potentillae* were reared on broad bean pollen and then starved for 20 hr, they responded to the volatile kairomones of *T. urticae* and *P. ulmi*. However, when they were reared on *T. urticae*, they did not respond to the kairomone of *T. urticae*, but only to that of *P. ulmi* (Table 1). The difference in response between predators reared on the two different diets could be accounted for either

TABLE 1. RESPONSE OF STARVED FEMALE *A. potentillae* TO KAIROMONES OF DIFFERENT SPIDER MITE SPECIES IN Y-TUBE OLFACTOMETER

Predator reared on	Content of arm 1	Content of arm 2	N^a	$N(1)$	$N(2)$	$N(0)$	Critical level
T. urticae	9 bean leaves infested with *T. urticae*	9 clean bean leaves	40	21	19	0	NS[b]
	18 apple leaves infested with *P. ulmi*	18 clean apple leaves	40	31	9	0	$P < 0.001$[b]
V. faba pollen	9 bean leaves infested with *T. urticae*	9 clean bean leaves	60	44	15	1	$P < 0.001$
	18 apple leaves infested with *P. ulmi*	18 clean apple leaves	53	38	14	1	$P < 0.001$

[a] N = number of predators tested; $N(1)$ = number of predators that walked to far end of arm 1; $N(2)$ = number of predators that walked to far end of arm 2; $N(0)$ = number of predators that did not walk to far end of one of the arms.
[b] Results of Sabelis and Van de Baan (1983).

by a genetic difference due to different selection pressures in the two cultures or by a physiological difference due to different diets. No differences in fecundity, developmental time, and mortality have been observed between *A. potentillae* reared on *T. urticae* or *V. faba* pollen (Overmeer, 1981). The predators can be reared equally well on both diets. However, it has been reported that predators reared on broad bean pollen cannot enter reproductive diapause, while those reared on *T. urticae* can (Overmeer and Van Zon, 1983). Because β-carotene is known to affect diapause in the two-spotted spider mite (Veerman and Helle, 1978), this compound was added to the broad bean pollen or to carotenoid-free eggs of albino *T. urticae* that *A. potentillae* were reared upon. This addition restored the predator's ability to enter diapause (Van Zon et al., 1981; Overmeer and Van Zon, 1983). Adding vitamin A, a derivative of β-carotene, to the diet of *A. potentiallae* also has the same effect (Veerman et al., 1983). Thus, a physiological difference between predators reared on the two different diets might cause the difference in response to the *T. urticae* kairomone.

When *A. potentillae* were reared for several generations on broad bean pollen mixed with β-carotene or vitamin A or just on broad bean pollen to which the carotenoid was only added five days prior to the experiment, the predators showed no response to the kairomone of *T. urticae* (Table 2). However, the response to the kairomone of *P. ulmi* was still present when the predators were reared for several generations on broad bean pollen to which crystalline β-carotene was added. Thus, the response of *A. potentillae* to the kairomones of *T. urticae* and *P. ulmi* is identical for predators reared on either *T. urticae* or pollen plus crystalline β-carotene as carotenoid source. These results show that β-

TABLE 2. RESPONSE IN Y-TUBE OLFACTOMETER OF STARVED FEMALE *A. potentillae* REARED ON DIET OF *V. faba* POLLEN TO WHICH CRYSTALLINE CAROTENOIDS WERE ADDED

Predators reared on	Content of arm 1	Content of arm 2	N^a	$N(1)$	$N(2)$	Critical level
V. faba pollen + β-carotene	9 bean leaves infested with *T. urticae*	9 clean bean leaves	40	20	20	NS
V. faba pollen (β-carotene was added 5 days prior to the experiment)	9 bean leaves infested with *T. urticae*	9 clean bean leaves	41	17	24	NS
V. faba pollen + vitamin A acetate	9 bean leaves infested with *T. urticae*	9 clean bean leaves	42	19	23	NS
V. faba pollen + β-carotene	18 apple leaves infested with *P. ulmi*	18 clean apple leaves	40	30	10	$P < 0.001$

[a] N = number of predators tested; $N(1)$ = number of predators that walked to far end of arm 1; $N(2)$ = number of predators that walked to far end of arm 2.

carotene and vitamin A affect the response to the *T. urticae* kairomone. Predators respond to this kairomone when they lack these carotenoids. Because vitamin A is an essential nutrient for inducing diapause and hence for hibernation, it is thus worth foraging for, since the ability to enter diapause is of selective advantage. Predators being under stress conditions (in this case carotenoid-deficiency) would do better by broadening their diet, and the response to the *T. urticae* kairomone by carotenoid-deficient predators can be interpreted in this context.

Amblyseius potentillae is found in orchards, where it is unlikely that the pollen of *V. faba* would be an important food source. Therefore one wonders under what circumstances carotenoid shortage could occur in the field. This question cannot be answered yet, but is of importance in concluding how frequently carotenoids are a limiting factor, and thus how decisive their role is in natural selection.

There are several ways in which *A. potentillae* can obtain carotenoids, and it would be interesting to study how they are affected by depriving *A. potentillae* of carotenoids: (1) Predation on any seizable phytophagous prey. When deprived of carotenoids *A. potentillae* responds to the kairomones of more prey species than after consumption of these compounds (Dicke and Groeneveld, 1986; this paper). (2) Cannibalism, a phenomenon often observed in cultures of *A. poten-*

tillae on broad bean pollen, but not in those on *T. urticae* (Overmeer and Van Zon, 1983; own observation). This only helps the carotenoid lack when the conspecific contains cartenoids. (3) Consumption of the types of pollent that have carotenoids available to the predator. (4) Consumption of red yeasts that are present on the host plant leaf. The phytoseiid mite *Amblyseius finlandicus* (Oudemans) has been found to feed and reproduce on fungal spores (Kropczynska, 1970), but so far *A. potentillae* has not been found to feed on fungi. (5) Phytophagy, a phenomenon reported for the predatory mite *A. hibisci* (Chant) (Porres et al., 1976) that has recently been confirmed for *A. potentillae* as well (Dicke, in preparation).

Thus, it might seem that, in the field, *A. potentillae* usually should have no problem in obtaining carotenoids. Whether this is true should be investigated by sampling predators in the field and testing their ability to enter diapause. However, if obtaining carotenoids is indeed not a problem for *A. potentillae*, this might be the result of the development in evolutionary time of one or more of the five above-mentioned strategies to obtain carotenoids. As carotenoids are unstable under light conditions, this might mean that the transparent *A. potentillae* has to replenish its supply regularly. Still, the amount of carotenoids needed for diapause induction seems to be small; after switching from a diet with carotenoids to one without, the offspring do not loose the ability to enter diapause until in the second generation (Overmeer and Van Zon, 1983). However, a low amount of carotenoids may be insufficient to detect the photoperiodic signal under dim light conditions, as has been reported for larvae of *Bombyx mori* (Shimizu and Kato, 1984).

Whether other functions of carotenoids in *A. potentillae* exist is not known. In other organisms, only photofunctions have been demonstrated for carotenoids (Krinsky, 1971), although many hypotheses about other functions have been put forward (Krinsky, 1971; Karnaukhov et al., 1977). Based on their isolation from bovine olfactory epithelium and the brownish-yellow color of olfactory mucosa, it has also been suggested that carotenoids function as receptors for the energy from odorous molecules (Briggs and Duncan, 1961; Kurihara, 1967). It was thus postulated that smell is impaired by vitamin A deficiency (Briggs and Duncan, 1961). In *A. potentillae*, vitamin A deficiency results in response to the kairomones of an increased number of prey species. In Y-tube olfactometer experiments in which different prey species were offered in each olfactometer arm, carotenoid-deficient *A. potentillae* distinguished between the kairomones of these prey species (Dicke and Groeneveld, 1986). This indicates that olfactory discrimination in *A. potentillae* is improved. The hypothesis of Briggs and Duncan (1961) would predict, however, an impaired olfactory function, and thus this hypothesis cannot hold for olfaction in *A. potentillae*.

A response to kairomones of a larger number of prey species can be thought to occur under stress situations other than carotenoid deficiency. Severe star-

vation can be such a stress situation. A response to the volatile kairomone of *T. urticae* may therefore be present in *T. urticae*-reared *A. potentillae* after starvation for a longer time than the 20 hr of food deprivation used in this study.

Conditioning due to experience with a host species has been observed to affect host recognition by hymenopterous parasitoids (Arthur, 1971; Vinson et al., 1977; Vet, 1983). In the present case, predators that were reared on two-spotted spider mites for many years do not respond to the volatile kairomone of this prey species (Sabelis and Van de Baan, 1983). Only predators that were reared on broad bean pollen and never had any contact with two-spotted spider mites respond to the volatile kairomone of this prey. The response of *A. potentillae* to the volatile kairomone of *T. urticae* is not dependent on previous contact with this prey species, but on whether the predator has available nutrients that are indispensable for diapause induction. The eyeless predatory mite *A. potentillae* can identify a deficiency in β-carotene or vitamin A in its body. As a result, the predator responds to a volatile kairomone of an unprofitable prey species when the alternative is no prey at all. The result of this response is that after finding and consuming this prey species, the predator will have carotenoids available and thus will be able to enter diapause.

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DEFENSIVE MECHANISMS OF LOBLOLLY AND SHORTLEAF PINE AGAINST ATTACK BY SOUTHERN PINE BEETLE, *Dendroctonus frontalis* ZIMMERMANN, AND ITS FUNGAL ASSOCIATE, *Ceratocystis minor* (HEDGECOCK) HUNT

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Abstract—Loblolly and shortleaf pine growing on a single site in the North Carolina piedmont were examined to determine similarities and differences in their defensive mechanisms against the southern pine beetle, *Dendroctonus frontalis* Zimmermann, and its fungal associate, *Ceratocystis minor* (Hedgecock) Hunt. Both species responded to wounding and fungal inoculation by forming a hypersensitive lesion around the wound site. There were significantly less soluble sugars and more monoterpenes in the lesion tissue than in unwounded inner bark. The two species were similar in resin flow rate and inner bark soluble sugar content, but the loblolly pines had thicker bark, longer hypersensitive lesions, and a higher concentration of inner bark monoterpenes. Inner bark monoterpene composition was also significantly different between the two pine species. It is hypothesized that two different defensive strategies against southern pine beetle attack may be utilized.

Key Words—*Dendroctonus frontalis*, *Coleoptera*, *Scolytidae*, *Ceratocystis minor*, *Pinus*, bark beetle, fungus, defense, monoterpenes, soluble sugars, resin flow, hypersensitive lesion.

INTRODUCTION

The southern pine beetle (SPB), *Dendroctonus frontalis* Zimmermann (Coleoptera: Scolytidae), is a serious pest of pines throughout the southern United States. It is capable of attacking and killing all four of the major southern pine species: loblolly, *Pinus taeda* L.; longleaf, *P. palustris* Mill; shortleaf, *P. echinata* Mill;

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and slash, *P. elliottii* Engelm.; but shortleaf and loblolly appear to be the two more susceptible hosts (St. George and Beal, 1929).

Upon attacking a tree, female SPB introduce several fungal species to the host phloem tissue (Bramble and Holst, 1935, 1940). One of these fungal associates is the blue-staining fungus, (*Ceratocystis minor* Hedgecock) Hunt (Rumbold, 1931). The role of this fungus in overcoming tree resistance is not fully understood, but it has been suggested to be a major factor in causing host mortality (Coulson, 1980). *C. minor* may overcome tree resistance by disrupting water movement within the tree (Caird, 1935; Bramble and Holst, 1940).

Berryman (1972) hypothesized a three-step defense sequence by which conifers defend themselves when attacked by bark beetles and their fungal associates. First, there is a wound cleansing step (primary resin flow), followed by an infection containment step (a hypersensitive reaction), and finally a wound healing step (periderm formation). In the southern pines, primary resin flow may act to slow down or prevent colonization by SPB, while the hypersensitive reaction may act to contain the associated fungi.

Primary resin flow may physically prevent successful bark beetle attack by "pitching out" the beetles (Vité and Wood, 1961). In the four major southern pines, tree resistance to SPB has been related to flow, viscosity, and crystalization time of the primary resins (Hodges et al., 1979).

The hypersensitive reaction has been more extensively studied in western conifers (i.e., Reid et al., 1967; Berryman, 1969). The reaction can be described as a rapid necrosis of cells surrounding the infection site, accompanied by the development of traumatic resin ducts and an increased concentration of monoterpenes and phenolics in the reaction zone (Reid et al., 1967). Shrimpton (1973) found a decrease in free-sugars and an increase in starch in the area surrounding a wound. The change in monoterpene composition may be an active defense against bark beetles by accumulating toxic, repellent, or inhibitory materials in the area of attack (Russell and Berryman, 1976). The change in carbohydrate composition may be an indirect defense by limiting a potential beetle/fungal nutrient (Wong and Berryman, 1977). Cook annd Hain (1985) have reported that a hypersensitive reaction also occurs in loblolly pine. They reported a change in monoterpene composition and a decrease in soluble sugars between unwounded phloem and phloem from the hypersensitive reaction zone. However, only qualitative data were presented for the monoterpenes, and the monoterpene and soluble sugar information were from different time periods after wounding. Thus, there was no quantitative measure of monoterpene accumulation in the hypersensitive lesion and no way to determine if monoterpene accumulation and soluble sugar decrease occurred simultaneously. The objective of this study was to quantitatively examine and compare the first two stages in host defense (resin flow and the hypersensitive reaction) of loblolly and shortleaf pine on a single study site in the North Carolina piedmont.

METHODS AND MATERIALS

Experimental work was conducted during the summers of 1983 and 1984 in the Hill Experimental Forest of North Carolina State University, located ca. 20 km north of Durham, North Carolina. The stand was predominantly pine, with loblolly, shortleaf, and virginia pine all being present. Equal numbers of loblolly and shortleaf pine (four in 1983 and eight in 1984) were examined beginning the first week of June, July, and August of each year. One shortleaf pine from the August 1984 sample was later infested with cerambycid larvae and was dropped from the analysis, making the total sample size 36 loblolly and 35 shortleaf pines. All trees used in this study were 50–65 years of age.

On the initial day of sampling during each month, tree physical characteristics (Table 1) were measured and inner bark samples removed for determination of soluble sugar and monoterpene content. The soluble sugar samples were removed with a No. 9 cork borer (13 mm diameter), wrapped in aluminum foil and placed on Dry Ice. The monoterpene samples were also removed with a No. 9 cork borer, placed in 2 ml of *n*-pentane or CH_2Cl_2 and placed on Dry Ice. Both of the inner bark samples were subsequently stored at −20°C until chemical determinations could be made. Also on this day, trees were wounded to the xylem at breast height with a No. 9 cork borer and inoculated with a 1 ml solution of *C. minor* in a 2.5% malt extract solution. The fungi were shake-cultured for 14 days prior to inoculation and were in the mycelial stage.

Two weeks later, the hypersensitive reaction resulting from the fungal inoculation was removed and the lesion length was measured. Two inner bark samples were removed from the hypersensitive lesion, one for soluble sugar determination and one for monoterpene determination. Samples were handled as described above. Also measured at this time was the resin flow rate. Flow was measured on an aspect of the tree perpendicular to the wound reaction. A 2-cm-deep hole with a diameter of 0.64 cm was made in the tree and a glass tube fit snugly into the hole. A calibrated receptacle was attached to the open end of the tube for capturing and measuring the amount of resin. Total flow was measured after 4–5 hr.

Inner bark samples were analyzed for soluble sugar content using the An-

TABLE 1. AVERAGE TREE PARAMETERS ±SD FOR LOBLOLLY AND SHORTLEAF PINES EXAMINED

Species	*N*	dbh (cm)	Height (m)	Bole ht (m)	Phloem (mm)	Bark (mm)
Loblolly	36	34.74 ± 4.56	25.85 ± 2.74	14.63 ± 2.63	1.38 ± 0.32	2.24 ± 0.55
Shortleaf	35	29.32 ± 4.46	24.05 ± 4.05	13.25 ± 2.72	1.26 ± 0.32	1.23 ± 0.29

throne colorimetric technique (Hodge and Hofreiter, 1962; Hazid and Neufeld, 1964). The inner bark samples were analyzed for monoterpenes using a modification of the gas chromatograph program used by Cook and Hain (1985). The program began with an initial temperature of 60°C which was held for 12 min. The temperature was then raised by 15°C/min until a final temperature of 200° was reached. This final temperature was held for 15 min. An internal standard of 1 μl ρ-cymeme was used in each sample. The monoterpene values reported herein are given as ρ-cymene equivalents.

The average resin flow rate (ml/hr), length of the hypersensitive reaction (mm), inner bark soluble sugar content (% dry wt), and inner bark monoterpene content (μl/g dry wt) and composition were calculated for each tree species. Student's t tests were conducted between species to determine if there were significant differences in resin flow or length of the hypersensitive reaction. A two-way analysis of variance was used to determine if differences existed between species or between unwounded and lesion inner bark tissue soluble sugar content. A two-way multivariate analysis of variance was conducted to determine if differences in inner bark monoterpene content or composition existed.

RESULTS

The loblolly pines examined had thicker bark ($t = 9.6163$, $df = 69$, $[P > t] \leq 0.0001$, two-tailed) than the shortleaf pines on this study site (Table 1). There was no significant difference in the average resin flow rate between the two species ($t = 0.9074$, $df = 69$, $[P > t] = 0.3674$, two-tailed); however, there was a large range in measurements within both species (Table 2). There was also a large range in the length of the hypersensitive reaction produced by the trees when wounded and inoculated with *C. minor* (Table 2), but overall, the loblolly pine produced a significantly longer lesion ($t = 2.2644$, $df = 69$, $[P > t] = 0.0267$, two-tailed) than did the shortleaf pines.

Unwounded inner bark soluble sugar content (percentage of the total inner bark dry weight) averaged 11.15 $\pm$ 5.08% ($n = 36$) in the loblolly pines and 10.36 $\pm$ 2.76% ($n = 33$) in the shortleaf pines. This was significantly greater

TABLE 2. MEASURES OF RESIN FLOW RATE AND LENGTH OF HYPERSENSITIVE LESION FOR LOBLOLLY AND SHORTLEAF PINES EXAMINED

		Resin flow rate (ml/hr)		Lesion length (mm)	
Species	N	$\bar{X} \pm$ SD	min, max	$\bar{X} \pm$ SD	min, max
Loblolly	36	0.29 $\pm$ 0.27	0.00, 1.11	100.8 $\pm$ 25.9	61.0, 150.0
Shortleaf	35	0.24 $\pm$ 0.20	0.00, 1.00	87.4 $\pm$ 24.0	47.0, 138.0

($F = 238.03$, $df = 1,125$, $[P > F] \leq 0.0001$) than the lesion soluble sugar content of the loblolly, $2.28 \pm 0.96\%$ ($n = 30$), and shortleaf, $2.09 \pm 1.37\%$ ($n = 29$), pines examined. The inner bark soluble sugar content was not significantly different between the two species ($F = 1.28$, $df = 1,125$, $[P > F] = 0.2596$).

The inner bark monoterpene concentrations (Table 3) were significantly higher in the hypersensitive lesion opposed to unwounded tissue ($\Lambda = 0.3286$, $F = 36.10$, $df = 6,106$, $[P > F] \leq 0.0001$) but, unlike the soluble sugars, there was also a significant difference between the monoterpene concentrations in loblolly and shortleaf pine ($\Lambda = 0.7657$, $F = 5.41$, $df = 6,106$, $[P > F] \leq 0.0001$). The between-species difference in inner bark monoterpene concentrations also represented a between-species difference in monoterpene composition ($\Lambda = 0.7254$, $F = 8.10$, $df = 5,107$, $[P > F] \leq 0.0001$). In loblolly pine, the change in inner bark monoterpenes from unwounded to lesion tissue resulted in a higher proportion of α-pinene being present and a lower proportion of β-pinene, while in shortleaf pine there was a lower proportion of α-pinene and a higher proportion of β-pinene in the lesion than in unwounded tissue (Figure 1). The monoterpene composition was significantly different between the unwounded and lesion tissue ($\Lambda = 0.6063$, $F = 13.90$, $df = 5,107$, $[P > F] \leq 0.0001$).

DISCUSSION

The loblolly and shortleaf pines responded similarly when wounded and inoculated with *C. minor*. Both species have well-developed resin canal systems and when severed, the resin flows down and out through the wound hole. Both species produced a necrotic lesion around the invasion site, and the lesion tissue contained significantly lower amounts of soluble sugars and higher concentrations of monoterpenes than was found in unwounded tissue. The results on the hypersensitive reaction agree with previous work on loblolly pine which reported decreases in reducing sugars following SPB introduction (Barras and Hodges, 1969) and in the hypersensitive lesion four weeks following fungal inoculations (Cook and Hain, 1985). The change in monoterpene content from unwounded to lesion tissue agrees with work by Cook and Hain (1985) on loblolly pine and Reid et al. (1967) on lodgepole pine. Russell and Berryman (1976) suggested that the change in monoterpene composition may be an active defense against bark beetles and their associated fungi. Based upon lethal dosage, Coyne and Lott (1976) ranked the toxicity of the six monoterpenes to SPB as limonene > myrcene > α-pinene > β-pinene > β-phellandrene > camphene. The increased concentration of all six of these monoterpenes in the lesion tissue and the changed composition of the monoterpenes appears to support the hypothesis of Russell and Berryman (1976).

TABLE 3. AVERAGE CONCENTRATIONS OF MONOTERPENES PRESENT IN UNWOUNDED TISSUE AND IN HYPERSENSITIVE LESIONS OF LOBLOLLY AND SHORTLEAF PINES EXAMINED

Species		N	Monoterpenes (μl/g inner bark dry wt $\pm$ SD)[a]					
			α-Pinene	Camphene	β-Pinene	Myrcene	Limonene	β-Phellandrene
Loblolly	Preformed	28	3.28 $\pm$ 4.97	0.18 $\pm$ 0.15	0.84 $\pm$ 1.39	0.06 $\pm$ 0.10	0.06 $\pm$ 0.12	0.02 $\pm$ 0.06
	Induced	30	665.56 $\pm$ 442.22	11.71 $\pm$ 9.34	159.22 $\pm$ 127.15	16.55 $\pm$ 23.79	9.41 $\pm$ 8.58	4.29 $\pm$ 4.77
Shortleaf	Preformed	28	2.88 $\pm$ 4.32	0.14 $\pm$ 0.14	0.83 $\pm$ 1.16	0.05 $\pm$ 0.10	0.06 $\pm$ 0.12	0.03 $\pm$ 0.08
	Induced	28	532.34 $\pm$ 349.13	8.31 $\pm$ 6.10	360.77 $\pm$ 297.13	2.28 $\pm$ 4.23	6.76 $\pm$ 7.10	5.29 $\pm$ 4.97

[a] Monoterpene values are presented in ρ-cymene equivalents.

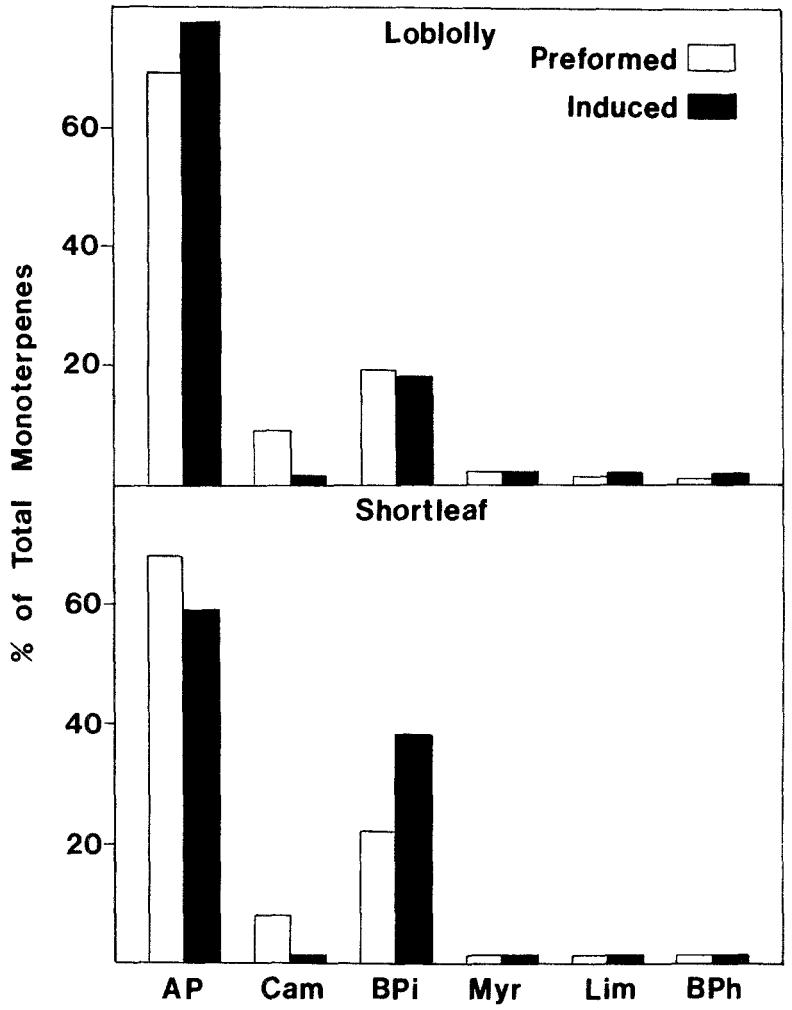

FIG. 1. Average composition of the inner bark monoterpenes from unwounded tissue (preformed) and the hypersensitive lesion (induced) for the loblolly and shortleaf pines examined. The monoterpenes were α-pinene (AP), camphene (Cam), β-pinene (BPi), myrcene (Myr), limonene (Lim), and β-phellandrene (BPh).

Preformed tree defenses against bark beetles and their associated fungi can be viewed as physical and/or chemical barriers which are present prior to attack and must be overcome by the invader. We examined two preformed physical barriers, bark thickness and resin flow rate, and one preformed chemical barrier, inner bark monoterpenes. Thicker fissure bark has previously been associated with SPB infestations where the preferred host was loblolly pine but not in areas

where shortleaf pine was the preferred host (Hicks, 1980). Although thick bark may constitute a physical barrier to attacking beetles and result in more intense predation (Franklin and Green, 1965), once attack is successful the SPB may benefit because bark thickness adversely affects some SPB parasitoids (Goyer and Finger, 1980). Also, if brood survival increases with bark thickness, the probability of adjacent trees, those that will be attacked next (Coster and Gara, 1968), being successfully attacked should increase because the number of potential attacking beetles would increase.

Resin flow rate was one of the variables used by Hodges et al. (1979) to separate resistant from susceptible southern pines. Higher flow rates can prevent beetles from becoming established in a tree and, even if the beetles are not "pitched out" (Vité and Wood, 1961), the rate of gallery construction is apparently related to the amount of resin encountered by the beetles (MacAndrews in Thatcher, 1960). Although not significantly different between species, the resin flow rates showed considerable tree-to-tree variation which may be a factor in an individual tree's susceptibility or resistance to SPB.

There were higher concentrations of α-pinene, camphene, β-pinene, and myrcene in the unwounded inner bark and higher concentrations of α-pinene, camphene, myrcene, and limonene in the hypersensitive lesion of loblolly compared to shortleaf pine. All of these substances are toxic to SPB (Coyne and Lott, 1976), and α-pinene, β-pinene, myrcene, and limonene are toxic to the western pine beetle, *D. brevicomis* LeConte (Smith, 1965). Along with being toxic, differences in monoterpene composition within the trees may be important. For example, Gollob (1980) suggested that loblolly pines with higher myrcene content may be viewed as nonhosts by SPB.

Host odors are important to attacking SPB populations, and α-pinene is believed to be the most important of these tree-produced volatiles (Renwick and Vité, 1969). By itself, α-pinene acts as a synergist for the SPB aggregation pheromone, frontalin (Kinzer et al., 1969; Renwick and Vité, 1969). Although turpentine appears to be a more effective pheromonal synergist (Payne et al., 1978), the lowered proportion of α-pinene in shortleaf tissue following attack may serve to slow mass aggregation of the beetle and allow the tree time to further respond with other defensive mechanisms. The decreased percentage of α-pinene is not large and possibly has no effect on the attacking SPB. However, we believe the hypothesis that the change does affect SPB attack behavior is plausible and could be tested.

The hypersensitive lesion produced in response to fungal inoculation was longer in loblolly than in shortleaf pine. The lesion area was resin soaked, high in monoterpene content, and low in soluble sugars in both host species. A larger reaction would force the beetles to expend more energy to reach suitable brood material and thus may be correlated with a higher probability of containing both the beetles and the associated fungi. Due to physiological differences between the two species, the difference in lesion size may or may not be important in

comparing species resistance to SPB attack. If the invading organisms are contained in the lesion, wound periderm will seal off the necrotic area, thereby protecting adjacent tissue from any possible adverse affects (Mullick, 1977).

Based upon the above information, we hypothesize two different strategies of host defense against SPB and its associated fungi. One strategy would be to rely upon strong quantitative defenses. The result would be a thick-barked tree with a high resin flow rate and a strong hypersensitive reaction. The second strategy would involve a qualitative defense. This may result in the production of nonattractive or less attractive host odors, as might be the result of a rapid change in monoterpene composition following beetle attack. Both loblolly and shortleaf pine possess characteristics within each defensive strategy. Both species have quantitative defenses of resin flow and a hypersensitive response with monoterpene accumulation, and both possess qualitative defenses as evidenced by the change in monoterpene composition. The loblolly pines examined appear to rely more heavily on a quantitative defense, while the shortleaf pines appear to have a better developed qualitative defense.

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NECRODOLS: Anti-insectan Terpenes from Defensive Secretion of Carrion Beetle (*Necrodes surinamensis*)[1]

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Abstract—Two compounds recently isolated from the defensive gland of *Necrodes surinamensis*, α- and β-necrodol, first representatives of a new category of monoterpenes (the necrodanes), are shown to be repellent to ants and other insects and irritating to cockroaches and flies. The compounds doubtless play a defensive role in *Necrodes*. The possible long-range applied significance of research on insect repellents is discussed.

Key Words—Coleoptera, Silphidae, *Necrodes surinamensis*, carrion beetle, monoterpenes, iridoids, necrodols, defensive secretion, insect repellent, insect irritant, bioassay.

INTRODUCTION

Monoterpenes, long recognized as major constituents of plant essential oils, play important roles as defensive agents and pheromones in the animal kingdom (Weatherston, 1973). The occurrence of over 30 acyclic, monocyclic, bicyclic, and tricyclic monoterpene skeletons, all enzymatically derived from geranyl pyrophosphate (1) (Figure 1), attests to the wide variety of functions served by these compounds (Devon and Scott, 1972; Iwasaki and Nozoe, 1974). Within this structural diversity, the iridoids, characterized by the 1-isopropyl-2,3-dimethylcyclopentane skeleton (2), first found in nepetalactone (3), are the only

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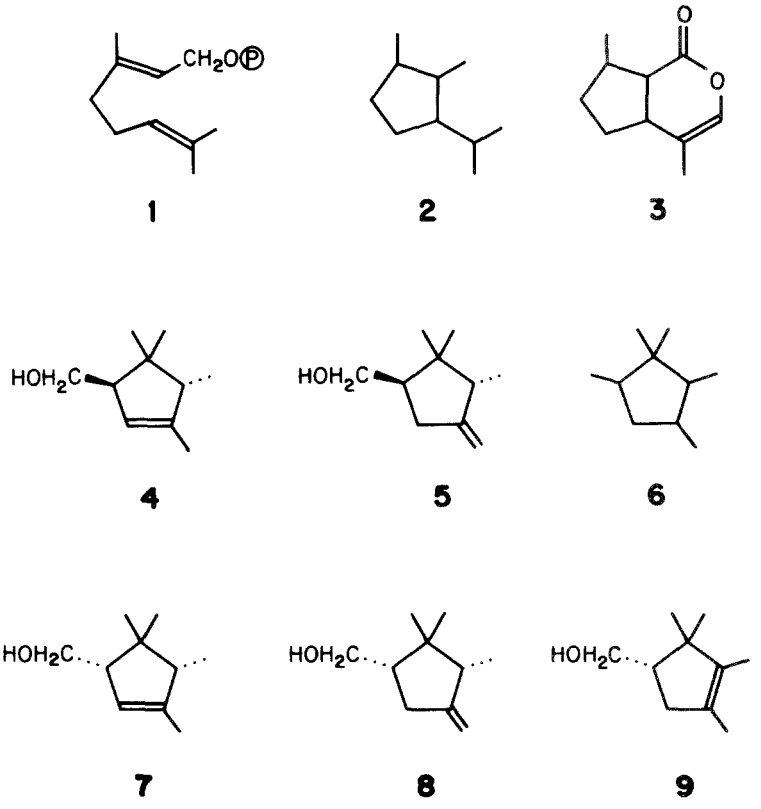

FIG. 1. Chemical structures

widely distributed monoterpenes based on the cyclopentane nucleus (Devon and Scott, 1972; Iwasaki and Nozoe, 1974).

In this context, the discovery of α- and β-necrodol (4, 5) from a silphid beetle, *Necrodes surinamensis*, is interesting (Eisner and Meinwald, 1982). These terpene alcohols appear to be the first representatives of a new family of cyclopentanoid monoterpenes, based on the 1,2,2,3,4-pentamethylcyclopentane nucleus (6), which we propose to call necrodanes. The necrodols are produced by the rectal gland of *Necrodes*. The products of this gland, together with enteric fluid, are ejected as an aimed spray when the beetle is under real or simulated attack. Predators such as ants are deterred by the discharges (Eisner and Meinwald, 1982). This in itself suggested a defensive role for the necrodols, but an insufficient quantity of the compounds was initially isolated from the beetles for biological testing. We have now synthesized milligram quantities of 4 and 5, together with some closely related structures, epi-α-necrodol (7), epi-β-necrodol (8), and γ-necrodol (9), and here present evidence that these compounds are repellent and topically irritating to insects.

METHODS AND MATERIALS

Syntheses of 4, 5, 7, 8, and 9 from (+)-camphoric acid, a readily available precursor of known absolute configuration (Beilstein, 1971), have been described (Jacobs, 1985) and will be published elsewhere. All compounds are liquids at ambient temperature.

In all the following tests, sample presentation was by way of capillary tubing of inner diameter = 619 ± 0.34 μm (mean ± SE; $N = 20$).

The general deterrency tests were carried out with β-necrodol only, the compound available in greater quantity. The tests were similar to those previously carried out with nepetalactone (Eisner, 1964) and consisted of observing the response of a variety of insects to the vapors emanating from the tip of a capillary tube filled with β-necrodol, presented to the insects manually at near contact range. The insects tested (Table 1) were a mixed array that had come to rest at night on an illuminated surface outdoors, on the grounds of the Archbold Biological Station, Lake Placid, Florida. The protocol involved initial presentation of a control (glass capillary tube filled with distilled water), first from one side of the body (5 sec), then the other (5 sec), followed by presentation of β-necrodol (5 sec on each side of body) if the response to the control was negative (no movement toward or away from capillary tube). A response was scored as aversive if the insect turned away from the necrodol both times. Respondents and nonrespondents were collected for subsequent identification.

The assay with ants (*Monomorium pharaonis*), also carried out at the Archbold Biological Station, involved testing for the repellency of β-necrodol vapors to workers feeding at a sugar bait. The tests were carried out near a natural colony of the ant, at a site to which foragers had laid a network of trails. The bait (10^{-1} M sucrose) was presented as a drop of fixed circumference (spread to the rim over the surface of a circular glass cover slip of 18 mm diameter). When ants had become tightly aligned to feed along the entire perimeter of the drop, a capillary tube with β-necrodol was abruptly swung into place with a mechanical arm that brought the tip of the capillary to a point 2 mm above ground just outside the margin of the drop. Presentation of the sample invariably induced dispersal of a substantial fraction of the feeding ants. The test was replicated eight times. For each test, counts of ants feeding at the drop were taken (from photographs) at 5-sec intervals, from 25 sec before to 25 sec after sample presentation, providing the basis for determination of the percentage of ants repelled as a function of time. Data for the five time transects prior to presentation of the sample showed no significant variability; these data were lumped for all tests ($N = 40$) for calculation of the base value of 0% repellency. Control presentations of empty capillaries, or of capillaries filled with inert chemicals (H_2O, glycerol), failed to induce ant dispersal.

Two tests were used for assessment of the topical irritancy of the necrodols, one with the cockroach *Periplaneta americana*, the other with the fly

TABLE 1. INSECTS REPELLED AND NOT REPELLED BY β-NECRODOL (VAPOR PHASE)[a]

		Repelled	Not repelled
Coleoptera	Helodidae	*Ora* sp.	
	Bostrichidae	*Prostephanus punctatus* (2)	
	Scolytidae	*Corthylus spinifer*	
	Chrysomelidae	*Chrysomela scripta*	
		Dysonycha conjugata	
	Curculionidae	*Neochetina eichhornia*	
	Tenebrionidae	*Leichenum seriehispidum*	
	Alleculidae	*Hymenorus* sp.	
	Anthicidae	*Tomoderus constrictus* (3)	
	Dytiscidae	*Celina angustata*	
	Hydrophilidae	*Berosus* sp.	
		Enochrus sp.	
	Carabidae	*Pentagonica picticornis*	
		Clivinia sp.	
		Stenolophus sp.	
		Anatrichus minutus	
	Cucujidae	unidentified	
	Staphylinidae	*Paederus floridanus*	
		unidentified	
		unidentified	
		unidentified (3)	
	Scarabaeidae	*Aphodius lividus* (3)	*Diplotaxis bidentata* (2)
		Ataenius gracilis	*Cyclocephala parallela*
		A. alternatus (2)	*Anomala* sp.
		A. imbricatus	
		A. simulator (2)	
	Cantharidae		*Polemius laticornis*
	Elateridae		*Conoderus falli*
			C. scissus (2)
Hymenoptera	Formicidae	*Monomorium pharaonis* (4)	
	Braconidae	*Cyllostome* sp.	*Rogas* sp.
	Cynipidae		unidentified
Hemiptera	Cydnidae	*Amnestus basidentatus* (2)	
		Pangaeus bilineatus (2)	
	Lygaeidae	Rhyparochrominae	*Nysius* sp.
	Corixidae		unidentified
Lepidoptera	Noctuidae		*Leucania* sp.
	Pyralidae		*Blepharomastix* sp.
			Paraponyx sp.
			unidentified
	Psychidae		*Platoeceticus* sp.
Ephemeroptera	Leptoceridae		*Oecetis* sp. (3)
	Caenidae		*Caenis* sp.
	Baetidae		*Callibaetis floridanus* (3)

[a] All tests based on single specimens, except where indicated by larger number in parentheses.

Phormia regina. The cockroach test was based on the observation (Eisner, 1961) that direct application of chemical irritants to *Periplaneta* induces preening reflexes. The responses are highly stereotyped, and the delay to their onset provides a measure of the irritancy of a sample. For assay purposes, late-instar decapitated nymphs are used, and the site of application of the chemical is the abdomen. We have used the test for assay of other chemicals (Eisner et al., 1961; Smolanoff et al., 1975), and the protocol is described in detail elsewhere (Eisner et al., 1976). Both α- and β-necrodol were assayed, as well as compounds 7–9, with 20 nymphs per sample. The compounds were tested in pure form, and stimulation was by near contact presentation (capillary tubing with sample held at less than 1 mm from the surface of the fourth or fifth abdominal tergite, to one side of midline). Delay to onset of scratching was timed to the nearest second.

The test with *Phormia* was also based on a preening reflex. When subjected to an irritant vapor, *Phormia* everts the proboscis, regurgitates a droplet onto its tip (the labellum), and proceeds to wipe the labellum against the substrate. In tethered flies, the substrate can be made inaccessible, and proboscis extension alone, either with or without regurgitation, can serve as an indication of the response. As with the *Periplaneta* test, delay to onset of extension provides a measure of the potency of the irritant. Details of the protocol are provided elsewhere (Eisner et al., 1976). Both α- and β-necrodol were tested, as well as compounds 7–9, with 20 flies per sample. Presentation was at close range (capillary tube held at 0.3–0.6 mm from labellum), and response was timed to nearest second.

Statistical comparisons were by the *K*-sample Smirnov test (Conover, 1971).

RESULTS

As is clear from Table 1, β-necrodol proved repellent to a diversity of insects (31 species), including Coleoptera (14 families), Hymenoptera (2 families), and Hemiptera (2 families). Among the Hymenoptera was *Monomorium pharaonis*, the ant also used in our feeding deterrency test. Unaffected by the compound were 18 species, comprising Coleoptera (three families), Hymenoptera (one family), Hemiptera (three families), Lepidoptera (three families), and Ephemeroptera (three families). Only three families, the Scarabaeidae, Lygaeidae, and Braconidae, are represented on both tables.

The potency of β-necrodol vis à vis *Monomorium* is evident from Figure 2. By 5 sec after presentation of the compound, the repellency had virtually peaked, indicating that it was essentially of immediate onset. Visual observation tended to confirm this. No sooner had the tube with the sample swung into place, than the ants turned away from the food and fled. Only ants positioned

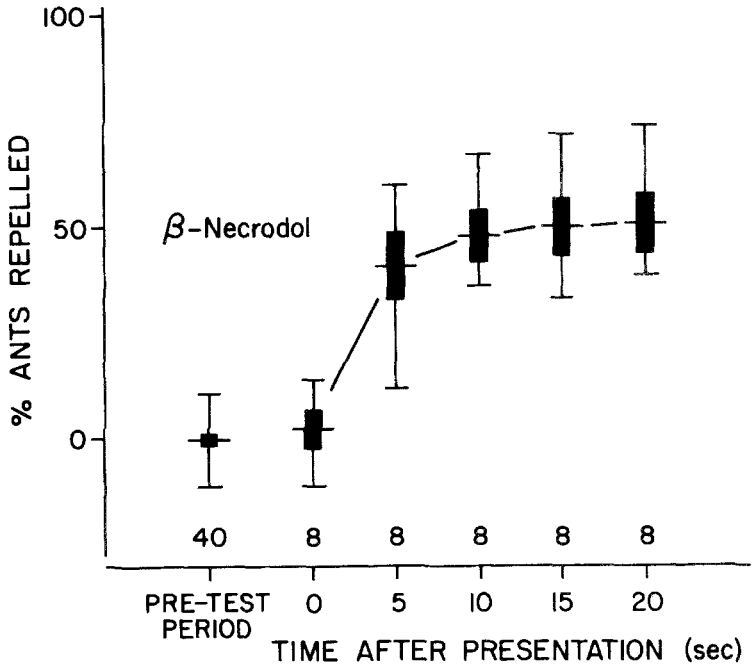

FIG. 2. Repellency of β-necrodol (vapor phase) to the ant *Monomorium*. Details in text. Horizontal lines, vertical lines, and vertical bars give means, ranges, and one standard error on each side of mean, respectively. Numbers above baseline give sample sizes.

furthest from the stimulus (those feeding from the drop across from the site of presentation of the tube) failed to respond.

The data on topical irritancy to roaches and flies are plotted for α- and β-necrodol only (Figure 3). The compounds proved equally active in each test ($P > 0.1$ for both cases). *Phormia* is more sensitive than *Periplaneta* to the compounds, a difference that we know to hold also for other topical irritants (e.g., *p*-benzoquinone, acetic acid, 2-hexenal; all tested in vapor form). Compounds 7, 8, and 9 (data not plotted) proved repellent in both tests. With *Periplaneta*, their activity matched that of the two necrodols ($P > 0.1$). With *Phormia*, 8 and 9 proved as active ($P > 0.1$), and 7 somewhat less active ($P < 0.05$), than the necrodols.

DISCUSSION

There seems little doubt that α- and β-necrodol contribute to the defensive action of the *Necrodes* spray. Although repellency data were obtained for β-necrodol only, one is tempted to presume α-necrodol to be similarly repellent,

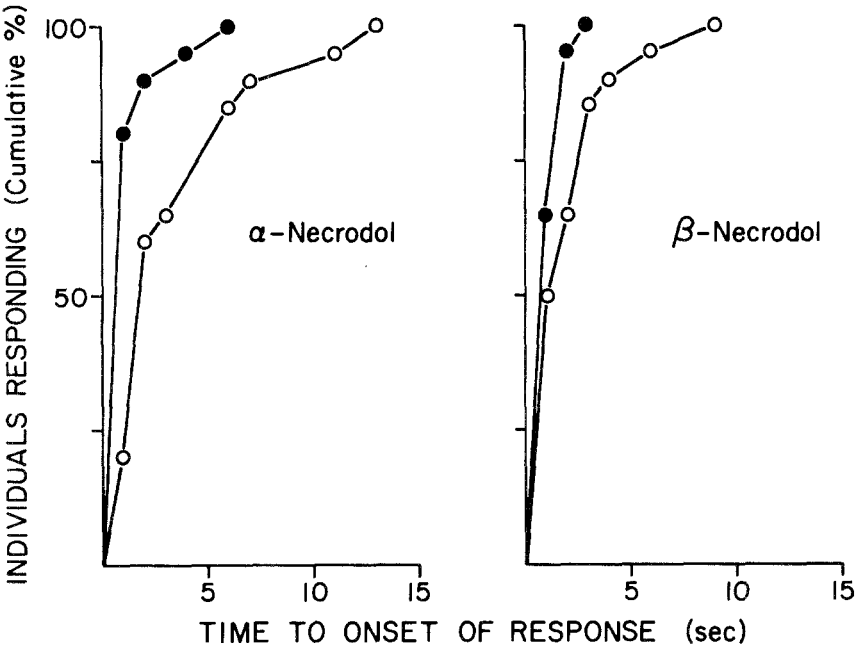

FIG. 3. Topical irritancy of α- and β-necrodol (vapor phase) to *Periplaneta americana* (open circles), and *Phormia regina* (closed circles). Details in text. Horizontal lines, vertical lines, and vertical bars give means, ranges, and one standard error on each side of mean, respectively; $N = 20$ for all four tests.

given the equal potency shown by the two compounds in the irritancy tests. To what extent the necrodols are supplemented or even synergized in their activity by some of the other components (Eisner and Meinwald, 1982) of the beetle's spray remains unknown. Certainly, one component, octanoic acid, has deterrent potential of its own. It is a proven topical irritant (*Periplaneta* test), and as part of the defensive secretion of another arthropod has been shown to have surfactant and penetration-promoting activity (Eisner et al., 1961). Another component, lavandulol, known also from essential oils of plants (Karrer, 1958), may be anti-insectan as well.

The fact that there were insects that failed to respond to β-necrodol should come as no surprise, since chemical repellents often have restricted rather than generalized activity.

The irritancy tests with *Periplaneta* and *Phormia* have become staples in our laboratories for the screening of natural products for insect deterrency. The cleansing responses elicited in these insects by chemical stimulation attests, we feel, to the existence in these species (as perhaps primitively in insects as a whole) of a "common chemical sense" (Keele and Armstrong, 1964; Mon-

crieff, 1944), a generalized, broadly attuned sensitivity to noxious chemical stimuli. The common chemical sense may, in fact, be the primary sensory input system that mediates insect repellency. The necrodols are not exceptional in being, at the same time, repellent to insects and effective in the *Periplaneta* and *Phormia* irritancy tests. We have extensive data on compounds (or chemical mixtures) of arthropodan and plant origin (for example, components of insect defensive secretions; plant essential oils) that support the contention that potency in the two irritancy tests correlates with repellency. The irritancy tests, therefore, provide a quick means for obtaining first-approximation quantitative data on the insect repellency of compounds.

Screening for repellency, fashionable before insecticides came into vogue, has long ceased to be a matter of priority. Naturally occurring repellents, one came to learn, are altogether too often ill-suited for purposes of insect control. For the chemical ecologist, the search for new repellents never lost its appeal, for with the characterization of novel compounds there often came the discovery of new biological roles for chemical factors in nature. But for use in conventional control programs, repellents were usually too volatile, unstable, or generally toxic. The prospects of biotechnological approaches to insect control put the search for insect repellents into new perspective. The possibility, for example, of conveying upon a plant the capacity to produce its own insect repellents, by transferring to that plant appropriate genes from another that already has the capacity, is no longer fanciful. Gene transfer from plant to plant is bound to become an applicable reality. The questions that will then arise are which genes and the capacity to produce which repellents. Chemical ecology, through discovery of appropriately active natural products, is now laying the groundwork that will help answer the second of these questions. Compounds such as cyclopentanoid terpenes, of which the necrodols are examples, which have evolved so often and proven themselves in so many different natural defensive contexts, may well be prime candidates for eventual exploitation in the context of biotechnological control.

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SEX PHEROMONE OF *Eupoecilia ambiguella* FEMALE: ANALYSIS AND MALE RESPONSE TO TERNARY BLEND

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Abstract—Sex gland extracts and washes of *Eupoecilia ambiguella* contain 10–20 ng/female of the primary sex pheromone component *Z*-9-dodecenyl acetate (*Z*9–12 : Ac), accompanied by a number of related compounds. These are *E*-9-dodecenyl acetate (*E*9–12 : Ac), *Z*-9-dodecen-1-ol (*Z*9–12 : OH), saturated acetates of 12, 16, 18, and 20 carbons, and traces of a doubly unsaturated acetate, tentatively identified as a 9,11-dodecadienyl acetate. Octadecyl acetate predominates among the pheromone-related components, making up 1–2, occasionally 20–30 times the amount of *Z*9–12 : Ac. The same compounds were also found in field-collected females and in effluvia. *Z*-9-Undecenyl acetate, which is a male attractant on its own, was also found in a sample of female effluvia. A hierarchy is observed in the ethological function of the pheromone components. *Z*9–12 : Ac is an attractant for *E. ambiguella* males. Dodecyl acetate (12 : Ac) is not attractive on its own but augments male catch when added to the main attractant. Addition of 18 : Ac augments attraction only when both *Z*9–12 : Ac and 12 : Ac are present. Wind-tunnel tests demonstrate that 18 : Ac also raises the disorientation threshold, as previously shown for 12 : Ac. Other compounds, with the possible exception of additional saturated acetates, had either no effect on trap catch or, in the case of *E*9–12 : Ac, *Z*9–12 : OH, and *E*-9,11-dodecadienyl acetate, were inhibitory above a certain level. A blend of roughly equal parts of *Z*9–12 : Ac, 12 : Ac, and 18 : Ac provides the best attractant blend for *E. ambiguella* known to date.

Key Words—*Eupoecilia ambiguella*, Lepidoptera, Tortricidae, sex pheromone, sex attractant, synergist, Z-9-dodecenyl acetate, dodecyl acetate, octadecyl acetate.

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INTRODUCTION

The tortricid moths *Eupoecilia ambiguella* Hbn. and *Lobesia botrana* Den. et Schiff. are the most important insect pests of European vineyards (Bovey, 1966). Although they coexist in many regions, *L. botrana* predominates in the Mediterranean countries while *E. ambiguella* is mainly found in more Northern, humid climates, particularly along the Rhine valley. Sex pheromone research on these two species has a long history, highlighted by the invention of the clock trap (Götz, 1941b) and early claims of control by mass trapping (Götz, 1941a).

The identification of Z-9-dodecenyl acetate (Z9–12:Ac; for other short forms see Table 1) as a sex pheromone component and male attractant was reported by Arn et al. (1976) and Saglio et al. (1977). The synergistic effect of 12:Ac was demonstrated by Arn et al. (1979); wind-tunnel and field studies involving this compound and Z-10-tridecenyl acetate were presented by Rauscher et al. (1984). Various reports (Roehrich et al., 1979, Arn et al., 1981, Winkelmann-Vogt and Schropp, 1984) deal with communication disruption as a means of grape moth control. Here we present the results of more detailed analyses of the female blend and of field and wind-tunnel tests with the compounds identified.

METHODS AND MATERIALS

Insects. A continuous culture of *E. ambiguella* was maintained in the laboratory (Rauscher et al., 1984). About 100 wild females were obtained from collection of second-generation larvae which were kept in the insectarium until spring. Sexed pupae and adults (provided with 10% sucrose solution) were held in an 18:6 hr photoperiod at 24°C, 56% relative humidity with 3000–6000 lux in the photophase. Pheromone extracts were made from 3-day-old females in the middle of the scotophase.

Preparation of Extracts. Female wash was prepared in near darkness (4 lux) by sucking calling females through a vacuum tube into a flask containing 0.5–1 ml methylene chloride (Merck, analytical grade). Each collection lasted a maximum of 30 sec (10–20 females) after which the solution was transferred to another vial. Typically, an extract from 100–200 females was obtained in one session. The pooled solution was filtered and reduced to 100 μl. Sex gland extracts were prepared as described by Guerin et al. (1986) by excising ovipositor tips in a minimum of hexane (Merck, for residue analysis, 1 μl/tip). For collection of effluvia, 50–100 females were placed in a 1-liter glass bottle lined with nylon gauze as a resting site and supplied with synthetic air at ca. 50 ml/min through a metal-bellows regulator. The air from the bottle passed through a Pasteur pipet with ca. 1 ml glass beads (1 mm diam.) for ca. 3 hr. These were

then extracted with 0.5 ml hexane and the eluate concentrated at room temperature.

Gas Chromatography–Mass Spectrometry. GC-MS was carried out on a Finnigan 4015 instrument using 25- and 50-m Silar 10c high-resolution glass capillary columns. Spectra were recorded using electron-impact (EI, 70 eV, 240°C, *m/z* 35–535) and chemical ionization (CI, isobutane 0.4 torr, 180°C, *m/z* 85–585). For trace level analyses of acetates, selected ion monitoring was carried out using *m/z* 61 ($CH_3COOH_2^+$ and M^+-60 (CH_3COOH) ions. Typically, the columns were temperature programmed as follows: 60°C, 2 min, 20°/min to 100°C, 4°/min to 250°C.

For determination of double-bond positions, a sex gland extract of 100 females in 0.1 ml hexane was first reacted with 20 μl trifluoroacetic anhydride for 1 hr at 50°C in a sealed ampoule for esterification of alcohols. After blowing off the reagent with nitrogen, the sample was derivatized with dimethyl disulfide (DMDS) as previously described (Buser et al., 1983).

Gas chromatography with electroantennographic detection (GC-EAD) was done as described (Arn et al., 1975; Guerin et al., 1985).

Field Tests. These were made in various vineyards in Switzerland. Two most suitable locations where *E. ambiguella* was abundant were Yvorne VD and Maienfeld GR. The tetra trap (base 9.5 × 16.5 cm and 9.5 cm high, Figure 1A), which can be folded from a rectangular sheet of laminated paper, and the rubber cap used as dispenser have already been described (Arn et al., 1979).

Trap Placement. In numerous tests with orchard and vineyard tortricids we have observed a high variability in trap catch which appears to stem from at least four sources, (1) long-range differences, apparently connected with pop-

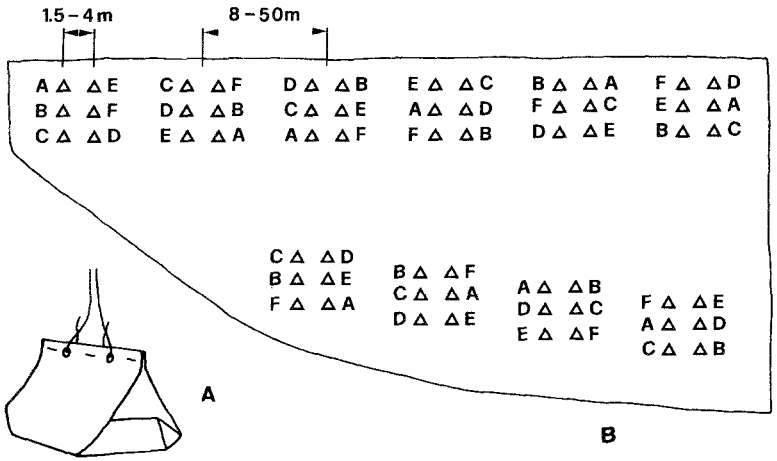

FIG. 1. (A) The tetra trap. (B) Arrangement of traps in a field test with six treatments and 10 replicates.

ulation distribution, often visible as a gradient across a given area and effective at distances as low as 5–10 m; (2) a border effect arising from a higher source of moths outside than inside the experimental area (competition effect); (3) effects of wind direction visible from the fact that traps closest to and farthest away from the wind receive the highest catch; and (4) microenvironment effects: some traps repeatedly attract many more insects than their neighbors, although their bait is the same and the surroundings supposedly identical.

In a replicated comparison of attractant blends, placing the treatments at the fairly large distances used by Perry et al. (1980) was not feasible due to lack of space and variations in moth density. On the other hand, rotation of traps during moth flight was impractical because of travel distances. We therefore adopted the following procedure: The traps to be compared (treatments) are placed in a group at distances of 1.5–4 m, but the groups of traps (replicates) are spaced apart as widely as possible (8–50 m) to make the best use of the moths available (Figure 1B). The position of a given treatment within replicates is systematically varied so that it occupies every position as often as possible. Neighboring of the same two treatments with respect to wind direction is kept to a minimum.

A convenient arrangement for a test with six treatments is shown in Figure 1B. Even with 10 replicates, differences in catch of a factor of two are often required for significance. As an alternative to the above design, we have, on occasion, placed all treatments and replicates in one long row. In this case, at least four buffer traps whose counts are disregarded are required at each end. Dose–response curves with individual compounds added to the main attractant or a blend were established with steps of factors of four to five or less. Trap catches were usually recorded once or twice a week; counts were transformed to log $(x + 1)$ and submitted to two-way analysis of variance followed by Duncan's multiple-range test ($P = 0.05$).

Wind-tunnel tests were made with laboratory-reared males as previously described (Rauscher et al., 1984).

RESULTS

Chemical Analysis

GC-EAD. All types of female extracts revealed one major product active on the *E. ambiguella* male antenna, coinciding with Z9–12 : Ac. Occasionally, with highly sensitive antennal preparations, small but reproducible responses were obtained at the elution times of 12 : Ac, *E*9–12 : Ac, and 18 : Ac (Figure 2).

GC-MS. The pheromone-related components identified in the female material are listed in Table 1. The main unsaturated component is Z9–12 : Ac, as

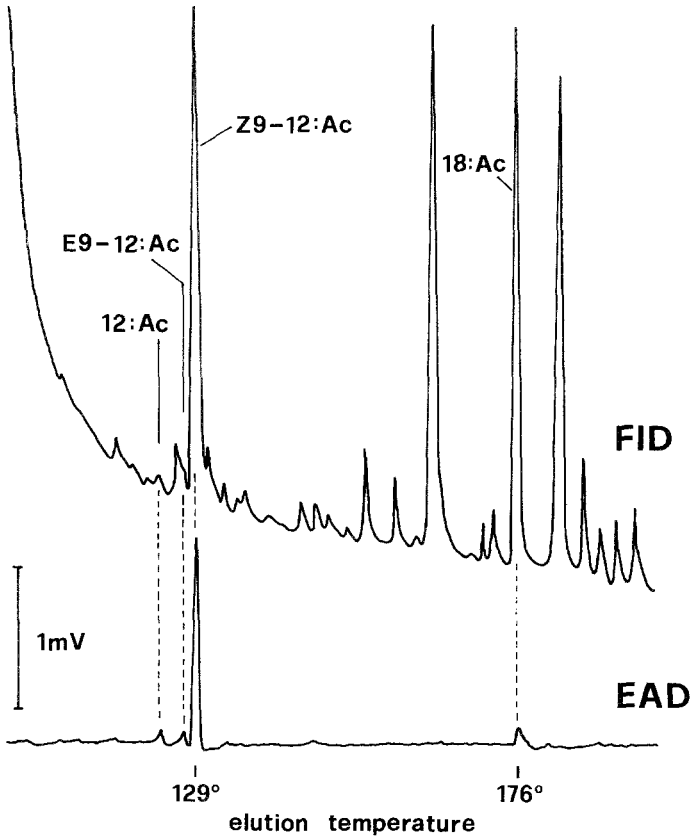

FIG. 2. Gas chromatogram of *Eupoecilia ambiguella* female wash (two female equivalents) using flame ionization (FID) and electroantennographic (EAD) detection with conspecific male antenna. Silar 10c glass capillary column (0.25 mm × 25 m); 2 min at 60°C, 20°/min to 100°C, 5°/min to 250°C.

reported previously (Arn et al., 1976, Buser et al., 1983). *E*9–12 : Ac was found in all preparations in amounts between 0.1 and 1% of *Z*9–12 : Ac. Its identity was confirmed by GC-EAD using a male antenna of *Sparganothis pilleriana* Den. & Schiff. which gave a response matching that of a similar amount of synthetic standard.

Saturated acetates were found in all samples with 18 : Ac predominating, followed by 20 : Ac, 16 : Ac, and 12 : Ac. There were indications for the presence of trace amounts of decyl acetate (less than 0.01% of *Z*9–12 : Ac); the level of tetradecyl acetate, if present, was below 0.002% of *Z*9–12 : Ac.

A compound with the mass spectrum and retention time of a 9,11-dodecadienyl acetate (*E* and *Z* not separated) was detected in most female extracts

TABLE 1. PHEROMONE-RELATED COMPOUNDS IDENTIFIED IN *E. ambiguella* FEMALES

Compound	Short form	Identification criteria	Female wash (ng/female)		Gland extract (ng/female)	Effluvia, (relative amount)
			Reared	Wild		
Z-9-Undecenyl acetate	Z9–11 : Ac	GC-MS	ND[b]	ND	ND	0.5
Dodecyl acetate	12 : Ac	GC-MS	0.2–1	0.4	3	0.8
Z-9-Dodecenyl acetate	Z9–12 : Ac	GC-MS, DMDS	15–30	10	100	100
E-9-Dodecenyl acetate	E9–12 : Ac	GC-MS, GC-EAD[a]	0.02–0.1	0.1	0.5	0.1
Z-9,11- or *E*-9,11-Dodecadienyl acetate or similar	Z9,11–12 : Ac	GC-MS	0.02	0.01	0.2	0.1
Z-9-Dodecen-1-ol	Z9–12 : OH	GC-MS, TFA-DMDS	1	ND	8	ND
Hexadecyl acetate	16 : Ac	GC-MS	2	1	10	0.2
Octadecyl acetate	18 : Ac	GC-MS	20–2000	20	1000–2000	30
Eicosyl acetate	20 : Ac	GC-MS	4	0.2	40	5–10

[a] *Sparganothis pilleriana* antenna.
[b] ND = not detected.

at a fraction of a percent of Z9–12 : Ac. While the 7,9 and 8,10 isomers showed significant differences in the lower mass range, the spectrum of the diene found in *E. ambiguella* closely matched that of Z9,11–12 : Ac.

An undecenyl acetate was detected in one sample of female effluvia at less than a percent of Z9–12 : Ac. From its elution just prior to 12 : Ac on the Silar column it was identified as Z9–11 : Ac.

Evidence for the presence of trace amounts (less than 0.01 % of Z9–12 : Ac) of hexadecenyl and octadecenyl acetates was obtained occasionally. Occurrence of Z-10-tridecenyl acetate, a synergist of male attraction (Rauscher et al., 1984), in the females could not be substantiated.

A novel approach was followed for the identification of a dodecenol found in all washes of laboratory-reared females. After trifluoroacetylation and DMDS adduct formation (TFA-DMDS) of a gland extract, we observed a component with the molecular ion $M^+ = 374$ corresponding to a bis(methylthio)dodecenyl trifluoroacetate. Prominent ions were at *m/z* 89 and 285, as expected for the two fragments A^+ and B^+, respectively, of a compound showing a double bond in position 9. Since other trifluoroacetates such as octadecyl were not observed, we concluded that this compound was a reaction product of the native alcohol and not a transesterification product of Z9–12 : Ac. It was assigned *Z* because the native product coeluted with Z9–12 : OH and gave no signal with the *S. pilleriana* antenna which is specifically sensitive to both *E*9–12 : Ac and *E*-9-dodecen-1-ol (Guerin et al., 1985).

Insects collected from the wild were found to contain the same components as the laboratory material except for Z9–12 : OH, which was below detection limits. Their Z9–12 : Ac content was reduced, possibly because they were less well adapted to our laboratory conditions.

The three methods of obtaining pheromone extracts provided similar results. Differences were obtained with the amount of 18 : Ac which was lower in the effluvia than in the wash, as expected from volatilities, and comparatively high in the gland extracts (20–30 times the amount of Z9–12 : Ac).

Other compounds identified in the gland extract were hydrocarbons of 23–29 carbons with the odd-numbered chains predominant, as reported from other insects (e.g., Descoins et al., 1985); saturated alcohols of 18, 20, and 22 carbons; saturated fatty acids of 14 and 16 carbons; and a monounsaturated 16-carbon acid.

Field and Wind-Tunnel Tests

Attractiveness of Z9–12 : Ac and Z9–11 : Ac. As previously reported, Z9–12 : Ac attracts *E. ambiguella* males in the field and the same was found true for Z9–11 : Ac, equally attractive at the same dose. Tests with mixtures of Z9–12 : Ac and Z9–11 : Ac at steps of 20% gave the same catches as the parent compounds, indicating simply an additive effect.

Effect of Saturated Acetates. In a first test series, 12 : Ac, 16 : Ac, 18 : Ac, and 20 : Ac were added singly to Z9–12 : Ac at levels between 0.2 and 5 times the proportion found in the females. An increase of catch was obtained with 12 : Ac at amounts of at least four times that found in the female blend as reported earlier (Arn et al., 1979, Rauscher et al., 1984). No such effect was found for the others. On the other hand, a significant improvement was obtained when Z9–12 : Ac was combined with all four saturated acetates even at the proportions found in the female wash. This indicated an interaction between the components. Systematic omission of each of the saturated acetates from the blend did not suggest that any component other than 12 : Ac was important for optimum catch (Table 2, test A). However, the 16- to 20-carbon acetates together augmented the catch significantly as long as 12 : Ac was present (Table 2, test B).

Combined Effect of 18 : Ac and 12 : Ac. Systematic tests of binary and ternary blends later revealed that addition of 18 : Ac alone to Z9–12 : Ac strongly augments *E. ambiguella* attraction provided that 12 : Ac is already present at a minimum of 20% (Figure 3). The optimized mixture from these tests, a 1 : 1 : 2 blend of Z9–12 : Ac, 12 : Ac, and 18 : Ac represents an improvement of a factor of two over our previous best (Rauscher et al., 1984).

E9–12 : Ac as an Inhibitor. As reported previously, presence of *E*9–12 : Ac in the lure inhibits grape moth catch (Arn et al., 1979). Further tests now show

TABLE 2. CATCHES OF *Eupoecilia ambiguella* IN TRAPS CONTAINING Z9–12 : AC AND SATURATED ACETATES

	Blend compositions[a]					
	Z9–12 : Ac	12 : Ac	16 : Ac	18 : Ac	20 : Ac	Total catch[b]
Test A	100	5	10	200	1	210 a
	100		10	200	1	127 bc
	100	5		200	1	251 a
	100	5	10		1	172 ab
	100	5	10	200		188 ab
	100					81 c
Test B	100	100	10	200	1	478 a
	100	100				335 b
	100	20	10	200	1	195 b
	100	20				142 cd
	100	5	10	200	1	141 bc
	100	5				105 d
	100		10	200	1	131 cd
	100					104 d

[a] Micrograms per rubber cap.
[b] Figures in the same test followed by the same letter not significantly different at $P = 0.05$.

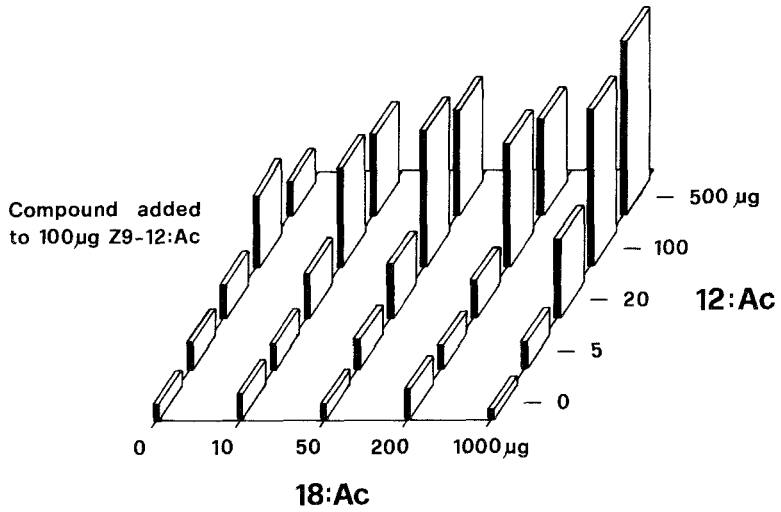

FIG. 3. Relative catches of *Eupoecilia ambiguella* with Z-9-dodecenyl acetate (Z9-12:Ac) and various amounts of dodecyl acetate (12:Ac) and octadecyl acetate (18:Ac) added. Combined results from four tests with overlap at (12:Ac/18:Ac) 0/0, 5/0, 100/0 and 100/200; total catch with the best treatment in a test with six replicates involving all 12:Ac and 18:Ac doses above 5 µg was 445 males. Data for 18:Ac = 0 are from Rauscher et al. (1984).

that 12:Ac reduces this sensitivity to the inhibitor. In a binary mixture, the presence of 0.2 to 2% *E* is sufficient for significant catch suppression of Z9-12:Ac; however, in a ternary blend containing 12:Ac and Z9-12:Ac at a 1:1 ratio, the threshold is 10% *E*. Adding more 12:Ac to a blend containing 20% *E* brought the attractiveness up to that of a sample with only 10% of the geometric isomer (Figure 4). From the dose-response curves obtained earlier (Rauscher et al., 1984) dilution by 12:Ac was not responsible for this change. This apparent desensitizing effect of 12:Ac against the inhibitor may be important when preparing formulations on a large scale where purification of isomers is impractical.

Other Components. Components detected or supposed at one point as part of the pheromone blend were tested at various levels in combination with Z9-12:Ac, the four saturated acetates, and Z9-12:OH (the latter at 5%). No positive effect on trap catch could be observed except as reported for Z10-13:Ac (Rauscher et al., 1984). No effect on catch was obtained from Z9,11-12:Ac (added at levels of 0.2-10% of the amount of Z9-12:Ac), Z9-14:Ac (0.5-10%), and Z11-16:Ac (5-100%), although *E*9,11-12:Ac was inhibitory at a level of 2% and above, and Z9-12:OH at 10%.

Diel Periodicity. Götz (1941b) reported that *E. ambiguella* males fly into female-baited traps in the early morning. We have found that, in line with ob-

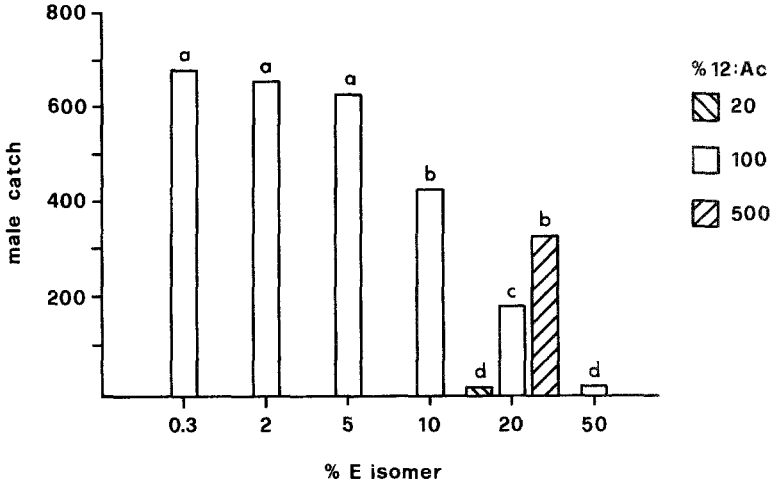

FIG. 4. Catches of *Eupoecilia ambiguella* with Z-9-dodecenyl acetate (1000 μg/cap) and varying amounts of *E* isomer and dodecyl acetate added. Totals of 10 replicates. Values marked with the same letter not significantly different at $P = 0.05$ (Duncan's multiple-range test).

servations in the laboratory (Arn et al., 1975; Rauscher et al., 1984), our populations fly during darkness; activity began before midnight (geographical time) and was over at 2:30 AM.

Wind-Tunnel Tests. These were done with the 1 : 1 : 2 blend of Z9–12 : Ac, 12 : Ac, and 18 : Ac which had given optimal field attraction. Compared with the previous results (Rauscher et al., 1984), addition of 18 : Ac to the binary blend results in a higher proportion of males reaching the source and increases the range over which caps are attractive (Figure 5).

DISCUSSION

Three components of the female secretion of *E. ambiguella* contribute to male attraction and can, at present, be considered part of the sex pheromone of this species: Z9–12 : Ac, 12 : Ac, and 18 : Ac. The type of hierarchy existing among the three compounds seems to be novel to the pheromone field: Z9–12 : Ac is required for attraction while addition of 12 : Ac augments male catch. Octadecyl acetate is neither attractive on its own nor does it augment the catch when added to Z9–12 : Ac unless 12 : Ac is present at a minimum level. As shown earlier in the wind tunnel (Rauscher et al., 1984), the effect of 12 : Ac is not so much to improve attractiveness—with Z9–12 : Ac alone at optimal dose about the same number of males reach the source as with calling females—as to raise the concentration level at which attraction to the source is still possible.

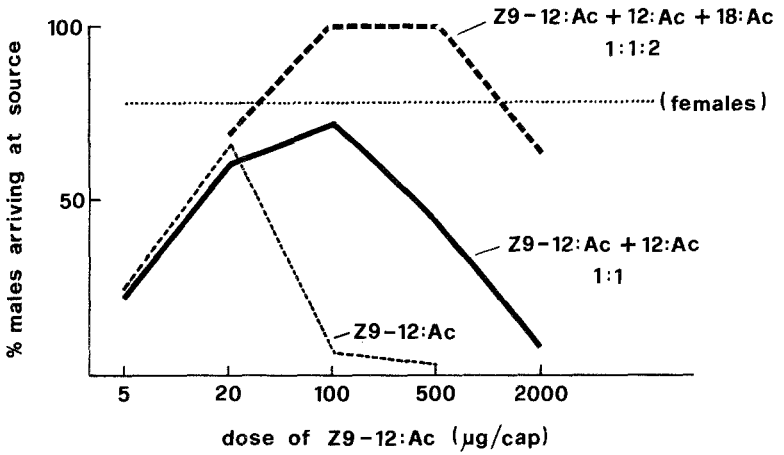

FIG. 5. Response of male *Eupoecilia ambiguella* in the wind tunnel to varying doses of *Z*-9-dodecenyl acetate in blends with dodecyl acetate and dodecyl plus octadecyl acetate; 25 males per data point. Differences between curves at doses of 100 μg and above significant at $P = 0.05$ (Duncan's multiple-range test). Results for Z-9-dodecenyl acetate alone and females are from Rauscher et al. (1984).

Octadecyl acetate appears to act in the same way by increasing the disorientation threshold even further.

The "best blend" of these three components contains a larger proportion of 12 : Ac than the female secretion. Although wind-tunnel and some field tests indicate that this blend is superior to calling females, other experiments were not quite conclusive. Since use of a suboptimal ratio by the females is unusual, we are left with the question if yet additional components, identified or unknown, contribute to male attraction by *E. ambiguella* females. Substitution of one pheromone component by another has been demonstrated in the cabbage looper (Linn et al., 1984). It is possible that 16 : Ac and 20 : Ac show a similar effect as the other saturated acetates. With complex interactions of blend components, behavioral experimentation is clearly becoming difficult and time-consuming.

No biological functions could be found for the other components of the female secretion, notably the *E* isomer and the parent alcohol of the main attractant which in many other tortricid pheromone blends act as coattractants. Equally unclear is the role of the other unsaturated products which were present in amounts too low for final identification, namely a dodecadienyl acetate showing characteristics of the 9,11 isomers.

Very curious also is the case of *Z*9–11 : Ac which was found in our sample of effluvia but neither in the sex gland extract nor in any of the numerous samples of female wash. The reason for this may be that its higher vapor pressure

raised its level just above detection limit. This chemical is as good an attractant for *E. ambiguella* males as its higher homolog and might be produced by an aberrant biosynthetic pathway. Fluctuation of pheromone blend composition with generations has been demonstrated in *Utetheisa ornatrix* (Jain et al., 1982). On the other hand, our laboratory culture, which has been inbred for over 100 generations, did not show any signs of altered or diminished pheromone production as was shown by Minks (1971) for *Adoxophes orana* F. v. R.

The practical use of pheromones for insect control has, in the past, been hampered by cost of chemicals. Additives such as the saturated compounds reported here enhancing biological activity and, as shown for 12 : Ac, permitting the use of attractants of technical purity could dramatically improve this situation. Further experiments will be needed to show if the effects we have demonstrated here for attraction are applicable to disruption.

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ALLELOPATHIC RESEARCH OF SUBTROPICAL VEGETATION IN TAIWAN
III. Allelopathic Exclusion of Understory by *Leucaena leucocephala* (Lam.) de Wit[1]

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Abstract—*Leucaena leucocephala* plantations in Kaoshu, southern Taiwan, exhibit, after several years of growth, a unique pattern of weed exclusion beneath *Leucaena* canopy. The pattern has been observed in many *Leucaena* plantations in Taiwan and is particularly pronounced in the area where a substantial amount of *Leucaena* litter has accumulated on the ground. Field data showed that the phenomenon was primarily not due to physical competition involving light, soil moisture, pH, and nutrients. Instead, aqueous extracts of *Leucaena* fresh leaves, litter, soil, and seed exudate showed significantly phytotoxic effects on many test species, including rice, lettuce, *Acacia confusa, Alnus formosana, Casuarina glauca, Liquidambar formosana*, and *Mimosa pudica*. However, the extracts were not toxic to the growth of *Leucaena* seedlings. The decomposing leaves of *Leucaena* also suppressed the growth of the aforementioned plants grown in pots but did not inhibit that of *Leucaena* plants. By means of paper and thin-layer chromatography, UV-visible spectrophotometry, and high-performance liquid chromatography, 10 phytotoxins were identified. They included mimosine, quercetin, and gallic, protocatechuic, *p*-hydroxybenzoic, *p*-hydroxyphenylacetic, vanillic, ferulic, caffeic, and *p*-coumaric acids. The mature leaves of *Leucaena* possess about 5% dry weight of mimosine, the amount varying with varieties. The seed germination and radicle growth of lettuce, rice, and rye grass were significantly inhibited by aqueous mimosine solution at a concentration of 20 ppm, while that of the forest species mentioned was suppressed by the mimosine solution at 50 ppm or above. However, the growth of *Miscanthus floridulus* and *Pinus*

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taiwanensis was not suppressed by the mimosine solution at 200 ppm. The seedlings of *Ageratum conzoides* died in mimosine solution at 50 ppm within seven days and wilted at 300 ppm within three days. It was concluded that the exclusion of understory plants was evidently due to the allelopathic effect of compounds produced by *Leucaena*. The allelopathic pattern was clearly shown in the area with a heavy accumulation of *Leucaena* leaf litter, which was a result of drought and heavy wind influence.

Key Words—Allelopathy, weed exclusion, *Leucaena leucocephala*, phytotoxins, mimosine, phytotoxic phenolics, forest plantation.

INTRODUCTION

It is often asserted uncritically that a deleterious effect of one plant upon another is ascribable to competition for light, soil moisture, or mineral nutrients. Muller (1969) therefore emphasized additionally that allelopathy should be recognized as one of the environmental factors to be considered in analyzing the mechanisms of plant interactions. Under some circumstances allelopathy may also interact with other natural habitat factors (Muller, 1966, 1974; Gliessman and Muller, 1978; Börner, 1971; Koeppe et al., 1976; Anaya and del Amo, 1978; Rice, 1984; Chou, 1983; Chou et al., 1981; 1984).

Since 1972, Chou and his associates have demonstrated several cases of allelopathy in Taiwan (Chou and Chung, 1974; Chou and Chiou, 1979; Chou and Hou, 1981; Chou and Lin, 1976; Chou and Yang, 1982; Chou and Young, 1975). They have dealt with the mechanisms of autointoxication of *Oryza sativa*, the allelopathic exclusion of weeds and pasture grasses, comparative allelopathy of natural vegetation and agricultural plantations, and the roles of allelopathy in tropical and subtropical agroecosystems. More recently the authors have focused on forest plantations; for example, Chou and Yang (1982) reported that the regulation mechanism of understory species in stands of *Phyllostachys edulis* was primarily due to the allelopathic effect of the bamboo leaf and leaf litter. Additionally, the tree *Leucaena leucocephala* has been widely planted in Taiwan because of its high economic value for producing nutritious forage, firewood, and timber. Generally, after a few years, the floors of these plantations are relatively bare of understory plants, except *Leucaena* seedlings. This pronounced pattern of weed exclusion beneath *Leucaena* trees is particularly pronounced in areas having a drought season. Nakasuga and Yamada (1979) indicated that only a few weeds were able to grow beneath *Leucaena* plantations in Okinawa. The exclusion of weeds by *Leucaena* plants was thought to be due to either a physical competition or a biochemical inhibition by *Leucaena* leachate, or both. The aim of this study was thus to elucidate the mechanism of allelopathic interference of *Leucaena leucocephala* interacting with environmental stresses.

METHODS AND MATERIALS

Study Site. Leucaena plantations chosen for the present study are located at Kaoshu, Pingtung County of southern Taiwan. In the area, six varieties of Salvado-type, namely K8, K28, K29, K67, K72, and S1 and one variety of native Hawaiian-type *Leucaena* were planted in different densities: 2500, 5000, 10,000, 20,000, and 40,000 plants per hectare. The monthly mean weather data (1978–1982) in the Kaoshu area are given in Table 1. The monthly mean temperature ranges from 19.4°C (January) to 28.6°C (July) and the annual precipitation is 2578.1 mm, of which 90.9% of rainfall (2343.4 mm) is concentrated in the summer season, from May to October, and about 9.1% of rainfall (234.7 mm) is distributed in the remaining seasons. In Kaoshu, there is a severe drought and heavy wind in winter, from December to February, and less than 40 mm of precipitation during the period. Correlated with these weather patterns, there has developed a pronounced pattern of weed exclusion beneath *Leucaena* plants (Figure 1). The density and diversity of understory species on the *Leucaena* plantation floor varied with planting density. However, in the present study we selected a study site with a planting density of 10,000 plants per hectare.

Materials. Fresh leaves and litter of *Leucaena leucocephala* varieties (listed above) were collected from the Kaoshu study site. The plant materials were air-dried in the field in sunlight and further dried out in a laboratory hood. Soils were collected from the *Leucaena* and grassland control sites from the surface

TABLE 1. AVERAGE MONTHLY WEATHER DATA (1978–1982) IN KAOSHU STUDY SITE[a]

Month	Temperature (°C)	Precipitation (mm)	Sunny day (hr)
January	19.4	13.0	186.6
February	20.8	19.1	153.0
March	23.3	72.5	170.8
April	25.5	78.1	167.0
May	27.6	213.7	158.2
June	28.4	254.1	201.4
July	28.6	590.0	203.6
August	28.3	688.6	162.4
September	27.8	383.4	162.1
October	26.6	213.6	162.8
November	23.6	46.2	133.6
December	20.2	5.8	159.4
Sum		2578.1	2020.8
Average	25.0		

[a]Data were obtained from the Agrometerological Bulletin, Vol. 25–29, Central Weather Bureau of R.O.C.

FIG. 1. Different understory patterns shown on the *Leucaena* floors at the Kaoshu site of Taiwan.

to 15 cm depth. The soils were air-dried and screened through a 2-mm sieve to remove all visible plant residues. For bioassay purposes, seeds of lettuce (*Lactuca sativa* var. Great Lakes 366), rice (*Oryza sativa* Taichung 65), and rye grass (*Lolium multiflorum*) were used. In addition, seedlings of *Acacia confusa, Alnus formosana, Casuarina glauca, Pinus taiwanensis, Liquidambar formosana, Miscanthus floridulus, Mimosa pudica*, and *Ageratum conzoides* were used for testing the phytotoxic effects of *Leucaena* leachates and extracts.

Field Measurements. Light intensity was measured under the canopy of *Leucaena* trees in different planting densities after three to four years of growth and in the open grassland control area in sunny days of September 1981, February 1982, and on May 1–3, 1985 in the Kaoshu study site. In the light measurement, at least 10 replications for each treatment were made. The biomass and litter on the *Leucaena* and the grassland control floors were sampled from plots of each treatment with three replications.

Preparation of Aqueous Extracts of Leucaena *Leaves, Litter, and Soil.* A 1% aqueous extract of air-dried leaves was prepared by soaking 2 g of air-dried leaves in 198 ml distilled water, shaking the mixture for 2 hr, and suction filtration. A 2.5% aqueous extract of leaf litter was prepared in the same way. However, soil extracts were prepared by shaking 150 g of each soil with 500 ml of distilled water for 2 hr and centrifuging the mixture at 3000 rpm

(2369 *g*). The aqueous extracts of leaves, litter, and soil were stored in a refrigerator at 4°C before assaying.

Physicochemical Analyses of Extracts and Soils. The aqueous extracts of *Leucaena* leaves, litter, and soil were subjected to osmotic concentration determination by using an osmometer (Fiske G-66). Other physicochemical properties, such as soil texture, soil pH, soil moisture, organic matter content, and percent of nitrogen and phosphorus were determined by methods described by Rice (1974).

Bioassay Techniques. Two bioassay techniques were employed to determine the phytotoxic potential of leaf, litter, and soil extracts. The sponge bioassay techniques described by Chou and Young (1975) and Chou and Lin (1976) were employed using the seeds listed in the Materials section. In every bioassay, distilled water served as a control. Tests and controls were set up in triplicate. The water culture bioassay was also used; it consisted of planting seedlings of *Ageratum conzoides* in aqueous mimosine solutions at concentrations of 50, 100, 200, and 300 ppm. The growth of seedlings was recorded every day until the 14th day. The bioassay data were subjected to statistical analysis by using the Student's *t* test.

Pot Experiment. Styrene plastic containers of 150 ml capacity were filled with about 150 g of air-dried soil intermixed with 1–2 g of *Leucaena* dried leaf. The soil without addition of *Leucaena* leaf served as control. Seedlings of *Acacia, Alnus, Casuarina, Pinus, Liquidambar, Miscanthus, Mimosa,* and *Ageratum* were planted in treatment and control containers, each replicated 30 times. The experiment was conducted in a phytotron with temperatures of 25°C for daytime and 20°C for nighttime.

Isolation and Identification of Phytotoxins. We hypothesized that mimosine and some phytotoxic phenolics were present in the leaves of *Leucaena.* The isolation and quantitative determination of mimosine in *Leucaena* leaves followed the techniques described by Brewbaker and Kaye (1981). In addition, 5 g of air-dried leaves of each *Leucaena* variety were extracted and the extracts analyzed for phytotoxic phenolics by using the techniques described by Chou and Young (1975). The extracts were further quantitatively analyzed by using high-performance liquid chromatography (HPLC) (Waters Associates, model 6000A) with a C-18 reversed phase column and solvent system of methanol–water–glacial acetic acid (20:80:1, v/v/v). The flow rate of solvent was 1.0 ml/min and chart speed was 0.2 in./min.

RESULTS

Field Measurements. In the summer period (July–September) in the Kaoshu study site, the average light intensity in an open grassland control area was 28,000 lux, while that under the canopy of *Leucaena* trees ranged from

4000 to 6000 lux (14.2–21.4% of the control). However, in the winter period (December–February), the average light intensity in the control area was 33,000 lux, while that under the canopy of *Leucaena* trees was 8000 lux (24% of the control). The relatively higher amount of light intensity in winter than in summer was primarily due to the *Leucaena* leaf fall as a result of a dry winter season and heavy wind. Additionally, the light intensity also differed with *Leucaena* varieties (Chou and Kuo, unpublished data).

The amount of solar radiation in a year is relatively high in the Kaoshu area (2020.8 hr), and the annual rainfall is also high (2578.1 mm). Since rainfall was particularly concentrated in the summer in the Kaoshu area (Table 1), the soil moisture was relatively higher in summer (usually above 15%) than in winter (below 11%) (Chou and Kuo, unpublished data). For example, in a recent field study during May 1–3, 1985, we found that the mean soil moisture of the *Leucaena* soil in the Kaoshu area was below 2%, indicating that the soil was very dry before the rainfall season. Nevertheless, the soil moisture content is not significantly different between the *Leucaena* soil and the control grassland soil during the drought season. Additionally, data of soil pH, soil texture, organic matter, and total nitrogen content were also not significantly different between the two soils (Table 2). However, the phosphate content was obviously higher in the *Leucaena* soil. An overall comparison of soil characteristics between the control grassland and the *Leucaena* soils showed that the latter provided better soil conditions for the growth of understory plants.

In the Kaoshu site, the biomass of ground cover beneath *Leucaena* plantations was relatively low as compared to the grassland control, being 9.3 g/m^2 on the *Leucaena* floor and 330 g/m^2 in the grassland control area, showing about 80% bare ground on the *Leucaena* floor. However, the amount of litter accumulated on the *Leucaena* floor was remarkably high (1027 g/m^2), being significantly higher than that of the biomass (Figure 2). The floristic composition in

TABLE 2. SOIL CHARACTERISTICS IN CONTROL GRASSLAND AND *Leucaena* PLANTATION AT KAOSHU STUDY SITE, PINGTUNG COUNTY, TAIWAN[a]

Characteristics	Grassland soil	*Leucaena* soil
Texture	Sandy loam	Sandy loam to loam
pH	5.9	6.5
Organic matter (%)	2.1	2.3
Total nitrogen content (%)	0.1	0.1
Total phosphate (meq/100 g)	61.0	68.0

[a] Data are the mean of 10 replicates of each soil analysis and are not statistically significant between the two soils.

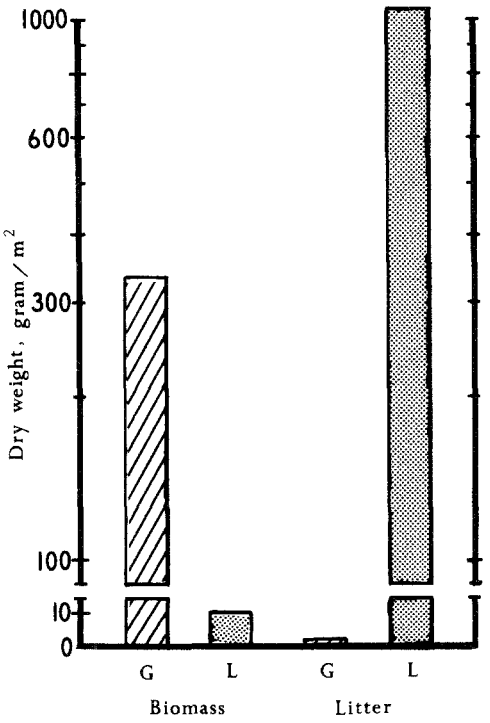

FIG. 2. Quantitative comparisons of biomass collected beneath the *Leucaena* trees (L) and in the open control grassland (G) and that of litter on the *Leucaena* floors in the Kaoshu study site.

the *Leucaena* understory at the Kaoshu site included eight species, and 16–23 species were found in the control grassland. The dominant species are different between the control and the *Leucaena* areas. The most abundant species in the control area are *Hyptis suaveolens, Paspalum* sp., and *Imperata cylindrica*, while those in the *Leucaena* plantation are *Leucaena leucocephala* itself, *Axonopus compressus*, and *Ageratum conzoides* (Kuo, 1983). It should be mentioned that *Ageratum conzoides* and *Rhynchelytrum repens* are shade-intolerant species. Their appearance in the *Leucaena* plantation may serve as an indication that low light intensity is not a causal factor of weed exclusion in this plantation. In addition, in the same area, *Acacia* plantations produce ground-level light intensity lower than that in *Leucaena* plantations; nevertheless the biomass production of understory plants is significantly higher in the former than the latter, indicating that the lower density of biomass under the *Leucaena* stand is likely not due to competition for light.

An overall review of the phenomenon occurring in the study site strongly indicates that the relatively lower density of weeds beneath the *Leucaena* plan-

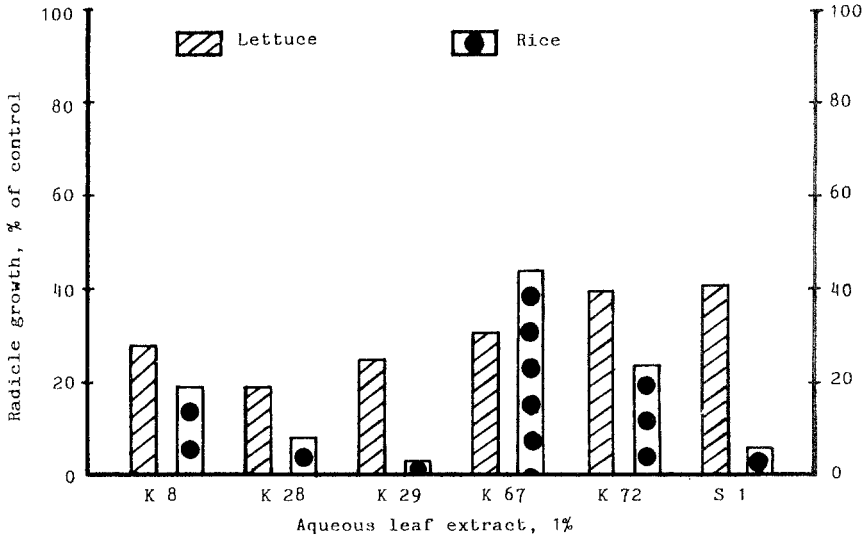

FIG. 3. Effects of 1% aqueous extracts of various *Leucaena* leaves on the radicle growth of lettuce and rice seedlings.

tation of the Kaoshu area is not due to the physical competition for light, soil moisture, and nutrients, but is rather due to severe winters of low precipitation and strong wind causing the leaves and other plant parts of *Leucaena* to fall. This difference may result from secondary plant metabolites leached from the *Leucaena* leaves and litter producing an allelopathic effect. This hypothesis requires further investigation.

Phytotoxicity of Aqueous Extracts of Plant Parts and Soils. The 1% aqueous extracts of *Leucaena* leaves significantly reduced the radicle growth of lettuce and rice (Figure 3). The phytotoxicity ranged from 60% to 96%, being significantly higher on rice than lettuce. Among six varieties, K29 and K28 generally gave the highest inhibition of radicle growth of the two tested plants. This inhibition is thought to be due to phytotoxic substances present in the extract instead of osmotic inhibition because the osmotic concentrations of the extracts (below 20 mosmol) are too low to cause any osmotic inhibition (Chou and Young, 1974). The phytotoxicity was not significantly different between varieties but was significantly reduced by the subsequent extractions. The second subsequent extracts of the leaves revealed no phytotoxicity, indicating that most of the phytotoxic substances present in the leaves were hydrophilic and easily leached out by water (Chou and Kuo, unpublished data).

Since there was a substantial amount of *Leucaena* litter accumulated on the floor, further bioassay of 2.5% aqueous extracts of litter of K8, K72, and

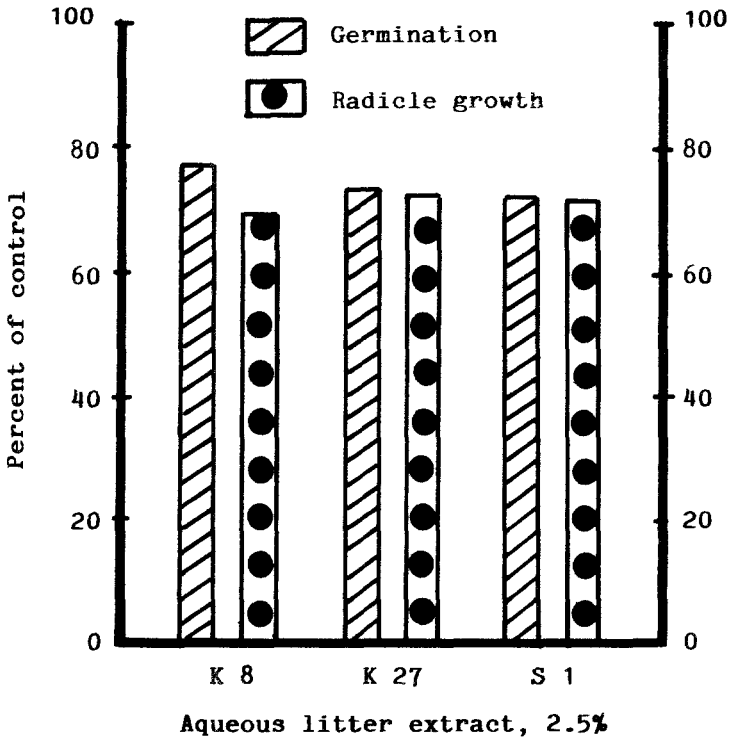

FIG. 4. Effects of 2.5% aqueous litter extracts of *Leucaena* on the seed germination and radicle growth of rye grass.

S1 was also conducted by using rye grass as the test plant. Seed germination and radicle growth of rye grass were inhibited by the extracts to 70% of the distilled water control, and the inhibition was not significantly different among *Leucaena* varieties (Figure 4). Furthermore, aqueous extracts of *Leucaena* soil (150 g soil/500 ml) and its grassland control soil were also bioassayed against lettuce. The original extract of *Leucaena* soil (1× solution) caused only a 20% inhibition, while the control soil exhibited 0%. However, when the 1× solutions were concentrated five times, the *Leucaena* soil extract caused 45% inhibition, indicating that the phytotoxicity was present in the *Leucaena* soil. However, the control soil showed 22% inhibition (the osmotic concentration of 5× solution is 30 mosmol) which was possibly due to an osmotic effect instead of phytotoxicity (Chou and Young, 1974).

We found that the *Leucaena* litter included *Leucaena* seeds also, and therefore we conducted an experiment to test the phytotoxicity of seed exudates. The exudates were obtained by soaking the seeds (200 *Leucaena* seeds in 100 ml of distilled water) for different time durations, such as 12, 24, 36, 48, 60, and 72

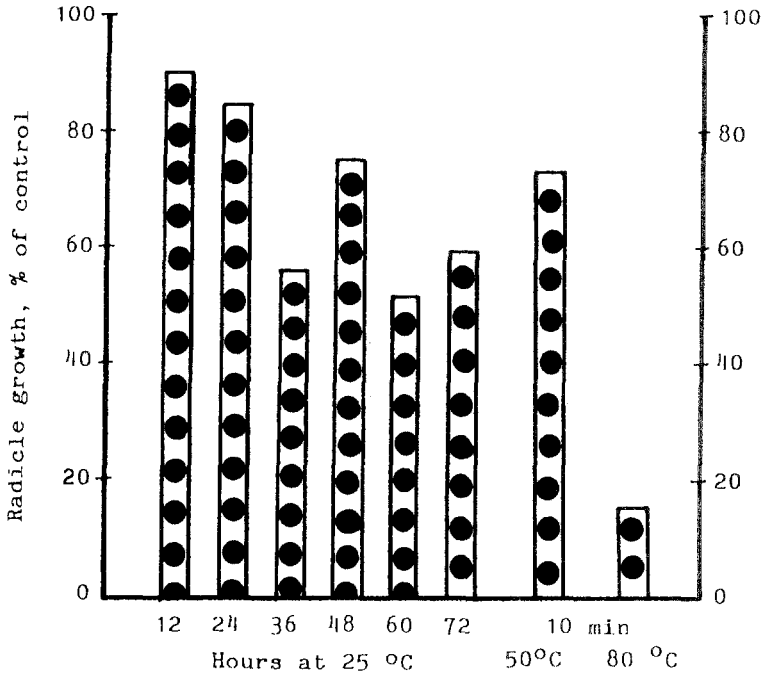

FIG. 5. Effects of aqueous exudates of *Leucaena* seeds on the radicle growth of lettuce seedlings. The exudates were obtained by soaking 200 *Leucaena* seeds with 100 ml distilled water for different time intervals and at various temperatures.

hr at room temperature, and for 10 min at 50°C and 80°C. No visible microorganism activity was found during the period of seed exudation. The bioassay results of lettuce showed that the phytotoxicity of seed exudate obtained at room temperature increased with increasing soaking time. The exudate obtained at the end of 24 hr caused 14% inhibition while that at 60 hr produced 47% inhibition. The toxicity of seed exudate was extremely high, above 80%, when seeds were soaked at 80°C for 10 min, while the toxicity was only 26% when the seeds were soaked at 50°C for 10 min (Figure 5).

It is concluded that the fresh leaves, litter, seeds, and soils of *Leucaena* exhibit significant phytotoxicity on the growth of lettuce, rice, and rye grass, indicating that phytotoxins are present in the plant parts and litter and that the phytotoxins are likely water soluble.

Effects of Aqueous Leaf Extracts of Leucaena *on Forest Species.* It is possible that *Leucaena* may be planted with other forest species. It was therefore desirable to evaluate the allelopathic inhibition of other forest species by *Leucaena*. Five forest species and an aggressive grass, *Miscanthus floridulus*, were

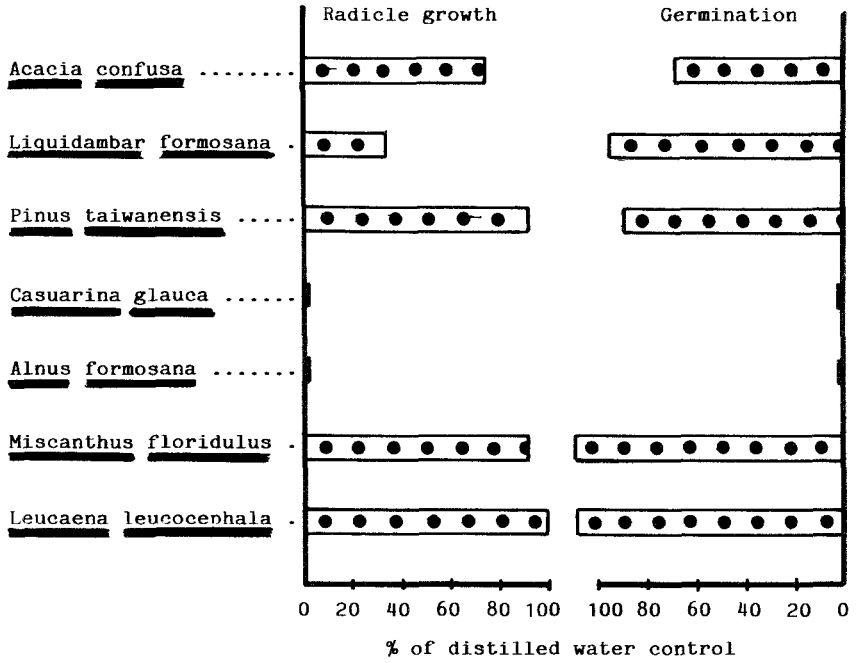

FIG. 6. Effects of 1% aqueous extract of *Leucaena* K28 leaves on the seed germination and radicle growth of seven plant species.

chosen for the study. The radicle growth of *Acacia confusa, Liquidambar formosana, Casuarina glauca*, and *Alnus formosana* were significantly inhibited by 1% aqueous extract of K28 leaves and that of *Pinus taiwanensis* and *Miscanthus floridulus* were not affected (Figure 6), indicating that these two latter species can tolerate the leached *Leucaena* metabolites. Moreover, in order to see whether *Leucaena leucocephala* was an autotoxic plant, its seeds were bioassayed by 1% *Leucaena* leaf extracts of varieties K8, K28, K29, K67, and S1. No inhibitory effect on the germination and radicle growth of *Leucaena* was found, indicating that *Leucaena leucocephala* can tolerate the leaching metabolites produced by itself. This finding agreed with field observations that *Leucaena* seedlings occur abundantly and are healthy under *Leucaena* canopy.

Effects of Decomposing Leucaena *Leaves in Soil on Growth of Plants.* Either 1 or 2 g of *Leucaena* leaves were mixed well with 150 g soil in a plastic container and planted with seedlings of *Leucaena leucocephala, Acacia confusa, Alnus formosana, Casuarina glauca, Liquidambar formosana*, and *mimosa pudica*. The survival rate of these seedlings at 60 days after transplanting is shown in Table 3. The results indicate that the seedlings of *Leucaena leucocephala* grow very well with 100% survival, while the seedlings of *Alnus formosana* and *Acacia confusa* did not grow so well and the survival rate was

TABLE 3. EFFECT OF *Leucaena* LEAF MIXED WITH SOIL OR MULCHED ON SOIL SURFACE ON GROWTH OF FOREST PLANTS[a]

	Survival (% of the control)		
	Leaf intermixed		Leaf mulched
Species	1 g	2 g	5 g
Leucaena leucocephala	100	100	86.7
Alnus formosana	72.2	44.4	36.8
Acacia confusa	29.6	18.5	14.0
Liquidambar formosana	4.5	9.0	30.6
Casuarina glauca	0	0	0
Mimosa pudica	0	0	0

[a] Data express the percent survival relative to survival in the soil alone.

about 50%. The remaining two species, *Casuarina glauca* and *Mimosa pudica*, were totally killed by the decomposing *Leucaena* leaves in soil. In addition, when the *Leucaena* leaves were deposited on the surface of pot soil instead of being mixed with soil, the results were similar to those of the former experiment (Table 3). This indicates that the decomposing *Leucaena* leaves mixed in soil produced some toxins that suppressed the growth of some forest species tested.

Quantitative Analysis of Phytotoxins in Leucaena *Leaves.* The mimosine content in different varieties was determined by spectroscopic analysis. The contents varied with *Leucaena* varieties, revealing that K29 had the highest mimosine content of 5.75%, followed by K28 (5.27%), K8 (4.95%), S1 (4.61%), K72 (4.42%), and K67 (2.77%). It is interesting to note that juvenile leaves contained higher amounts of mimosine than normal mature leaves. Harvested leaves exposed to sunlight at noon lost over 50% of total mimosine as compared to the air-dried leaves. The mimosine contents of *Leucaena* leaves significantly varied with season and location of *Leucaena* plantations (Yeh, 1983).

In addition to mimosine, phytotoxic phenolics present in *Leucaena* leaves were mainly identified by paper chromatography and HPLC. The HPLC spectrum of nine authentic phenolic acids is given in Figure 7A, which is used for the quantitative comparison of phenolic acids isolated from the *Leucaena* leaves (Figure 7B). The contents of phenolic acids in leaves of *Leucaena* K28 are given in Table 4. They are gallic, protocatechuic, *p*-hydroxybenzoic, *p*-hydroxyphenylacetic, vanillic, caffeic, *p*-hydroxycinnamic, and ferulic acids. The amount of these phenolic compounds was significantly higher in young leaves than in mature leaves. Of these, protocatechuic and *p*-hydroxycinnamic acids had higher concentrations than the remaining compounds. Moreover, quercetin

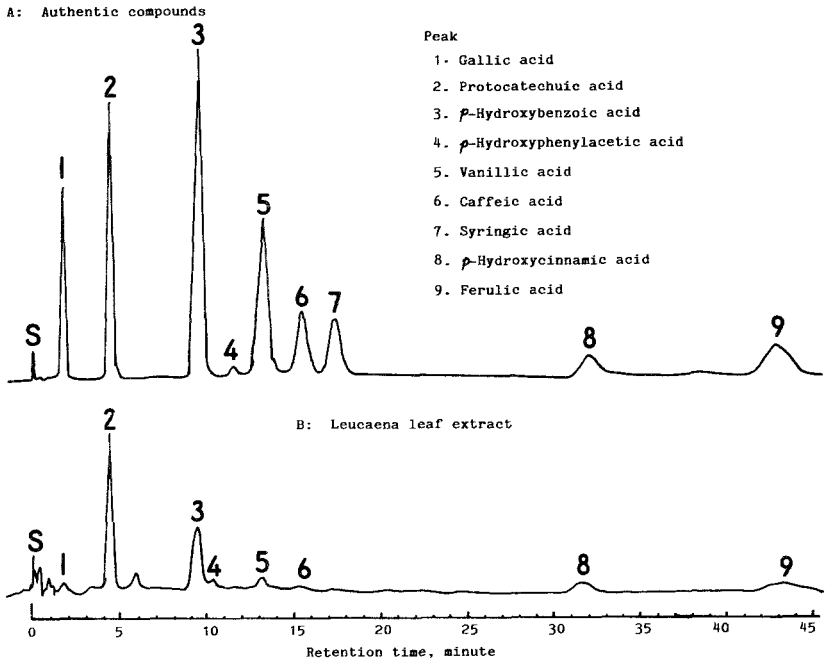

FIG. 7. The spectrum of high-performance liquid chromatography of known authentic compounds (A) and that of *Leucaena* K28 leaf extract (B). S indicates the solvent peak of the starting point.

and 18 other flavonoids were found in seven varieties of *Leucaena* leaves (Kuo, 1983); some of those flavonoids might be phytotoxic in nature.

Inhibitory Effects of Mimosine on Plant Growth. The aqueous solutions of mimosine at concentrations of 10, 20, 30, 40, 50, 60, 70, and 80 ppm were bioassayed using lettuce and rice seeds as test materials. Results are shown in Figure 8. Mimosine exhibited significant suppression of the radicle growth of lettuce and rice at concentrations of 10 and 20 ppm. Above 50 ppm, mimosine almost prevented the growth of both kinds of seedlings and significantly suppressed the growth of *Acacia confusa* and *Casuarina glauca*. In addition, forest species such as *Acacia confusa, Casuarina glauca, Liquidambar formosana, Pinus taiwanensis*, and *Alnus formosana*, and an aggressive weed, *Miscanthus floridulus*, were also bioassayed. At the 200 ppm concentration, mimosine significantly inhibited seed germination and radicle growth of the tested plants, except *Pinus taiwanensis* and *Miscanthus floridulus* (Figure 9). This agrees with results shown in Figure 6, indicating that the inhibition of the forest plants is apparently due to mimosine and the phenolics mentioned. However, the *Leucaena* seedlings were not suppressed by either plant extracts or the mimosine solution at 200 ppm. Furthermore, seedlings of *Ageratum conyzoides* could sur-

TABLE 4. QUANTITATIVE COMPARISON OF PHYTOTOXIC PHENOLICS PRESENT IN LEAVES OF *Leucaena leucocephala* VARIETY K28

Phytotoxic phenolics	Amount ($\times 10^{-4}$ μmol)	
	Young	Mature
Gallic acid	4.215	2.153
Protocatechuic acid	12.05	1.816
p-Hydroxybenzoic acid	2.439	1.551
p-Hydroxyphenylacetic acid	ND[a]	2.685
Vanillic acid	0.467	0.492
Caffeic acid	3.722	2.637
p-Hydroxycinnamic acid	9.528	4.015
Ferulic acid	1.323	0.343
Total	33.746	15.692

ND: not detectable

vive in an aqueous mimosine solution at 50 ppm for six days, but died at 300 ppm within four days (Table 5). These findings of bioassays of known mimosine agree well with those of previous bioassays of the aqueous extracts of *Leucaena* plant parts, and overall bioassay findings strongly support the allelopathic hypothesis of weed exclusion by *Leucaena* plants.

DISCUSSION

It was hypothesized that *Leucaena leucocephala* plantations exhibit an allelopathic pattern of weed exclusion, and this hypothesis was supported by the findings of field observations and experiments, greenhouse pot experiments, and laboratory assays. The *Leucaena* leaves and litter contained a substantial quantity of mimosine, which showed strong phytotoxic activity on many species used in this study. However, the extracts of *Leucaena* leaves and aqueous solutions of mimosine at concentrations below 200 ppm had no phytotoxic effect on the growth of *Leucaena* itself. In addition to mimosine and quercetin, eight phytotoxic phenolics were also identified. Among 10 phytotoxins found in the plant, mimosine is the most toxic to the test species, of which some are excluded from *Leucaena* plantations. Mimosine is a well-known toxic nonprotein amino acid present in the seeds and foliage of the plant. The toxic effect of mimosine on animals has been reported by various authors (Hegarty et al., 1976; Jones, 1981; Reis et al., 1975). Mimosine can cause inhibition of mungbean (Ling et al., 1969; Smith and Fowden, 1966) and hypocotyl or radicle growth of lettuce (Wilson and Bell, 1978). Reisner and Bucholtz (1979) found

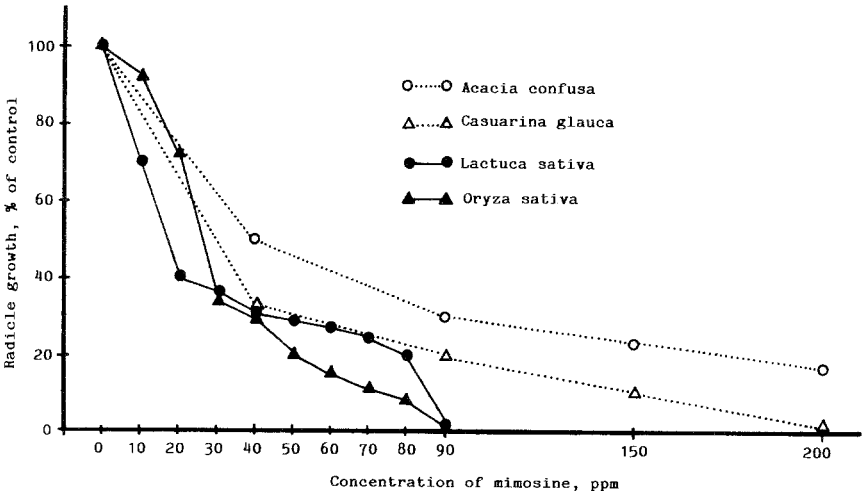

FIG. 8. Effects of aqueous solution of mimosine in various concentrations on the radicle growth of four tested species.

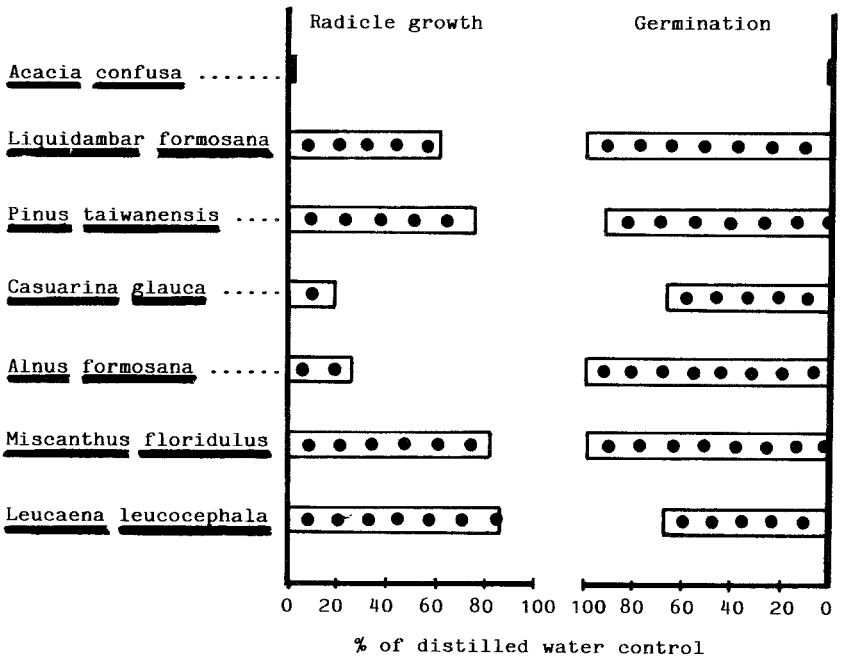

FIG. 9. Effects of 200 ppm mimosine in distilled water on the seed germination and radicle growth of seven plant species.

TABLE 5. EFFECTS OF AQUEOUS SOLUTION OF MIMOSINE IN VARIOUS CONCENTRATIONS ON SEEDLING SURVIVAL OF *Ageratum conzoides* GROWN IN WATER CULTURE

Days after transplanting	Survival (as % of control)				
	0 ppm (control)	50 ppm	100 ppm	200 ppm	300 ppm
3	100	100	100	100	100
4	100	80(20)[a]	20(80)	20(80)	0
5	100	60(40)	0	0	0
6	100	20(80)	0	0	0
7	100	0	0	0	0
14	100	0	0	0	0

[a]Data in parentheses indicate the percent injury of test seedlings.

that mimosine completely inhibited cell division and synthesis of DNA, RNA, and protein in *Paramecium tetraurellia* at submillimolar concentration. It is an interesting question why mimosine significantly inhibits the growth of many plants tested yet hardly affects the growth of *Leucaena* seedlings. According to Smith and Fowden (1966), *Leucaena* may have a detoxification mechanism that degrades mimosine into 3,4-dihydroxypyridine and then converts 3,4-dihydroxypyridine into nontoxic metabolites. This might be the reason why the growth of young *Leucaena* seedlings was not retarded under mature *Leucaena* trees.

The allelopathic phenomenon has often been found in areas under a stress environment. It is possible in the present study that the differential field pattern of various study sites could be influenced by environmental conditions. Winter drought is common in the Kaoshu site but not in the other sites. The drought season caused a significant amount of *Leucaena* leaves to fall; consequently, the litter of *Leucaena* released phytotoxic phenolics and mimosine to the soil and suppressed the growth of understory plants. The suppression of understory plants by fallen *Leucaena* leaves was also seen locally in the Chialin site, where a *Leucaena* tree had died and fallen, leading to a significant amount of fallen leaves and to an allelopathic phenomenon. Thus, these ten allelopathic compounds of mimosine, quercetin, and phenolic acids present in the *Leucaena leucocephala* plant parts and other unidentified compounds are likely responsible for the allelopathic effects of *Leucaena* plantations.

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ISOLATION AND CHARACTERIZATION OF GLUCOCAPPARIN IN *Isomeris arborea* NUTT.

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Abstract—*Isomeris arborea* (Capparaceae), is the only woody caper endemic to southern California and northern Baja. Methylglucosinolate, also known as glucocapparin, was the only glucosinolate found in *I. arborea* organs by paper chromatography of the thiourea derivatives and was quantitatively determined by gas chromatography by hydrolytic products. The concentration of glucocapparin ranged from an average of 4.6 mg/g wet weight in mature leaves to 5.2 mg/g wet weight in immature leaves. Buds averaged 6.2 mg/g wet weight and capsule walls 1.8 mg/g wet weight. Seeds contained an average of 14.3 mg/g wet weight of glucocapparin. Glucocapparin concentration was found to vary significantly among the mature leaves of individuals within a single population. This compound is known to be deleterious to nonadapted herbivores and may be implicated in the chemical defense mechanism of *I. arborea*.

Key Words—Capparaceae, chemical defense, allelochemics, glucocapparin, glucosinolate, *Isomeris arborea*, caper.

INTRODUCTION

Glucosinolates are secondary metabolites most notably responsible for the flavors and pungent scents of cruciferous crops such as cabbage, mustard, and radish, among others (MacLeod, 1976). They also provide a degree of protection against fungal infection and nonspecialized herbivores (Stoessl, 1970; Feeny, 1977). However, their role in caper–insect interactions is unexplored at present.

Glucosinolates are found as cytoplasmic components of most, if not all, plant tissues and organs of the species containing them and are especially concentrated in seeds (Van Etten et al., 1979; Van Etten and Tooky, 1979). Glu-

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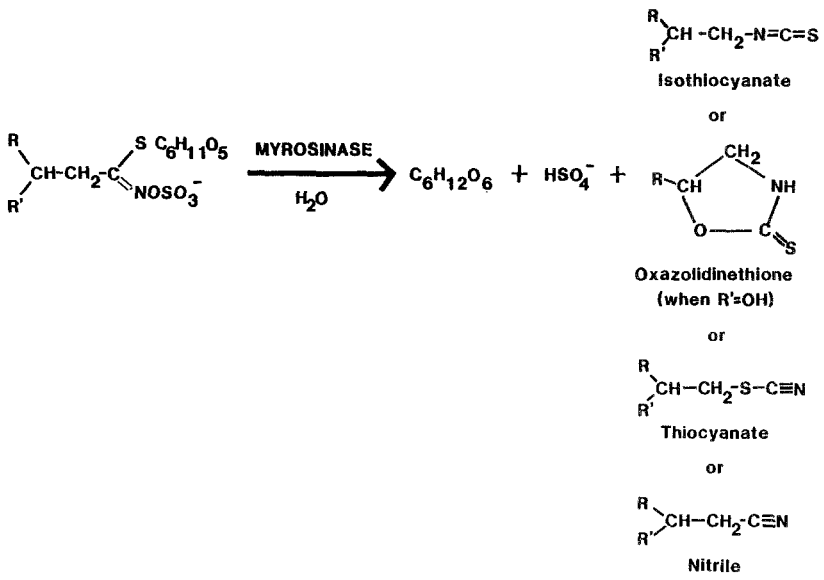


FIG. 1. Enzymatic hydrolysis of a generalized glucosinolate.

cosinolates undergo a hydrolysis reaction catalyzed by the enzyme myrosinase (thioglucosidase glucohydrolase, EC 3.2.3.1) to generate glucose, a sulfate ion, and a toxic nitrogenous hydrolytic product (Figure 1). The class of nitrogenous hydrolytic product generated depends on hydrolytic conditions as well as the "R" group of the reacting glucosinolate (Cole, 1976; Gil and MacLeod, 1980). It is generally accepted that isothiocyanates are the predominate class of toxin formed. Other toxins generated are oxazolidinethiones, nitriles, and thiocyanates (see Figure 1). Ingestion of these nitrogenous hydrolytic products and of the intact glucosinolates by nonadapted insects results in reduced growth and development (Erickson and Feeny, 1974).

Although glucosinolates and their nitrogenous hydrolytic products are known to be repulsive to generalist herbivores, they have also been shown to be oviposition and feeding stimulants to specialized herbivores having evolved mechanisms to circumvent the effects of these toxins (Feeny et al. 1970; Schoonhoven, 1972). *Isomeris arborea* Nutt. is a perennial shrub of the Capparaceae native to desert, valley grassland, and coastal sage scrub plant communities of southern and central California. *I. arborea* has high photosynthetic rates and contains levels of organic nitrogen which are as high as many cultivated plants and are higher than most shrubs in the plant communities in which it is found. Because of its high photosynthetic rates, high nitrogen levels, and a lack of any physical defense, *I. arborea* would be especially susceptible to exploitation by herbivores if endogenous secondary compounds (e.g., glucosinolates) did not impose a strong chemical barrier against herbivory.

The pattern of secondary compounds in *I. arborea* does not follow the scenario outlined by Rhoades and Cates (1976) and Feeny (1976). *I. arborea* individuals usually live in relatively dense populations, have a woody perennial growth form, and often support dense foliage throughout all seasons (Iltis, 1957). These characteristics lead us to expect quantitative defense compounds in *I. arborea*. However, *I. arborea* contains glucosinolate, which is a qualitative, physiological toxin. Generally toxins and other qualitative defense compounds are found in nonapparent plants. *I. arborea* has not been reported to contain quantitative defense compounds.

Rhoades (1979) points out that consumption by an adapted herbivore is limited to an amount that will not saturate the herbivore's ability to detoxify or otherwise tolerate the defensive chemical. In controlled experiments and field observations, *Murgantia histrionica*, an adapted herbivore, preferentially fed on *I. arborea* flower buds and capsules even though all plant parts were equally available (Nuss, 1983). *M. histrionica* had a much greater mortality rate when forced to feed on leaves rather than on buds only or capsules only (English, 1983). Based on these observations, we hypothesized that either some aspect of nutritional quality was lower in leaves than in buds or capsules, or that leaves, buds, and capsules contained different concentrations of defensive compounds. Perhaps both factors worked in combination to produce the observed pattern. Also, it is common to see some *I. arborea* individuals having as many as 150 herbivores while others in the same population have few or none (B. Collier, personal communication). These observations suggest that glucosinolate concentration among *I. arborea* individuals might vary significantly within a population.

In the first part of this study we identified the glucosinolate component of *I. arborea* and developed the methods necessary to accurately quantify glucosinolate in many small samples of *I. arborea* organs. In the second part of this study we analyzed for differences in glucosinolate complement among organs and among individuals within a population of *I. arborea*.

METHODS AND MATERIALS

Collection of Plant Material. Plant material from *Isomeris arborea* Nutt. was collected from Sorrento Valley, a coastal sage scrub plant community along Flint Kote Road, Torrey Pines State Preserve, San Diego County, California. Immature (not fully expanded) and mature leaves, flower buds, capsule walls, and seeds were collected from five plants for studies comparing glucosinolate concentration among organs. In a separate study to compare glucosinolate concentration in leaves among plants within the population, mature leaves were collected from three branches from each of 10 plants. In each case fresh plant material was picked and immediately stored on Dry Ice before being transferred to a freezer for storage prior to analysis.

Glucosinolate Identification. Preliminary spot test, in conjunction with paper chromatography (Rodman, 1978), were employed as the primary method of identification of glucosinolate in *Isomeris arborea*. Sinigrin (allylglucosinolate), a standard glucosinolate, was also tested as a control, because it produces an isothiocyanate upon enzymatic hydrolysis. In addition, glucosinolate was extracted from seeds of *Cleome spinosa*, a caper closely related to *I. arborea* reported to generate isothiocyanate and oxazolidinethione upon hydrolysis (Kjaer and Thomson, 1963); *Brassica napus*, known to generate isothiocyanate and oxazolidinethione upon hydrolysis (Youngs and Wetter, 1967); *Brassica oleracea*, known to generate all three types of aglucons (Van Etten et al., 1976); and pea (*Pisum sativum* L.), which does not contain glucosinolates.

Thiourea derivatives from the hydrolytic products of *I. arborea* glucosinolate were chromatographed in six different solvent systems on 30-cm^2 Whatman No. 1 chromatography paper spotted 4 cm from the bottom. Phenylthiourea and methylthiourea, derivatized with ammonia from methylisothiocyanate, a mustard oil common in the Capparaceae, were simultaneously chromatographed. The six solvent systems used for paper chromatographic studies were: (1) *n*-butanol–toluene–water (3 : 1 : 1); (2) chloroform–water (5 : 3); (3) toluene–ethanol–water (5 : 1 : 2); (4) toluene–*n*-butanol–water (3 : 1 : 2); (5) toluene–*n*-butanol–water (10 : 1 : 2); (6) toluene–acetic acid–water (5 : 2 : 4).

In each system the aqueous phase was used to condition the chromatography chamber. Ascending chromatography was terminated after the solvent front ran to ca. 5 cm from the top of the chromatography paper. After drying the paper, spots were made visible by spraying the paper with Grote's reagent and suspending it over steam. Resulting blue or violet spots were circled with pencil and their mobility calculated relative to that of the phenylthiourea standard.

Gas Chromatographic Analysis. Quantification of *I. arborea* isothiocyanate was done by comparing retention times and area counts to those of standard isothiocyanate (Pfaltz and Baur Inc., Stamford, Connecticut). Gas chromatographic analysis was done on a Varian Vista 6000 gas chromatograph using a 6-ft, 2-mm ID, glass chromatography column packed with 10% Carbowax 20 M on 100/120 mesh Chromosorb WAW. Injection and FID temperatures were set at 150°C and 200°C, respectively. Nitrogen flow was 30 ml/min. Oven temperature was programmed to maintain 60°C for 2 min and then increased to a final temperature of 120°C at a rate of 10°C/min.

An extraction procedure that proved to be adaptable to as many as 40 analyses per week was developed from the methods of other researchers (see Daxenbichler and Van Etten, 1977; MacLeod and MacLeod, 1977; Van Etten and Daxenbichler, 1977; Rodman, 1978). Typically, 50 ml of hot 70% methanol was blended with 0.30–1.00 g of frozen, crushed plant tissue for 1 min in a tissue homogenator (Sorvall Omni-Mixer No. 17150) at high speed. The mix-

ture was boiled and condensed to approximately 30 ml. The liquid was filtered through Whatman No. 2 filter paper, and the plant tissue was mixed with ca. 10 ml of distilled water followed by similar filtration with rinses. The combined liquid extract was then condensed by vacuum evaporation to < 10 ml. Water was added to a final volume of 10 ml. The 10-ml glucosinolate extract was decanted into screw-cap centrifuge tubes (Pyrex No. 8422) with 10 ml of 0.05 M sodium phosphate buffer, pH 7.5, 0.10 ml of 4.2% ascorbate in water, 15.0 ml methylene chloride, and 1.0 ml myrosinase extract, prepared as described by Rodman (1978). Caps with Teflon liners were fixed tightly to the tubes which were placed on a table top shaker set at approximately 50 oscillations per minute. The tubes were allowed to mix overnight (ca. 12 h) at room temperature.

After hydrolysis, the aqueous phase and the methylene chloride phase were cleanly separated by centrifugation for 20 min at 1500 rpm on a table-top centrifuge (Sorvall RT 6000). Twelve milliliters of the methylene chloride phase were removed by syringe for gas chromatographic analysis of nitrogenous hydrolytic products. An Evapo-Mix vacuum evaporator (Buchler Instruments) with an ice-water bath was used to gently concentrate the 12.0 ml methylene chloride extracts to approximately 1 ml each. The concentrated extracts were transfered with methylene chloride rinses to 2-ml vials having Teflon-lined screw caps. Next, undecane was added as an internal standard to make a final concentration of 0.05% before methylene chloride was added gravimetrically to a final volume of 1.50 ml. The concentration of glucosinolate is given in milligrams of glucocapparin per gram of plant material, fresh weight. Because milligrams of methylisothiocyanate per milliliter of methylene chloride is actually measured by gas chromatography, appropriate conversion factors are used. Standard solutions containing 0.50, 1.00, 5.00, 10.00, and 15.00 mg of sinigrin were used with an extract from 1.00 g of bean leaves to determine percent mean recovery and standard deviation for this method of quantitative analysis. In each case $N = 10$. Mean recovery ranged from ca. 103% for small amounts to ca. 97% for larger amounts of sinigrin analyzed (Table 1).

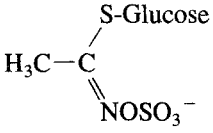
TABLE 1. RECOVERY OF ALLYLISOTHIOCYANATE UPON ENZYMATIC HYDROLYSIS OF SINIGRIN (In each case, $N = 10$)

Sinigrin hydrolyzed (mg)	Mean % recovery ± SD
0.50	103.3 ± 3.5
1.00	99.6 ± 1.5
5.00	96.7 ± 1.2
10.00	97.3 ± 2.3
15.00	97.0 ± 1.4

RESULTS

Glucosinolate Identification. The three major types of nitrogenous hydrolytic products generated by glucosinolates found in members of the Capparaceae are isothiocyanates, thiocyanates, and oxazolidinethione (Kjaer and Thomson, 1963; Gmelin and Kjaer, 1970; Kjaer and Schuster, 1971; Gil and MacLeod, 1980). Spot tests allowed us to determine which of these aglucons predominates in *Isomeris arborea*.

Paper chromatographic studies of the derivatized isothiocyanate showed that extracts from *I. arborea* leaves, flower buds, capsule walls, and seeds generated only one isothiocyanate, the thiourea analog of which has the same R_{Ph} values as methylthiourea. This indicates that *I. arborea* synthesizes the methylisothiocynate-generating glucosinolate known as glucocapparin (methylglucosinolate).



(1)

Gas chromatography confirmed identification of methylisothiocyanate and allowed quantitative determination of glucocapparin in the various plant parts of *I. arborea*.

Comparative Concentrations. A significant difference in glucocapparin concentration was found among immature and mature leaves, buds, capsule walls, and seeds ($P < 0.001$). Glucocapparin levels ranged from an average of 1.8 mg/g fresh weight of capsule walls to 14.3 mg/g fresh weight of seeds. Buds (6.2 mg/g), immature leaves (5.2 mg/g), and mature leaves (4.6 mg/g) had intermediate levels which were not statistically different (Table 2). These results confirm the hypothesis that there are quantitative differences in the glucocapparin content among organs fed upon by specialist herbivores, such as *Murgantia histrionica*.

TABLE 2. MEAN MILLIGRAMS OF GLUCOCAPPARIN PER GRAM FRESH WEIGHT OF PLANT ORGAN ± STANDARD DEVIATION OF FIVE *Isomeris arborea* INDIVIDUALS

Immature leaves	Mature leaves	Buds	Capsule walls	Seeds
5.2 ± 1.4ab	4.6 ± 0.8ab	6.2 ± 0.7b	1.8 ± 1.0a	14.3 ± 4.8c

[a] $F = 20.80$, $P < 0.001$. Means followed by the same letter are not significantly different at the 0.01 level (Student-Newman-Keuls test for multiple comparisons).

TABLE 3. MEAN MILLIGRAMS GLUCOCAPPARIN PER GRAM FRESH WEIGHT OF MATURE LEAVES FROM TEN *Isomeris arborea* INDIVIDUALS[a]

Plant number	Glucocapparin (mean mg/g fresh wt mature leaves ± SD)
1	4.7 ± 1.1
2	6.4 ± 0.4
3	4.3 ± 1.3
4	5.3 ± 0.6
5	3.6 ± 0.2
6	5.3 ± 0.8
7	4.1 ± 1.0
8	3.9 ± 0.3
9	3.3 ± 0.1
10	5.5 ± 0.6

[a] $F = 2.72$, $P < 0.025$. Samples were from three separate branches from each plant.

A comparison of glucocapparin concentration among the mature leaves of 10 individuals from the Sorrento Valley population showed a significant variability at $P < 0.025$ (Table 3).

DISCUSSION

In this study we used a method that allows simultaneous qualitative and quantitative analysis of glucosinolate and that is adaptable to a large number of samples, such as would be required by ecological studies. Because only one glucosinolate is quantified, involved derivatization like the analytical techniques of Olsen and Sorensen (1979) or time-consuming ion-exchange chromatography (Van Etten and Daxenbichler, 1977) is not required. The percentage of recovery of allylisothiocyanate from sinigrin when using this method was greater than 96% and standard deviation due to methodology was quite small (<3.5%).

Almost all capers studied contain glucocapparin as the most abundant glucosinolate (Kjaer and Thomson, 1963). Glucocleomin (2-hydroxy-2-methylbutylglucosinolate), which yields 5-ethyl-5-methyl-2-oxazolidinethione upon enzymatic hydrolysis, is usually found in conjunction with glucocapparin but was not found in *I. arborea*; glucocleomin is the second most common glucosinolate in the Capparaceae. Oxazolidinethione- and thiocyanate-producing glu-

cosinolates, compounds generally found in other capers, are notably lacking in *I. arborea*.

Surprisingly, the organs predicted to be the best defended in *I. arborea* do not have the highest glucocapparin concentration. Rhoades (1979) predicts that reproductive structures should be more highly defended than other organs because damage to them would represent a greater loss of plant fitness than similar damage to other organs. Also, McKey (1974) suggests that defense compounds are distributed and redistributed among plant parts according to a balance between vulnerability to attack by herbivores and the relative value of a plant part in terms of overall plant fitness. Of the organs studied, capsule walls always contained the lowest levels of glucocapparin (Table 2). Preliminary studies indicated that immature seeds contained glucocapparin levels which were one third to one half of the levels of mature seeds (Stillinger, unpublished data).

Glucosinolate is generally most concentrated in the mature seeds of plants equipped with this defense system (Rodman, 1978). The highest concentration of glucocapparin in *I. arborea* was found in mature seeds. As a further protection, mature seeds have a hard seed coat which generally present a physical barrier to phytophagus insects.

The difference in glucocapparin levels of mature leaves among plants (Table 3) is an important aspect of the defensive system of *I. arborea* and is currently being studied more extensively. Rhoades (1979) points out that quantitative variability of defensive chemicals among plants should be advantageous because it reduces the ability for herbivores to counteradapt.

The concentration of glucosinolate has been found to respond to environmental factors such as water stress, available nitrogen, soil sulfate, and soil type (Freeman and Mossadeghi, 1972, 1973; Josefsson, 1970; Ju et al., 1980; Louda et al., 1985). These factors are not expected to be identical for all individuals at any one time and may contribute, along with heritability, to the observed variation among plants in glucocapparin and vulnerability to insect herbivores.

Studies on the defensive "strategies" of plants typically consider only one aspect of a complex suite of characteristics that limit herbivory. Our observations suggest that glucocapparin is part of a defense system which alone restricts feeding by generalist herbivores, and in conjunction with other facets of defensive physiology, limits feeding by specialist herbivores. These other facets, which are under investigation, include fluctuations of nitrogen concentration, variability of individual phenology, and the possible presence of other defensive compounds in *I. arborea*.

Because *I. arborea* is a perennial plant with one easily quantifiable glucosinolate, it is an excellent candidate for studies which relate its physiology and phenology to such things as coevolution and plant defense theory.

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EUPHYDRYAS ANICIA (Lepidoptera: Nymphalidae) UTILIZATION OF IRIDOID GLYCOSIDES FROM *Castilleja* AND *Besseya* (SCROPHULARIACEAE) HOST PLANTS[1]

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Abstract—Iridoid glycosides were found to be sequestered by natural populations of *Euphydryas anicia* after ingestion from the host plants *Besseya alpina*, *B. plantaginea*, and *Castilleja integra*. Both major iridoids of *B. alpina*, catalpol and aucubin, were found in butterfly populations where this was the only host plant. The catalpol–aucubin ratio was higher in the butterflies than in the host plant. An *E. anicia* population which uses both *B. plantaginea* and *C. integra* as host plants was found to sequester catalpol as well as another iridoid, macfadienoside. Macfadienoside was the major iridoid of *C. integra*, while catalpol esters were the major iridoids of *B. plantaginea*. Although it was a major sequestered iridoid, catalpol was a minor constituent in both host plants. The macfadienoside–catalpol ratio in the butterflies from this population was highly variable, and there appeared to be both sex and individual variation in host plant and/or iridoid glucoside utilization by *E. anicia*. Although other iridoids were present in the host plants, none was sequestered in more than trace amounts.

Key Words—*Euphydryas anicia*, Lepidoptera, Nymphalidae, *Castilleja integra*, *Besseya plantaginea*, *Besseya alpina*, iridoid glycosides, catalpol, macfadienoside, sequestration, plant-insect interactions, herbivory, ecological chemistry.

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INTRODUCTION

Larval host plant specificity in checkerspot butterflies (*Euphydryas*, Nymphalidae) was interpreted in terms of the iridoid glycoside content of the host plant, and this was then definitely established as an important paradigm in a number of butterfly–host plant interactions (Bowers, 1981, 1983; Bowers and Puttick, 1986). The later work (Bowers and Puttick, 1986) showed sequestration of the bitter and presumably emetic iridoid catalpol by *Euphydryas phaeton* and a variety of iridoid glucoside fates in other herbivores.

Populations of *E. anicia* occur throughout the Colorado mountains where they are hosted by *Besseya* and *Castilleja* (Scrophulariaceae) species. We hypothesized that chemical analyses of *E. anicia* and its host plants would extend the generality of the iridoid glucoside paradigm for Scrophulariaceae utilization to natural *Euphydryas* populations and could provide data of value in studying the evolution of host plant specialization by *Euphydryas* butterflies. Various populations and species exhibit a range of behavior, from strict monophagy to utilization of as many as four host plants in a given population (Ehrlich et al., 1975; Singer, 1983).

In the long term, we also want to test the idea that host plant chemical factors might play an important part in providing differential selection pressures between populations at ecologically variant sites and that this could eventually lead to speciation among *E. anicia* populations.

No chemical studies have previously been reported on any of the host plants, and their analyses would be of value to our ongoing investigation of the Scrophulariaceae as well as to the plant–insect interaction. Detailed chemical results will be published at a later date, with the present report focusing on the establishment of iridoid ingestion by *Euphydryas* larvae and sequestration in the adult butterflies.

METHODS AND MATERIALS

Collections. For the first study we chose *E. anicia* using *Besseya alpina* as a larval host at three high-altitude (about 3700 m) sites (Cumberland Pass, Gothic Mt., and Bellview Mt.) and the same species using both *B. plantaginea* and *Castilleja integra* at a lower (2700 m) high-plains site (Red Hill). A detailed study on the ecology and population genetics of *E. anicia* at Cumberland Pass has been published (Cullenward et al., 1979). More recently, adult nectar source, larval host plant distribution, and adult butterfly distribution have been mapped for the Red Hill site (Odendaal, unpublished work). In addition, cross-breeding studies among *E. anicia* populations at each of the above sites have been carried out to study genetic differentiation and mate choice (Odendaal and Ehrlich, unpublished work).

B. alpina (Gray) Rydb. (FRS 239; CSU Herbarium No. 14530) was collected at Bellview Mt., Gothic Mt., and Cumberland Pass (all Gunnison County, Colorado); *B. plantaginea* (James) Rydb. (FRS 240; CSU No. 18338) and *C. integra* Gray (FRS 230A; CSU No. 66099) were collected north of highway 285 between Como, Colorado, and Red Hill Pass, Park County. Collections were all made in late June or early July 1984 and specimens were identified by Professor Dieter Wilkin, Department of Botany, Colorado State University. *E. anicia* Doubleday and Hewitson were collected at the same sites and identified by PRE and FJO. The *Besseya* species grow as broad-leaved rosettes, and entire above-ground material was used for the analyses, since the larvae are leaf consumers. With *C. integra*, leaves and bracts are the major portions consumed. Whole above-ground material was again used for the basic analyses. Separate TLC comparisons were performed on stems, leaves, bracts, and seeds and showed that they contained essentially the same iridoids, although there were some relative concentration differences. *Euphydryas* from each site were collected live in late June or early July, placed in methanol, and the mixture held in the freezer until isolation of iridoids and analysis, which were all carried out within six weeks. Individuals were collected, placed in killing jars briefly, removed, and stored at room temperature until analysis. Dried specimens were weighed, and the average weight was then used to establish a dry weight basis for the pooled *Euphydryas*, which were extracted fresh. Female weights were 34.2 ± 4.2 mg ($N = 3$) and males were 20.8 ± 3.0 mg ($N = 9$). The presence or absence of eggs in the females was not determined.

General Chemical and Analytical Methods. Thin-layer chromatography (TLC) was conducted on Silica gel with 6:4 $CHCl_3$–MeOH or 65:35:2 $CHCl_3$–MeOH–H_2O eluting solvents, both double developed. For visualization, plates were sprayed with a solution made up of 90 ml 50% EtOH, 10 ml of conc. H_2SO_4, and 0.5 ml of *p*-anisaldehyde, followed by heating at about 110° for 3–5 min. Iridoids without substitution at C-4 were gray, brown, or blackish, while those with acid, ester, or aldehyde functional groups at C-4 were pink. Iridoid detection limit was about 1 μg. Gas–liquid chromatography (GLC) of the iridoid trimethylsilane (TMS) ether derivatives was similar to that reported (Inouye et al., 1976). A 30-m DB-1 capillary column was used isothermally at 270° with a column head pressure of 18 psi, and FID detection. Under these conditions typical retention times were sucrose 7.0, aucubin 8.5, trehalose 8.6, catalpol 10.4, and macfadienoside 11.4 min. Macfadienoside was not quantitatively eluted from the column, but a reproducible detection of 40% was established by injection of known amounts as well as by comparison with TLC and, particularly, [^{1}H]NMR analysis. Analyses by [^{1}H]NMR were conducted on Bruker 270 or Nicolet 360 MHz spectrometers.

Plant Analysis. A detailed description will be given of the *B. plantaginea* iridoid isolation and analysis and others will then be summarized. Lab-bench

dried plant material (32 g) was stirred in 200 ml of MeOH for 6 hr, filtered, and the solution evaporated to dryness. The residue was triturated well with $CHCl_3$, the $CHCl_3$ removed, and the remaining residue triturated with H_2O. The aqueous solution was extracted with $CHCl_3$ and the aqueous portion evaporated to dryness to leave 2.5 g of a mixture of sugars and iridoid glucosides. This mixture was again triturated well several times with a minimum of methanol, concentrating the iridoids in the methanol and leaving much of the insoluble sugars behind. The methanol was evaporated to leave 1.0 g of crude iridoid–sugar mixture. The sugar content (mostly sucrose) was estimated at 10–15% from GLC and the isolation procedure below.

The iridoid mixture (500 mg) was separated into components using medium-pressure liquid chromatography (MPLC) on C_{18} reverse-phase Silica gel and 65:35 H_2O–MeOH elution. Ten fractions were obtained which were then evaporated, and the residues were further purified by an additional MPLC, prep. TLC, or crystallization. The pure iridoids were then identified by [^{1}H]- and/or [^{13}C]NMR and (in part) by comparison with standard samples.

Insect Analyses. Because of the high iridoid content and relatively low content of interfering polar compounds in the butterflies, a somewhat simplified extraction and isolation scheme was used, particularly in the single butterfly analyses. An example isolation is given here. One 35.2-mg air-dried *E. anicia* female collected at Red Hill (July 10, 1984) was crushed in 15 ml MeOH, and the mixture was stirred (magnetic stirrer) for 4 hr. The solution remaining after filtration was evaporated in vacuo and the residue triturated five times each with 0.5 ml $CHCl_3$, followed by four triturations (0.5 ml each) with MeOH. The MeOH solution was evaporated to yield a crude iridoid and sugar mixture, 4.0 mg, 11%. The total was then analyzed for iridoid content (about 30% of the mixture) by TLC, GLC of the TMS derivatives, and 360 MHz [^{1}H]NMR.

RESULTS

Each of the host plants was found to contain an array of iridoid glucosides, with the total content 1–2.5% of the dry weight. Catalpol, 1 (Figure 1) was a major iridoid (40–50%) of *B. alpina*, less so (10–15%) for *B. plantaginea*, but was a very minor iridoid (about 3%) of *C. integra*. Aucubin, 2, was present in *B. alpina* at only a slightly lower concentration than catalpol, but was not found in *B. plantaginea* nor in *C. integra*. The major iridoid (about 60%) of *C. integra* was macfadienoside, 3.

For the *Besseya plantaginea* isolation (see Methods and Materials), for example, the relative iridoid content of the plant was assessed from the ten original MPLC fractions (major iridoid underlined in a mixture): Fraction 1 (sugars, catalpol, and 8-epiloganic acid), 90 mg; 2 (8-epiloganic acid and mussaenoside), 10 mg; 3 (mussaenoside), 4 mg; 4 (mussaenoside and verproside),

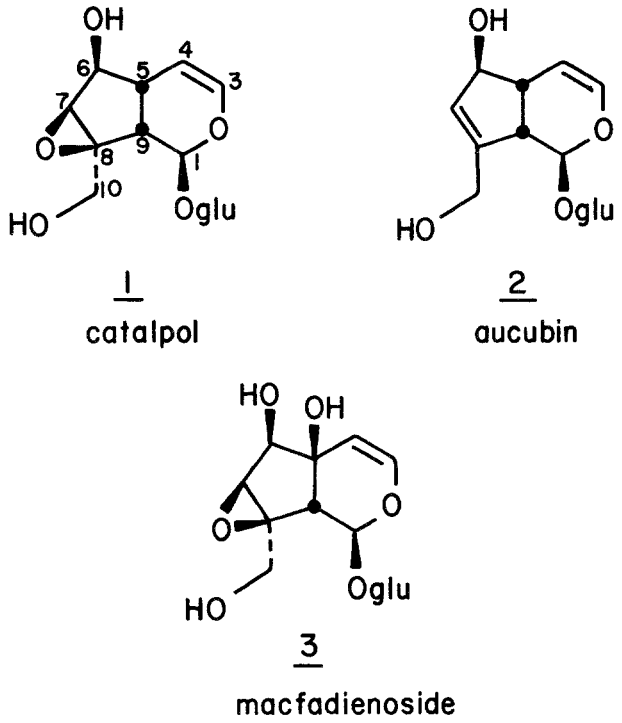

FIG. 1. Host plant iridoid glycosides.

24 mg; 5 (mussaenoside and verproside), 122 mg; 6 (verproside), 32 mg; 7 (verproside and amphicoside), 12 mg; 8 (amphicoside), 73 mg; 9 (amphicoside and veronicoside), 24 mg; 10 (veronicoside), 37 mg. These data, along with GLC of the crude TMS-derivative mixture indicated the following relative percentages: 8-epiloganic acid (5%), mussaenoside (5%), veronicoside (15%), amphicoside (20%), catalpol (12%), verproside (43%). Veronicoside is 6-benzoylcatalpol, amphicoside is 6-veratrylcatalpol, and verproside is 6-dihydroxybenzoylcatalpol. None of these was found in the analyzed butterflies. Details of structure determinations will be published elsewhere.

Complete iridoid analysis for the *B. alpina* and *C. integra* host plants is not as yet complete, but major components were determined. For *C. integra*, 75 g of dried above-ground plant material yielded 2.3 g of purified iridoid and sugar mixture, whose TLC indicated the presence of nine iridoids. Isolation work showed the mixture to consist of about 60% macfadienoside, with the second major iridoid being adoxoside. Catalpol represented about 3% of the iridoid content, and we could not identify aucubin as being present. Adoxoside represented about 15% of the total, but was not found in any of the adult butterflies. The *B. alpina* host plant yielded about the same quantity (1.5%) total

iridoids, and TLC of plants from each site showed them to be virtually identical in iridoid content (six spots observed). TLC and GLC data showed the major iridoids to be catalpol and aucubin. This combination represented about 80% of the total, with the remainder about equally distributed among four minor iridoids.

Iridoid content of the adult butterflies was about 1–4% of the dry weight of the insect. Analyses for individual iridoids revealed that catalpol, aucubin, and macfadienoside were sequestered in varying amounts by *E. anicia*. There was TLC and GLC evidence for the presence in minor amounts of some of the other iridoids from the host plants as well as iridoids (or metabolites) which were not noted in the host plants, but these were not investigated further. In order to compare insect utilization of host plants at the various sites, we determined relative amounts of the three major iridoids in the host plants and adult butterfly groups (along with a few individuals) and the data are presented in Table 1.

The NMR analyses were of particular value in accurately assessing the

TABLE 1. COMPARISON OF SEQUESTERED IRIDOID CONTENT OF *Euphydryas anicia* AND HOST PLANTS

Site	Iridoids (Catalpol = 1)	
	Aucubin–Catalpol	Macfadienoside–Catalpol
Cumberland Pass		
Besseya alpina host plant	0.92	no macfadienoside
Euphydryas anicia (31 males)	<0.10	no macfadienoside
E. anicia (6 females)	0.63	no macfadienoside
Belleview Mt.		
B. alpina host plant	0.68	no macfadienoside
E. anicia (27 males)	0.15	no macfadienoside
E. anicia (8 females)	0.18	no macfadienoside
Gothic Mt.		
B. alpina host plant	0.65	no macfadienoside
E. anicia (15 males)	0.29	no macfadienoside
Red Hill		
B. plantaginea host plant	no aucubin	no macfadienoside
Castilleja integra host plant	no aucubin	20
E. anicia (33 males)	no aucubin	0.15
E. anicia (31 females	no aucubin	0.70
E. anicia (1 male)	no aucubin	0.40
E. anicia (1 male)	no aucubin	0.44
E. anicia (1 female)	no aucubin	4.6
E. anicia (1 female)	no aucubin	2.9

butterfly aucubin–catalpol and macfadienoside–catalpol ratios of Table 1. The C-3 proton resonances (aucubin, dd, 6.21 ppm; catalpol, dd, 6.33 ppm; macfadienoside, d, 6.40 ppm) were sufficiently separate so that the relative iridoid content could be determined directly from integrals observed in the partially purified iridoid mixture. These ratios closely matched those for aucubin–catalpol and macfadienoside–catalpol established from the GLC method. In a typical example, the macfadienoside–catalpol ratio was determined to be 2.9 by the NMR analysis and 3.0 by the GLC analysis. The GLC showed the presence of small amounts of the insect sugar trehalose, as well as trace amounts of other iridoids, which were also detected by TLC. The total of these were estimated at less than 10% of the total purified iridoid mixture and could not be positively identified.

With the butterflies from the Gothic, Cumberland, and Belleview sites similar methods were used to determine aucubin–catalpol ratios. No macfadienoside was found in these butterflies. In the TLC analyses, all butterflies showed a diffuse gray iridoid-like spot more polar (lower R_f) than the major iridoids, which did not appear in any of the host plant analyses. This substance could not be correlated with any extraneous peaks in the GLC or [^{1}H]NMR analyses, and its genesis is unknown at this time. Collections and groupings were made prior to the analyses and hence are not those of an optimally designed experiment. They should, rather, be viewed as exploratory in nature. The results did yield initial data of value as well as information on which to build hypotheses to be tested in future field and laboratory studies.

DISCUSSION

The results clearly show iridoid ingestion and sequestration by natural populations of *E. anicia* hosted by three different plant species. In most cases, catalpol was the major iridoid of the insects, although aucubin and macfadienoside were also found; macfadienoside was, however, the major iridoid in two Red Hill females. In one case, that of female butterflies at Cumberland Pass, there was little, if any, selective sequestration of catalpol over aucubin. Male butterflies at Cumberland Pass and Belleview Mt. did selectively sequester catalpol, but somewhat less so at Gothic Mt.

Detailed conclusions cannot yet be drawn from all the data from the Red Hill population since *E. anicia* is known to use both host plants for pre- and postdiapause larval feeding (Odendaal, unpublished data). One immediate conclusion is that all the individuals analyzed spent part of their life cycle on *C. integra* since they all contained macfadienoside, which is not present in *B. plantaginea*. The two females were either less efficient at sequestering catalpol over macfadienoside than were the two males or else spent a greater part of their life cycle on *C. integra* than did the males. Although the total macfadien-

oside content of both sexes was lower in the population average than in the individuals, a female vs. male difference still was maintained. The low macfadienoside content of the pooled butterflies at Red Hill (as compared to the individuals) suggests that some do not use *C. integra* at all. A differential usage among pre- and postdiapause larvae in this univoltine species might also account for some of the variability. The data from the Red Hill population is perhaps most easily explained by both sex and individual variations in butterfly utilization of host plants and/or iridoid glucosides.

It is notable that the catalpol content of both the *B. plantaginea* and *C. integra* host plants is quite low compared to other iridoids, and hence a very specific sequestration appears to be taking place. The *B. plantaginea* does contain large quantities of esterified catalpol derivatives (veronicoside, amphicoside, and verproside), and the possibility remains that the insect is hydrolyzing these materials in order to obtain catalpol. Labeling or feeding experiments and frass analysis will be necessary to definitely establish or exclude the utilization of these catalpol esters. Since catalpol could be hydroxylated in the insect to yield macfadienoside, a likely biosynthetic step in the plant, the butterfly could be producing macfadienoside, rather than sequestering it. This seems unlikely, however, in view of the results at the Cumberland, Belleview, and Gothic sites. Catalpol was sequestered by all the butterflies analyzed, but in no case was macfadienoside found. It is not likely that *E. anicia* at Red Hill would metabolize catalpol to macfadienoside, while *E. anicia* at the other sites would not.

In summary, we have further established iridoid ingestion and sequestration as an important paradigm in the interaction of *Euphydryas* and their host plants and extended the work to some natural populations of *Euphydryas anicia*. It seems likely that iridoid utilization will be of general applicability in the Melitaeine group as has been suggested (Bowers, 1983). Preliminary results from our laboratory show that *E. editha* (*C. linariifolia* host plant) and *Poladryas arachne* (*Penstemon virgatus* host plant) also sequester catalpol. How far this paradigm can be extended to other lepidopteran taxa has yet to be determined, although its utility in understanding the feeding strategies of *Junonia coenia* (Nymphalidae) and *Certomia catalpae* (Sphingidae) has been established (Bowers, 1984; Bowers and Puttick, 1986).

The importance of iridoid glycosides in other plant–insect herbivore relationships is suggested by two further examples. Thus, the observed (Owen and Whiteway, 1980; Moran and Southwood, 1982) shift of three phytophagous insect specialists from their normal iridoid-containing *Verbascum* (Scrophulariaceae) host to the introduced *Buddleja davidii* (Buddlejaceae) need not in itself "support radical views on plant classification" (Strong et al., 1984). The shift is logical in that the Buddlejaceae are also know to contain iridoid glucosides, and the insects in question may simply be chemists rather than botanists. The second example deals with a white admiral butterfly, *Limenitis* (*Ladoga*) *cam-*

illa (Limenitinae), which is monophagous on *Lonicera periclymenum* in England and has a restricted range, although the host plant is ubiquitous. The restricted range was interpreted (Pollard, 1979) in terms of the increased relative importance of bird predation at cooler latitudes where larval and pupal populations are slow to develop. All *Lonicera* species have high iridoid glucoside content and, in view of the established relationship among iridoid glucosides, palatability, and bird feeding behavior (Bowers, 1981), it is possible that variations in host plant iridoid glucoside content and levels of sequestered iridoids could also play a role in the *Limenitis* range restriction. If iridoid glucoside content of larvae at cooler latitudes is low, this could account for increased predation.

Ingestion of iridoids does not automatically mean sequestration, but it may be worthwhile to test the hypothesis that all aposematic insects monophagous on iridoid-containing plants sequester these bitter substances. In view of the fact that iridoids appear to be stable in dry Lepidoptera specimens and are sequestered in reasonable concentrations, it should be possible to verify this hypothesis by analysis of individual insects from present collections.

Our results at this point are not sufficiently detailed or quantitative, and extensive comparisons cannot yet be made with the detailed studies on cardenolide sequestration by *Danaus plexippus* (Brower et al., 1984) and *D. gilippus* (Cohen, 1985). Some trends, however, can be noted. In the *Danaus* systems, cardenolide TLC profiles of the adult butterflies were generally comparable to those of the host plants. In one case, 20 of 23 cardenolides of the plant were also found in the adult butterfly. Our TLC and GLC iridoid profiles of adult *E. anicia* and its larval plants are markedly different from each other because of the propensity of the butterflies to preferentially sequester catalpol (and somewhat less so macfadienoside), while excreting or metabolizing other iridoids. The fortuitous occurrence of macfadienoside in only one of the host plants has, however, allowed us to determine something of plant usage based upon adult butterfly analyses. This was successful in the *D. plexippus* work since cardenolide profiles were different in different plant hosts, and these were reflected in the adult butterflies.

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POTENTIALLY DEFENSIVE PROTEINS IN MATURE SEEDS OF 59 SPECIES OF TROPICAL LEGUMINOSAE[1]

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Abstract—A survey of 59 species of tropical legume seeds revealed high interspecific variation in proteinaceous capacity to inhibit bovine trypsin (a digestive enzyme) and to agglutinate human (type B, Rh positive) and laboratory rabbit red blood cells. The legume subfamily Mimosoideae was conspicuous for the absence of seeds with very weak trypsin inhibition. Congenerics sometimes differed strongly from each other with respect to both trypsin inhibition and phytohemagglutination. Half the species of seeds displayed no hemagglutinating capacity with one or the other kinds of red blood cells, and in only 27% of the 30 cases where there was some activity did the same species of seed actively agglutinate both species of red blood cells. A species of seed that had hemagglutinating capacity was almost invariably associated with moderate to high levels of trypsin inactivation. While it has been long known that a great diversity of small toxic and potentially defensive molecules occur in legume seeds and that one species of seed often contains several of them, we now feel that it is reasonable to consider legume seeds as also containing a high diversity of potentially toxic protein molecules. A single seed is likely to contain, at the least, three to four classes of defensive compounds, any or all of which, or some in combination, may be the cause of a seed being rejected by a potential seed predator.

Key Words—Seed predation, seed chemistry, lectins, protease inhibitors, phytohemagglutins, Costa Rica, seed defenses, Leguminosae.

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INTRODUCTION

A seed is a bag lunch for a seedling. However, there has been strong selection for seed traits that lower the probability that the seed will become lunch for a seed predator. Since seeds are rich in nutrients of value to a wide variety of animals, we expect that natural selection will have led to each species of seed having a variety of defenses, some of which function against certain potential seed predators, and others that function against others. Some seed proteins, e.g., lectins or phytohemagglutinins (Liener, 1979; Rudiger, 1984; Janzen, 1981) and proteinase inhibitors (Ryan, 1979), are among these potential defense traits (e.g., Janzen, 1977, 1978, 1981; Janzen et al., 1976; Lee, 1979; Olsnes and Pihl, 1973; Laskowski and Kato, 1980; Gatehouse et al., 1979; Adam 1974; Stripe et al., 1976; Warsy et al., 1974; Jayne-Williams and Burgess, 1974; Tannous and Ullah, 1969; Pearch et al., 1979).

One important step in understanding the defenses of a seed is identifying the potentially toxic compounds in that seed. This is less simple that it would appear for three major reasons. First, "toxicity" is a dosage- and animal-specific trait, although, of course, certain classes of compounds (e.g., alkaloids, cyanogenic glycosides) have a higher chance of being toxic than do others. This means that simply identifying a compound in a seed does not disclose the seed's toxicity to animals (although some quite educated guesses are possible). Second, a given phytochemist tends to be specialized with respect to identification of a certain class of compounds. It is therefore almost impossible for the ecologist to send a bag of seeds to a single phytochemist and ask for a profile of all the potentially defensive compounds in that seed. Third, there are two major groups of potentially defensive compounds in seeds.

There are relatively small molecules that are generally straightforward to describe, identify, and isolate. Their mode of action on animal systems in generally very specific, and these compounds have a long tradition of examination by pharmacologists; caffeine, strychnine, L-dopa, canavanine, and cyanogenic glycosides are some well-known examples. If seed defenses were constituted only of compounds such as these, we would undoubtedly understand seed relationships with seed predators much better than we do. However, seeds also contain large molecules made up of repeating patterns of sugars, phenols, and amino acids. These carbohydrates, tannins, lignins, phytohemagglutinins (lectins), and protease inhibitors (to name but a few) are difficult to describe, identify, and isolate. Their mode of action on animal systems is often generalized (e.g., one may be a "digestion inhibitor," another "inhibit uptake of amino acids by the intestine," etc.). These large molecules have been known for a very long time in a general sort of way; tanning leather with polyphenols so as to prevent access to collagen by bacterial enzymes, and boiling beans to denature toxic phytohemagglutinins may be just as ancient to humans as is chewing plants for alkaloid pain-killers and mashing rotenone-rich foliage into streams

to stupefy fish. However, these large molecules have been largely the despair of phytochemists. An additional complication in interpreting large molecules is that they are often major parts of the biomass of a seed and clearly serve also as sources of raw biosynthetic construction materials for the developing seedling. However, given their physiological activity against animals, they are additionally a potential part of the defense repertoire of a seed.

One of the more profitable ways of determining the defense repertoire of a seed is to test the seed contents against potentially susceptible biological systems. In fact, two of the large and potentially defensive types of proteinaceous molecules found in seeds are traditionally recognized by their physiological activity against animal systems rather than by chemical characterization. Here we report for 59 species of tropical legume seeds the results of challenging trypsin, a protease enzyme, with the finely ground contents of live seeds. We also report the results of asking if the same seed contents will agglutinate red blood cells of the human (type B, Rh positive) and the laboratory rabbit (*Oryctolagus cuniculus*).

As will be discussed below, we wish to emphasize that in such a general screening only positive results are useful; protease inhibitors and phytohemagglutinins display a wide variety of specificities, and a seed that neither inhibits trypsin nor agglutinates rabbit or human red blood cells may still contain a very potent proteinase inhibitor or phytohemagglutinin. For example, *Phaseolus lunatus* seeds showed no hemagglutination with type B blood (Table 1) but contain a powerful hemagglutinating factor for type A human blood (anonymous reviewer). The species of seeds to be screened were chosen in great part because they are the subjects of intensive feeding tests against seed predators in the Costa Rican habitats where they grow (e.g., Janzen, 1981; Janzen et al., 1986) and because they are subjects of an intensive long-term effort to characterize the entire defense repertoire of certain species of seeds (e.g., *Dioclea megacarpa*, Rosenthal et al., 1982; Rosenthal and Janzen, 1983, 1985; *Lonchocarpus* spp., Fellows et al., 1979; Janzen et al., 1986).

METHODS AND MATERIALS

Seed Sources. All the seeds listed in Table 1 were collected by D.H.J. between 1965 and 1980 in the lowlands of Guanacaste Province, Costa Rica except as itemized below. Exceptions: *Entada gigans* and *Oxyrhynchus trinervius* were beach drift seeds and *Mucuna mutisiana*, *Parkia pendula*, and *Pterocarpus officinalis* were rainforest seeds, from Corcovado National Park, southwestern Costa Rica; *Adenopodia polystachya* and *Canavalia bicarinata* came from mangrove forest margins near Puntarenas, Costa Rica; *Erythrina* spp. came from the area around San Jose, Costa Rica; *Mucuna andreana* came from rainforest edges at Finca La Selva, Heredia Province, Costa Rica; *Leucaena glauca* from St. John's Island, British Virgin Islands, Caribbean; *Ormosia* spp. were

TABLE 1. POTENTIALLY DEFENSIVE COMPOUNDS IN SEED CONTENTS OF 59 SPECIES OF TROPICAL LEGUMINOSAE

Plant	Dry weight (mg)		Protease inhibitor[c] (μg dry seed/μg trypsin at 50% inhibition)[d]	Hemagglutinin activity[e] (HU/mg dry weight)	
	Entire seed[a]	Seed contents[b]		Rabbit	Human[f]
Caesalpiniodeae					
Bauhinia glabra	101	72	11.9	12.0	0.0
Bauhinia pauletia	69	49	321	0.5	0.0
Bauhinia ungulata	30	15	393	1.0	0.0
Caesalpinia bonduc	4101	2297		0.0	0.0
Caesalpinia coriaria	30	15	476	0.0	0.0
Caesalpinia eriostachys	371	260	16.2	0.0	0.0
Caesalpinia pulcherrima	214	150	526	4.2	0.0
Cassia biflora	4	2.2	393	0.0	0.0
Cassia emarginata	28	17	1071	0.0	0.0
Cassia grandis	1152	772	19.0	1.8	7.0
Cassia leptocarpa	12	7	≫2500	0.0	0.0
Delonix regia	758	440	395	trace	0.0
Schizolobium parahybum	1208	737	≫2500	0.4	0.0
Mimosoideae					
Acacia angustissima	12	10	211	0.0	0.0
Acacia cornigera	236	130	71.4	trace	1.3
Acacia tenuifolia	42	21	57	0.0	0.0
Adenopodia polystachya	288	216	6.7	0.0	0.0
Albizzia adinocephala	16	10	55	0.0	0.0
Albizzia caribaea	33	20	38	trace	1.7
Albizzia longepedata	30	18	151	0.0	1.3
Entada gigans	23621	22440	27.6	0.0	0.0
Enterolobium cyclocarpum	1423	740	26.7	0.0	0.0
Leucaena glauca	48	27	147	0.0	0.0

Lysiloma divaricata	35	20	41	0.0	0.0
Mimosa quadrivalis	7	6	613	0.0	0.0
Parkia pendula	100	65	39	0.3	0.0
Pithecellobium mangense	35	18	41	1.1	0.0
Pithecellobium platylobum	203	152	28.6	trace	0.0
Faboideae					
Ateleia herbert-smithii	68	41	$\gg 2500$	0.0	0.0
Canavalia bicarinata	233	189	54.3	81.0	0.0
Canavalia brasiliensis	568	449	33.3	106.0	trace
Canavalia maritima	1225	919	161.9	157.0	0.0
Centrosema plumieri	94	73	105	0.0	0.0
Centrosema pubescens	44	36	267	0.0	0.0
Crotalaria incana	6	5	264	0.0	0.0
Crotalaria pumilio	4	3	208	0.4	2.0
Dalbergia retusa	56	44	72	0.0	0.0
Dioclea megacarpa	12701	9907	57.1	75.0	1.8
Oxyrhynchus trinervius	2200	1716	17.1	0.0	0.0
Erythrina berteroana	282	186	5.7	0.0	8.7
Erythrina poeppigiana	326	261	148	0.0	2.1
Gliricidia sepium	140	119	$>1000^g$	trace	0.0
Indigofera hirsuta	1	0.6	559	0.0	0.0
Lonchocarpus acuminatus	317	292	2200	0.0	0.0
Lonchocarpus costaricensis	309	250	1050	0.0	0.0
Lonchocarpus eriocarinalis	215	174	112.4	0.0	0.0
Lonchocarpus minimiflorus	68	58	1063	0.0	0.0
Lonchocarpus rugosus	51	41	648	trace	0.0
Lonchocarpus salvadorensis	110	105	>1500	0.0	0.0
Machaerium arboreum	321	305	17.1	0.5	0.0
Mucuna andreana	9205	8100	138.1	8.0	0.0
Mucuna mutisiana	9445	7367	85.7	27.0	0.0
Mucuna pruriens	379	315	235	0.0	0.0
Ormosia tovarensis	3419	2838	2480	0.2	0.0

TABLE 1. Continued

Plant	Dry weight (mg)		Protease inhibitor[c] (μg dry seed/μg trypsin at 50% inhibition)[d]	Hemagglutinin activity[e] (HU/mg dry weight)	
	Entire seed[a]	Seed contents[b]		Rabbit	Human[f]
Ormosia venezolana	1281	1089	933	0.0	0.0
Phaseolus lunatus	75	65	29.0	trace	0.0
Pterocarpus officinalis	792	752	10.9	0.2	70.1
Sesbania emerus	7	5	786	0.2	trace
Sophora macrocarpa	607	449	71.4	trace	0.0
Pure SBA[h]				28,000 ± 10%	540 ± 10%

[a] Dry weight of seed with seed coat. Derived from percent seed coat values in Janzen (1977).
[b] Dry weight of seed contents. Derived from percent seed coat values in Janzen (1977).
[c] Results from laboratory of C.A. Ryan, Department of Agricultural Chemistry, Washington State University, Pullman, Washington 99164 (1978). Chymotrypsin assays could not be run because seed carboxypeptidases split the chymotrypsin substrates.
[d] Large seeds had seed coats removed before grinding and testing; small seeds were ground entire and tested as such.
[e] Results from Laboratory of I.E. Liener, Department of Biochemistry, University of Minnesota, St. Paul, Minnesota, 55108.
[f] Human blood group: B, Rh positive.
[g] Precipitated out, could not get good results, very weak.
[h] Prepared as described in Uy and Wold (1977).

purchased in a native market in Merida, Venezuela; *Sophora macrocarpa* was collected to the north of Santiago, Chile.

These plant species are common, and the collections were unambiguously identified by D.H.J. Additionally, voucher specimens for all these species have been deposited in the herbarium of the Missouri Botanical Gardens, St. Louis, Missouri. All the Guanacaste plant names conform to Janzen and Liesner (1980) except that *Lysiloma seemannii* is now known as *Lysiloma divaricata*. D.H.J. identified all plants in the field and was certain that only mature and dormant seeds were collected for analysis.

Seed Treatment. Large seeds, those weighing more than 500 mg dry weight after removal of the seed coat, were ground and tested after removal of their seed coats. Smaller seeds were ground up entire for the tests. All seeds were living at the time of grinding and ranged between 5 and 10% water content (i.e., seemingly dry and hard). Seeds were finely ground in a Wiley mill before testing. This seed meal was not oven-dried before use or weighing for percentage determinations.

Proteinase Inhibitor Tests. Bovine trypsin was purchased from Worthington (Freehold, New Jersey) and was approximately 50% active as determined with the active site titrant *p*-nitrophenyl-*p'*-guanidinobenzoate (Chase and Shaw, 1967). Enzyme activity was determined by the method of Hummel (1959) using tosyl-L-arginine methyl ester as substrate. The powder from individual seeds was dissolved in 0.5 M KCl (50 mg powder per ml), soaked for 1 hr, and contrifuged at 10,000*g* for 10 min. The clear supernatants were employed for assays. Trypsin inhibitory activity was determined by the addition of increasing quantities of the extract to a standard quantity of trypsin and incubated for 5 min prior to assay. The data is reported as the quantity (dry wt) required to decrease the activity of 1 μg trypsin 50%.

Hemagglutinin tests. The finely ground seed was suspended in 10 volumes of physiological saline (0.9% NaCl) and vigorously shaken for 5 min. Any insoluble material was removed by centrifugation, and the hemagglutinating activity of the supernatant was determined by the photometric procedure of Liener (1955). In this method, serial dilutions of this extract (or a suitably diluted portion thereof) are added to an equal volume of a suspension of trypsinated rabbit or human red blood cells. The latter were prepared by prior treatment with glutaraldehyde, as described by Turner and Liener (1975), in order to increase their stability during storage. The mixture of each dilution with the red blood cell suspension was allowed to sit at room temperature for 2 $\frac{1}{2}$ hr, at which time the absorbance at 620 nm was measured in a Coleman Junior Spectrophotometer model 6A.

One hemagglutinating unit (HU) is arbitrarily defined as that amount of lectin which causes a 50% decrease in the absorbancy of the cell suspension under the conditions specified above. The reciprocal of the dilution (X) corre-

sponding to 1 HU may be calculated from the percentage of the cells remaining in suspension of those tubes having the closest values which are above (tube a) and below (tube b) the 50% endpoint. The following equation is employed:

$$\log x = \log c = (50 - b) \cdot \log 2$$

Where c = reciprocal of dilution in tube b, a = % of cells in suspension in tube a, and b = % of cells in suspension in tube b. The agglutinating activity of the seed extract is then calculated from the value of x and the weight of the seed material employed in making the original extract.

RESULTS

The results in Table 1 show clearly that trypsin inhibition among a large array of legume seeds can range from negligible (e.g., *Cassia leptocarpa*, *C. emarginata*, *Schizolobium parahybum*, *Ateleia herbert-smithii*, *Gliricidia sepium*, *Lonchocarpus acuminatus*, *L. costaricensis*, *L. minimiflorus*, *L. salvadorensis*, *Ormosia tovarensis*) to very intense (e.g., *Bauhinia glabra*, *Adenopodia polystachya*, *Erythrina berteroana*, *Pterocarpus officinalis*). Very intense activity was found in all three legume subfamilies, but the Mimosoideae are conspicuous for the absence of seeds with very weak trypsin inhibition. Congenerics may differ strongly from each other (e.g., different species of *Cassia* seeds ranged from very intense inhibition to virtually no effect) as well as be quite similar. Except for the two species of *Ormosia* (which are traditionally viewed as very rich in alkaloids that are toxic to vertebrates), all really large seeds showed inhibitor activity; small seeds display such a variety of intensity that no generalization can be made about the direction of their intensity.

Half the species of seeds displayed no hemagglutination activity against laboratory rabbit or human blood cells. The same seed was active against both kinds of red blood cells in only 27% of the 30 cases where there was some activity. Overall, there was more agglutination response by the rabbit than by the human red blood cells; this conforms with the direction of the "control" results with a purified hemagglutinin SBA. However, in both cases the response is relatively weak when compared with SBA. A high level of hemagglutinating activity against one kind of red blood cell is apparently randomly associated with the level of activity against the other.

A species of seed that showed hemagglutinating activity was almost invariably associated with comparatively moderate to high levels of trypsin inactivation. However, there were at least two cases of some hemagglutination by seeds that displayed virtually no trypsin inhibition (e.g., *Schizolobium parahybum*, *Ormosia tovarensis*).

DISCUSSION

The positive results listed in Table 1 are only the tip of the iceberg. It is likely that every legume seed contains one or more protease inhibitor and/or one or more phytohemagglutinin. However, demonstrating this will require screening against a wide variety of enzymes (and perhaps screening under a variety of environmental conditions such as different but biologically realistic levels of pH and temperature). Likewise, given the strong specificity of different kinds of phytohemagglutinins for different species of red blood cells, a seed will have to be screened against many kinds of blood cells and perhaps other sugar-containing substrates before it can be certified as free of phytohemagglutinins (lectins). For example, as mentioned earlier, lectins in *Phaseolus lunatus* seeds do not hemagglutinate type B human blood, but do hemagglutinate type A human blood.

Since virtually every species of seed listed in Table 1 contains one or more kinds of uncommon amino acid, alkaloid, or cyanogenic glycoside, the results of this screening add robustness to the hypothesis that each species of legume seed is likely to have a variety of defenses. For example, *Dioclea megacarpa* contains canavanine and canaline, and now it is clear that it also contains a trypsin inhibitor protein and a hemagglutinating protein. Conversely, when an animal refuses to feed on a seed, it is clear that one cannot automatically attribute such a refusal to the first potentially defensive compound that is easily isolated from the seed. For example, *Pterocarpus officinalis* seeds contain the alkaloid hypaphorine, a potent feeding deterrent to at least one species of small seed-eating rodent (Janzen et al., 1982). However, it is obvious from the results presented in Table 1 that the refusal of wild rainforest animals to harvest the large and abundant *P. officinalis* seeds may also be due to their strong trypsin inhibitor ability, strong lectin activity, or the combination of these obnoxious proteins with the alkaloid (or even with some as yet undetected other defensive compound).

When an animal bites into a seed, it gets the defensive compounds along with a large bulk of material that is either quite edible and digestible, or merely inert from the viewpoint of the animal. The material other than the defensive compounds plays two important roles in the context of this survey. First, when examining the defensive compound repertoire of a seed (or any other plant part), it is commonplace to think in terms of concentration (or activity) of the compounds in the seed contents. This quantitative measure is presumed to have some biological meaning. However, we must caution that concentrations (or intensity of activity) as measured by the biologist are extremely difficult to accurately interpret in terms that represent how the animal experiences them. Not only is there the problem that one beast's poison may be another beast's dinner, but additionally there may be strong threshold effects. For example, Gatehouse

et al. (1979) showed that the threshold level of trypsin inhibitor in *Callosobruchus*-resistant cowpeas (*Vigna unguiculata*) lies between 0.5 and 0.8%.

Furthermore, impact of the defense compound may simply be to lower the gain from the food being eaten, rather that do some direct damage to the animal. For example, if a trypsin inhibitor occurs at some low level, it may inactivate only part of the gut enzyme pool, leaving another portion of the gut's enzymes to function normally. If the seed happens to contain a large amount of the substrate, the remaining enzyme pool may be able to harvest so much resource that the trypsin inhibitory effect is inconsequential from the viewpoint of the potential seed predator; seed predators eat mouthfuls of seed content, rather than just the compounds being assayed. In the context of the present survey, the message is that high or low values of proteinase inhibitor or phytohemagglutinin activity may not translate well into the inedibility or toxicity of a meal of seeds, especially when one considers the highly positive gains that an animal may get from a bite of the seed. Of course, the greater the amounts and kinds of potential defense materials in the seed, and the lesser the amounts of traditional nutrients present, the greater the chance that a seed will be inedible or toxic to a seed predator that is not specialized on that species of seed. Equally, the larger the defense repertoire and the less the nutrient content, the more biochemically specialized is likely to be the specialist seed predator that regularly feeds on a seed that is toxic to other animals.

Second, the other portions of the seed affect the interpretation of our survey results. When a caterpillar takes a bite of a leaf, some of the defensive compounds in the leaf may function in making leaf material unavailable rather than hurting the caterpillar directly; likewise, the contents of the seeds surveyed here may affect the ability of the proteinase inhibitors and phytohemagglutinins to react with the test substrates. For example, when the seed is ground up and placed in a liquid medium, the trypsin inhibitors may well react with some of the other proteins in the seed rather than with the trypsin offered as an assay. Likewise, the phytohemagglutinins may cross-react with complex polysaccharides in the seed meal, and thus be prevented from participation in the bioassay. A number of such responses are to be expected if selection has operated to produce a seed whose nutrients become unavailable when the seed is eaten, rather than (or in addition to) to produce a seed that is actually toxic or otherwise inedible to the seed predator.

In other words, the positive results in Table 1 can be believed, but no result, including the zeros, can be taken to indicate the maximum amount of reactable proteinase inhibitors or phytohemagglutinins that may occur in the seed. As mentioned in the Introduction, it is even quite possible for there to be inhibitors present that do not react with bovine trypsin but will react with other enzymes. Likewise, the values obtained in Table 1 cannot be viewed as the maximum possible levels of reaction in the gut of an animal, since the gut adds

various other substrates and reactants to the mix, creating a reaction environment that is obviously different from that used in the screening for the data in Table 1 (see Becker, 1984, for a discussion of similar philosophies vis à vis tannins as digestion inhibitors for leaf-eating caterpillars).

It is tempting to expect an inverse relationship between the activities of the two quite different classes of proteins for which we assayed. However, the expectation disappears when one considers that these two classes of compounds are only two of at least three to five classes of potentially defensive compounds in a seed (and perhaps there are double that number of kinds of defensive compounds when one considers that it is commonplace for a single species of seed to contain several kinds of alkaloids or uncommon amino acids). Furthermore, the plant has other defenses such as seed hardness, seed number, phenology, dispersal mode, etc. (Janzen 1978). A seed that is short on any one these defenses may well be exceptionally well-endowed on some other defense axis (e.g., Janzen, 1969, 1977; Janzen and Higgins, 1979) rather than just increase one of the potential defenses that we happen to screen for here.

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TOXICITIES OF HOST SECONDARY COMPOUNDS TO EGGS OF THE *Brassica* SPECIALIST *Dasineura brassicae*[1]

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Abstract—The toxicities of selected host secondary plant substances to eggs of the *Brassica* specialist *Dasineura brassicae* were estimated in a laboratory test. Isothiocyanates (ITCs) and 1-cyano-2-phenylethane, autolysis products of glucosinolates, were more toxic than a "green leaf alcohol," *cis*-3-hexen-1-ol, found in numerous plant families. The most toxic compound, 2-phenylethyl ITC, occurs in all investigated *Brassica* hosts of *D. brassicae*. However, the less suitable *Brassica* hosts additionally release comparatively large amounts of allyl ITC. This suggests that even a crucifer specialist may be restricted in its use of particular hosts due to their compositions and concentrations of glucosinolate compounds.

Key Words—Secondary compounds, glucosinolate, isothiocyanates, toxicity, *Brassica*, Cruciferae, *Dasineura brassicae*, Diptera, Cecidomylidae.

INTRODUCTION

Glucosinolates are characteristic secondary compounds of cruciferous plants (Kjaer, 1976). Glucosinolate compounds (glucosinolates and/or their breakdown products) can have adverse effects on herbivores which, in nature, do not feed or feed only to a limited extent on crucifers (Feeny, 1977; Blau et al., 1978; Lerin, 1980). Deleterious effects have very seldom been shown against insects specialized for feeding on crucifers. However, very few experiments have been specifically designed for testing toxicities of glucosinolate compounds to crucifer specialists (Feeny, 1977). Much more information is available regarding their use of glucosinolate compounds as behavioral cues in connection with feeding and reproduction (Verschaffelt, 1911; Feeny, 1977; Lerin,

[1] Winn., Diptera: Cecidomyiidae.

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1980). Although glucosinolates are ubiquitous in crucifers, plant species differ with regard to composition, concentrations and breakdown routes of these compounds (Kjaer, 1960, 1976).

Since cruciferous species can be characterized by their glucosinolate profiles (Rodman & Chew, 1980), tolerances of a particular crucifer specialist to the various substances may help explain the extent to which it uses individual cruciferous species as hosts. In this study, the toxicities of selected compounds to eggs of the *Brassica* specialist *Dasineura brassicae* Winn. were estimated. The selection of test compounds was based on knowledge about host plant suitability (Åhman, 1981) and plant chemistry (Cole, 1976).

Host Relationships of D. brassicae. In Sweden, *D. brassicae* infestations have only been reported on *Brassica* spp. (Sylvén, 1949). *D. brassicae* females deposit their eggs through holes in the pod walls with their telescopic ovipositor. The oviposition holes are, to a large extent, feeding and oviposition punctures made by the weevil *Ceutorhynchus assimilis* Payk. (Speyer, 1921; Sylvén and Svenson, 1975). Eggs normally hatch after four days, and larvae feed inside the pods until they are full-grown (Sylvén, 1949). In a host plant suitability test (Åhman, 1981), both egg survival and larval weights were significantly lower on *B. juncea* Coss & Czern (brown mustard; Chinese–eastern European origin) and *B. nigra* Koch (black mustard) than on *B. napus* L. (rape) and *B. campestris* L. (turnip rape). In intact cruciferous plants, glucosinolates occur within the parenchymal tissues (Kjaer, 1960), and only minute amounts are broken down and released from the plants (Cole and Finch, 1978). However, when plants are damaged, glucosinolates hydrolyze enzymatically, and both volatile and nonvolatile aglucones are released (Kjaer, 1960).

Since *D. brassicae* females oviposit in the vicinity of damaged plant tissue, the eggs are probably exposed to comparatively high concentrations of aglucones. Cole (1976) made a survey of volatile glucosinolate aglucones released after autolytic hydrolysis of numerous crucifers and found that concentrations of isothiocyanates (ITCs) in *B. juncea* and *B. nigra* were considerably higher than in *B. napus* and *B. campestris* (Table 1). There were also differences in aglucone compositions between the suitable and less suitable hosts for *D. brassicae* (Table 1). Since the composition and concentrations of glucosinolate compounds may vary within a species with plant age, plant part, environmental conditions, and plant genotype (Delaveau, 1952, 1958; Kjaer, 1960; Lichtenstein et al., 1964; Josefsson, 1967, 1971; Josefsson and Appelqvist, 1968; Cole, 1978, 1980; Louda and Rodman 1983a,b), only major patterns were considered during selection of test compounds.

METHODS AND MATERIALS

Test Compounds. The compounds selected for testing were: sinigrin, the "mother" glucosinolate of allyl ITC; allyl ITC, predominant in the less suitable hosts *B. juncea* and *B. nigra*; 2-phenylethyl ITC, present in all *Brassica* species

TABLE 1. VOLATILE AGLUCONES FROM GLUCOSINOLATES IN 8-WEEK-OLD AUTOLYSED *Brassica* PLANTS (MODIFIED FROM COLE, 1976)

	B. napus	*B. campestris*		*B. juncea*[a]	*B. nigra*
Aglucone concentration (μg/g plant tissue)	9	11[b]	100[c]	35	38
Isothiocyanates (ITCs) (% of total aglucones)	56	36	12	97	97
Allyl ITC (% of total aglucones)	0	0	1	69	84
2-Phenylethyl ITC (% of total aglucones)	11	27	5	20	11
1-Cyano-2-phenylethane[d] (% of total aglucones)	11	9	19	0	0

[a]*B. juncea* shows polymorphism in glucosinolate composition (seed investigations) relative to geographical origin. Chinese–European forms contain mainly sinigrin, while gluconapin in the major glucosinolate in forms from Pakistan and India (Vaughan et al., 1963; Josefsson, 1972; Gland et al., 1981).
[b]Named *B. rapa* L. by Cole (1976); synonymous with *B. campestris* ssp. *rapifera*, turnip, a European form with swollen "roots."
[c]Named *B. chinensis* L. by Cole (1976); synonymous with *B. campestris* ssp. *chinensis*, an Asian leafy form.
[d]Named 2-phenylpropionitrile by Cole (1976).

investigated by Cole (1976); *n*-butyl ITC, which is not known to occur naturally; 1-cyano-2-phenylethane (i.e., the nitrile corresponding to the ITC, 2-phenylethyl ITC; Cole 1980), which was found in the suitable hosts but not in *B. juncea* and *B. nigra*, and finally, *cis*-3-hexen-1-ol, which occurs in *Brassica* spp. (MacLeod, 1976; Wallbank and Wheatley, 1976; Cole, 1978, 1980) and in numerous other plant species from various families (Visser et al., 1979). Relative concentrations of glucosinolate breakdown products to "green leaf compounds" (*cis*-3-hexenol and *trans*-2-hexenal) vary during plant development. In mature *B. campestris* plants, the ratio fluctuates around 1:1 (Cole, 1978, 1980).

The test compounds were obtained from various sources: sinigrin from Nutritional Biochemicals Corporation, Cleveland, Ohio; allyl ITC from Merck, Darmstadt, BRD; 2-phenylethyl ITC and *n*-butyl ITC from Maybridge Chemical Co. Ltd., Tintagel, Cornwall, UK; *cis*-3-hexen-1-ol from Northern Aromatics Ltd., Radcliffe, Manchester, UK, and 1-cyano-2-phenylethane was synthesized by the Department of Organic Chemistry, Royal Institute of Technology, Stockholm. The purity of the compounds was at least 95%.

Dilution Preparations. Series of 10-fold dilutions (v/v) of liquid test compounds in 0.09 M NaCl–water solution were prepared. Desired concentrations of the solid compound, sinigrin, were obtained by weighing and directly dissolving the compound in NaCl–water solution. Since most liquid compounds had low solubility in water, each dilution was homogenized just before pipetting, using an automatic shaker. Also, in some dilution preparations, the surfactant Tween 80 (0.1 ml/liter) was added to enhance mixing of the test compound with the NaCl–water solution. However, results from the bioassays with and without the surfactant were of the same order of magnitude. Preliminary tests were made to determine the approximate minimum lethal concentration for each compound. Dilutions with concentrations around this point were later tested in a standardized way.

Egg Treatments. Eggs were exposed to the compounds in the wells (volume: 300 μl) of polystyrene, microtiter plates. Into each of the 16 wells on a plate, 150 μl of one of the dilutions of a test compound or the control (NaCl–water solution) was pipetted. The fluid was soaked up by Sephadex G-25 Fine, which was carefully poured into the well. *D. brassicae* eggs were obtained from *B. napus* plants exposed to females in the field or in a cage in the laboratory. The egg transfer was carried out under a microscope, using a scalpel to open the pods. One egg at a time was picked up on a fine-tipped, moistened brush and placed on top of the Sephadex "bed" in a well. Eggs from each egg batch were distributed among the control plate and the plates with various concentrations of one of the test compounds. Afterwards, each plate was tightly wrapped in one layer of Parafilm and another of aluminum foil, isolating the wells from each other and from the surrounding air. The plates with eggs were kept at room temperature (20–22°C) for at least five days, after which egg mortality (i.e., proportion of eggs that had not hatched) was determined under a microscope.

Method Evaluation. Lethal doses determined by this method reflect not only the toxicities of the compounds, but also their transport characteristics such as their volatility and tendency to penetrate the egg. All the compounds other than the salt sinigrin may have a fumigant action. However, test conditions should be fairly similar to the natural situation occurring for eggs in pods. Furthermore, one must be aware that even if toxicity test results correspond to ITC release of the plants (Cole, 1976), other plant characteristics might have been responsible for the observed differences between *Brassica* spp. in *D. brassicae* mortality rates (Åhman, 1981).

RESULTS

All the tested breakdown products from glucosinolates and *n*-butyl ITC were lethal to *D. brassicae* eggs at doses lower than that of the "leaf alcohol" *cis*-3-hexen-1-ol (Figure 1). 2-Phenylethyl ITC had the lowest lethal dose of

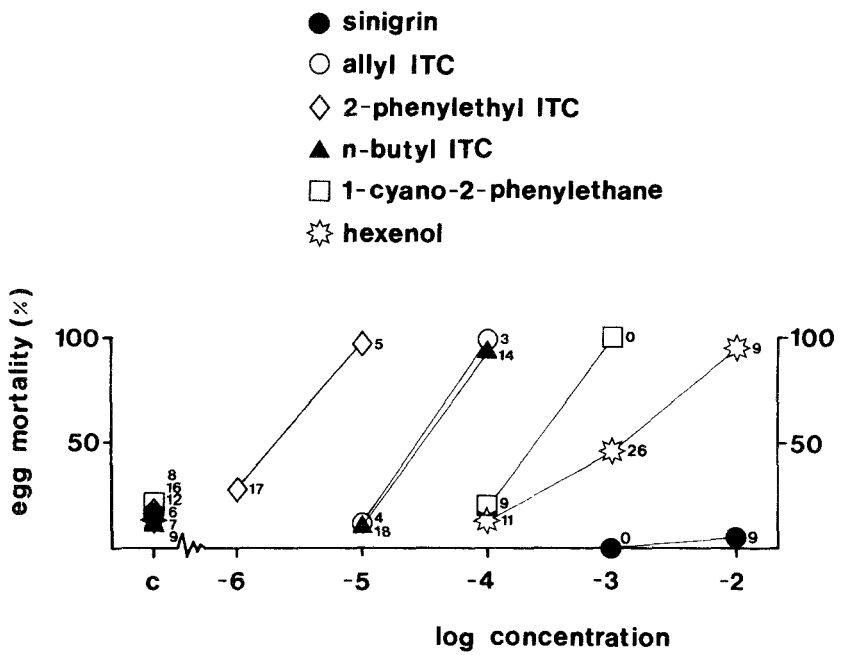

FIG. 1. Dose–mortality relationships for *Brassica* secondary compounds and *D. brassicae* eggs. Each point represents a mean from at least four replicates of 16 eggs each, except for sinigrin −2, −3 (two replicates). Concentrations of liquid compounds: v/v, and sinigrin: g/ml (c = controls). Figures represent ± standard deviations. Lines connect mean points.

all the test compounds. Lethal doses of allyl ITC and *n*-butyl ITC were lower than that of the nitrile 1-cyano-2-phenylethane, and no lethal effects were obtained with the glucosinolate sinigrin at the concentrations tested.

DISCUSSION

D. brassicae mortality rates were higher on *B. juncea* and *B. nigra* than on *B. napus* and *B. campestris* in a previous host suitability test (Åhman, 1981). Results from the present study imply that this differential mortality may have been due to the higher release of ITCs from *B. juncea* and *B. nigra* (Cole, 1976). The most toxic compound, 2-phenylethyl ITC, occurs in all four plant species, but *B. juncea* and *B. nigra* additionally release comparatively large amounts of allyl ITC (Table 1). However, female oviposition behavior might reduce egg exposure to potentially toxic hole exudates. For example, fewer eggs were laid on *B. juncea* and *B. nigra* than on *B. napus* and *B. campestris* in preference trials (Åhman, 1981, 1985b), implying that host use patterns may

be affected by the host's glucosinolate profile; however, differential oviposition behavior need not necessarily rely on such compounds as cues.

In addition, the female may reduce the exposure of her eggs to autolysis products from the damaged pods by using her long ovipositor to place eggs at a distance from the holes. Such behavior was suggested when two previous host suitability tests (Åhman, 1981, 1985a) using different egg transfer techniques were compared. In the study where mortality rates differed between *Brassica* spp. (Åhman, 1981), eggs in NaCl–water solution were injected into the pods with a syringe through small holes in the pod walls. In the other study, V-shaped scars were made in the pods, and the eggs were placed away from those scars with a fine-tipped brush (Åhman, 1985a). In this latter case, there was no significant difference in egg mortality between the two test species, *B. napus* and *B. juncea*. The eggs were probably more exposed to potentially toxic plant juices in the first injection-transfer than in the V-scar transfer. Furthermore, females may avoid oviposition in recently made holes, where concentrations of breakdown products are likely to be high.

2-Phenylethyl glucosinolate (or probably rather the ITC, produced when this glucosinolate is broken down) seems to be a particularly toxic compound to many insects, including crucifer specialists. It was one of the two glucosinolate compounds toxic to the crucifer specialist *Plutella maculipennis* Curtis when tested in diets at concentrations where other glucosinolates were not toxic (Nayar and Thorsteinson, 1963). Furthermore, in a field study, *Cardamine cordifolia* plants without crucifer-adapted chrysomelids on them were characterized by a particularly high content of that compound (Louda and Rodman, 1983a). Moreover, insecticidal actions of various macerated crucifer roots and root extracts, against insect species which do not feed on crucifers, have been attributed to the high release of 2-phenylethyl ITC from those roots (Lichtenstein et al., 1962, 1964; Lowe et al., 1971).

Not only was survival of *D. brassicae* eggs transferred to *B. nigra* and *B. juncea* lower than that of eggs transferred to *B. napus* and *B. campestris*, but larval weights were also lower on *B. juncea* and *B. nigra* (Åhman, 1981, 1985a). To ensure that the lower larval weights were not a result of delayed egg hatching, recently hatched larvae (instead of eggs) were transferred to *B. napus* and *B. juncea* using the V-scar technique. This test also showed larval weights to be significantly lower on *B. juncea* (I. Åhman, unpublished results). Thus, apart from being potentially lethal to *D. brassicae* eggs, *B. juncea* and *B. nigra* also have negative effects on larval growth. These adverse effects may be caused by their high ITC release and restrict *D. brassicae* from using them as hosts.

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IDENTIFICATION OF SEX PHEROMONE PRODUCED BY FEMALE SWEETPOTATO WEEVIL, *Cylas formicarius elegantulus* (SUMMERS)[1]

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Abstract—A sex pheromone of the sweetpotato weevil, *Cylas formicarius elegantulus* (Summers), was obtained from collections of volatiles from virgin females, and pheromone was isolated by means of liquid and gas chromatography. The purification procedure was monitored by quantitative laboratory and field bioassays and the compound was identified as (*Z*)-3-dodecen-1-ol (*E*)-2-butenoate by means of spectroscopic and microchemical methods. Synthesis, followed by laboratory and field bioassays, showed that the biological activity of the synthetic material was qualitatively and quantitatively indistinguishable from that of the purified natural product.

Key Words—*Cylas formicarius elegantulus*, sweetpotato weevil, sweet potato, (*Z*)-3-dodecen-1-ol (*E*)-2-butenoate, sex pheromone.

INTRODUCTION

Sweet potato is the sixth most important crop in the world (Anonymous, 1981) and is surpassed in importance as a root crop only by the potato (Martin, 1983). Most sweet potatoes are grown in the tropics, and there the sweetpotato weevil, *Cylas formicarius elegantulus* (Summers), is the limiting factor to both production and storage of this crop. This pest occurs in all parts of the tropics where sweet potatoes are grown and in many temperate zones as well. Very low level pre- or postharvest infestations reduce both quality and marketable yield (Pros-

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hold, 1983), and extremely bitter tasting and toxic sesquiterpenes produced by the potato tissue in response to insect feeding can render the commodity unfit for consumption (Akazawa et al., 1960; Uritani et al., 1975). Losses due to such infestations and diseases that often follow weevil attack are estimated at 35–95% (Anonymous, 1978).

Presently, there is no suitable method for the detection of low-level infestations; by the time insects are seen on the crop, considerable damage has already been done. The underground feeding habits of the larvae and the nocturnal activity of the adults make it difficult for farmers to make effective use of pesticides. Thus, there is a need for a sensitive detection tool to aid the grower in managing and controlling infestations of the sweetpotato weevil (Coffelt et al., 1978).

It is established that insect pheromones can often be used to locate, survey, or monitor pest populations at levels not otherwise detectable (see Klassen et al., 1982). We report here the isolation and identification of a sex pheromone, (*Z*)-3-dodecen-1-ol (*E*)-2-butenoate, obtained from female sweetpotato weevils and the response of conspecific males to the synthesized pheromone in the laboratory and in the field.

METHODS AND MATERIALS

Insect Rearing and Handling. Insects were reared in the laboratory on commercially obtained sweet potatoes at 26 ± 1°C and 60 ± 5% relative humidity under a 14:10 (light–dark) photoperiod using the procedures described by Coffelt et al. (1978). Virgin insects were obtained (females for pheromone collection and males for use in bioassays) by separating male and female weevils within 24 hr of eclosion. Adults were maintained, prior to testing, for 5–7 days in groups of 50–75 in 100 × 15-mm glass Petri dishes with bottoms lined with Whatman No. 1 filter paper. During this period, males and females were kept in separate growth chambers with environmental parameters identical to those used for insect rearing. Adults were provided a small slice of sweet potato every 48 hr.

Pheromone Collection. Pheromone collections were made by drawing air over virgin female weevils that were confined in a 10.5-liter (24.5 × 22.2 cm diam.) glass jar. Each jar contained 1500–2000 females and one sweet potato. House vacuum (120mm Hg) was used to pull ambient air through the unit at 2 liters/min. The units were kept in a laboratory hood within a room maintained at 26 ± 1°C and 65 ± 5% relative humidity and a 14:10 (light–dark) photoperiod. Volatiles emanating from the unit were entrained using two glass adsorption traps (1.5 × 8 cm) each containing 2.0 g Chromosorb 102 (60–80 mesh). Prior to use, the adsorbent was eluted with 100 ml of reagent-grade diethyl ether.

Volatiles were collected continuously over a 30-day period for a given

batch of insects. Pheromone was collected at 48-hr intervals by eluting the entrained volatiles with 5–7 ml of pentane–ether (90:10). A fresh potato was placed in the unit at this time. During the course of this study, approximately 850,000 female equivalent days (FED) were collected.

Laboratory Bioassay. Samples were bioassayed using the procedure described by Coffelt et al. (1978). Briefly, 3–5 μl of test extract were pipetted onto the flattened end of a glass rod and then exposed to a series of ten males that were held individually in 1.6 × 5-cm Teflon-coated shell vials. The treated rod was suspended within 1 cm of the test insect for a period not greater than 15 sec. A positive response, defined as antennal elevation and locomotion, was taken as evidence of the presence of pheromone. Rods treated with solvent only served as controls, and exposure of a single rod to 10 males constituted one replication. This bioassay was employed only after initial studies revealed that only those fractions that elicited the above responses were attractive to males in the field. All laboratory bioassays were conducted under Cool-White fluorescent room lighting at 26 ± 2°C during the latter half of the photophase using males that were between 7 and 30 days of age. Pheromone units are expressed in terms of FED, one FED being the estimated quantity of pheromone obtained from one female per 24-hr period. The collected volatile material and all fractions obtained in the purification of the pheromone were bioassayed at 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} FED.

Field Tests. Field bioassays were conducted in February–August 1984 in 0.2-hectare plots of sweet potato at the Federal Experiment Station, St. Croix, U.S. Virgin Islands.

In the first series of tests (February 1984), unpurified volatiles that contained pheromone, partially purified material (active fractions from HPLC) and purified material (from the Carbowax 20 M GC column) were compared to determine whether any loss of attractiveness to feral males occurred as a consequence of the indicated fractionations.

Doses of 0.0, 0.2, 2.0, and 20 FED in 50 μl hexane were applied to 22 × 22-mm glass cover slides. After solvent evaporation, the cover slides were placed in the center of a 100 × 15-mm Teflon-lined Petri dish (cover slides elevated 16 mm above floor of dish). Dishes were placed at the center of individual 50 × 50-cm wooden platforms raised to the plant canopy height (15–20 cm). The number of males on the platform and in the dish (< 15 mm from source) within 5 min of deployment of the sample were recorded and taken as evidence of response. All tests were conducted between 1930 and 2145 hr Atlantic ST at temperatures ranging from 20.5 to 22.2°C.

Platforms were deployed at 2-m intervals in a line approximately perpendicular to the prevailing easterly wind direction. Treatment locations were randomized within plots, and a total of nine replications were made in three different plots over several nights, with no plot being used for more than one replication per night. Cover slides treated with hexane only served as controls.

The second series of field bioassays was conducted in March and September 1984 to compare the relative attractiveness of the purified natural product with synthetic pheromone (>99% purity). Doses treated were 0.0, 0.08, 0.8, 8.0, and 80 ng/50 μl, and the material was formulated on glass slides. Additionally, the purified natural material was compared with synthetic pheromone when formulated on rubber septa (A.H. Thomas No. 8753-D22). The material was dissolved in 100 μl of hexane and pipetted into the large reservoir of the septum cap. Septa were loaded with 10, 30, 100, and 300 ng of material and aged a minimum of two days prior to use. Septa treated with hexane served as controls. Septa were positioned about 3 cm above the floor of the Petri dish with a paper clip. Test duration was 20 min. There were four replications at each dose in two different plots on different days. Within plots, treatment locations were randomized between replications. Treatments were randomized within plots, and two plots were used. Other parameters were as in the previously described field bioassay.

The third series of field bioassays was conducted in August 1984 to identify the nature of the relationship between the applied dose of synthetic pheromone (>99% purity) and the number of feral males attracted. Bioassays were similar to those previously described, and the pheromone was formulated on rubber septa in doses of 0, 1, 10, 100, or 1000 ng (eight replications).

Isolation. After excess solvent was removed from the collected volatiles at 25°C with N_2, the concentrate was chromatographed on a gravity-flow glass column (15 × 1 cm ID) prepared by slurry packing 5.0 g of 60–100 mesh silica (J.T. Baker Chemical Co., Phillipsburg, New Jersey). Solvents used for the gravity flow column included 5% ether–hexane (80 ml) and 10 and 20% ether–hexane (80 ml each). The active fractions from the gravity flow column were concentrated and injected onto a high-performance liquid chromatography (HPLC) column (250 × 4.4 mm ID) packed with 5 μm silica (Lichrosorb (Si-60). The HPLC column was eluted with 2% ether in hexane at a flow rate of 2.0 ml/min. Ten fractions (2 ml each) were collected. An additional 20 ml of 5% ether–hexane was used to remove the (inactive) polar material from the column. The active fractions from the HPLC column were combined, concentrated, and further purified by gas–liquid chromatography (GLC).

All micropreparative GLC was performed with a Varian model 1400 gas chromatograph equipped with a flame-ionization detector. The effluent from the packed columns was split with 2% of the effluent routed to the detector, and 98% collected in cooled, 30-cm glass capillary tubes (Brownlee and Silverstein, 1968).

The initial column used for further purification of the active material from HPLC was a 2 m × 2.3 mm ID glass column packed with 5% OV-101 on 80–100 mesh Chromosorb G-HP. Helium was used as the carrier gas at 20 ml/min, and the column temperature was programmed from 120 to 220° at 10°/min.

The active fraction collected from the OV-101 column was subsequently chromatographed on a 1.8 m × 2.0 mm ID glass column packed with 4.4% Carbowax 20 M on 120–140 mesh Chromosorb W operated isothermally at 180°C. Helium was used as the carrier gas at 20 ml/min.

All analytical GLC was performed with a Hewlett-Packard model 5790 gas chromatograph equipped with a flame ionization detector and a splitless capillary injector. Output from the detector was interfaced to a Nelson 4416 data system. The active fraction from the Carbowax packed column was analyzed on the following fused silica capillary columns: 50 m × 0.25 mm ID BP-1 methyl silicone (Scientific Glass Eng., SGE); 50 m × 0.25 mm ID Carbowax 20 M; 50 m × 0.25 mm ID CPS-1 (equivalent to SP-2340); and a 35 m × 0.25 mm ID soft glass column coated with cholesteryl-*p*-chlorocinnamate (Heath and Doolittle, 1983). Helium was used as the carrier gas for all columns at a linear flow of 18 cm/sec.

Identification. The active component was identified by chemical ionization mass spectrometry (CI-MS) and by Fourier transform proton nuclear magnetic resonance (FT-PMR). Additional structural information on the active compound was derived from the mass spectra of the products from microozonolysis.

Mass spectra were obtained with an upgraded Finnigan 1015/3200 chemical ionization mass spectrometer coupled with a Varian 1400 gas chromatograph and a user-designed splitless injector. Fused silica capillary columns used in the gas chromatographic inlet system included a 50 m × 0.25 mm (ID) methyl silicone (BP-1) and a 50 m × 0.25 mm (ID) Carbowax 20 M (Quadrex) column. Helium was used as the carrier gas at a linear flow of 18 cm/sec. Reagent gas, either methane or isobutane, was introduced as make up gas. The ['H]NMR spectra were obtained with a Nicolet 300-MHz Fourier transform spectrometer. Approximately 4 μg of the purified compound was placed in a 70-μl external capillary extension tube (Wilmad, Buena, New Jersey) containing 15 μl of deuterated benzene. Two thousand transients were collected over a 1-hr period using a 6-μsec pulse (90° tip angle). Proton decoupling was accomplished by standard decoupling techniques (decoupler power ca. $\frac{1}{2}$ watt) and 4000 transients spectra were obtained over a 2-hr period.

Microozonolysis of the pheromone (50 ng each experiment) was carried out in CS_2 or hexane at −70°C, and the ozonide was reduced with triphenylphosphine (Beroza and Bierl, 1967). The ozonolysis products were analyzed by CI-MS. Synthetic standards were used to confirm the ozonolysis products and also to verify the spectral data.

Synthesis The synthesis of (*Z*)-3-dodecen-1-ol (*E*)-2-butenoate, I, was initiated with the preparation of 3-dodecen-1-ol from 1 decyne (Figure 1).

For the synthesis of 3-dodecyn-1-ol, n-butyllithium II, (9.3 ml of 2.7 M in hexane) was injected into a cooled (≤0°C) solution of 1-decyne (4.5 ml, 25 mmol), in dry tetrahydrofuran (THF, 36 ml) under nitrogen. Ethylene oxide

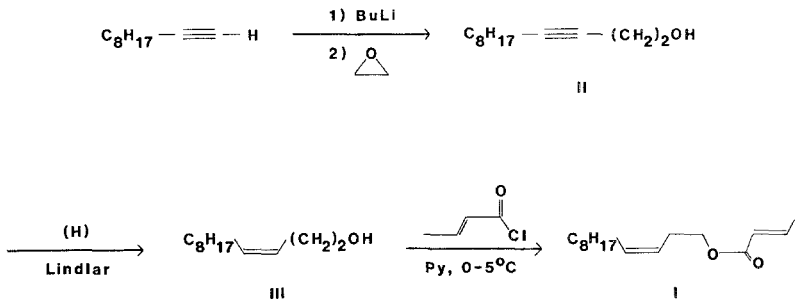

FIG. 1. Synthesis of (*Z*)-3-dodecen-1-ol (*E*)-2-butenoate.

(1.5 ml, 30 mmol) was injected from a precooled syringe, and then hexamethylphosphoric triamide (9 ml, ca. 2 equiv.) was injected. The resulting mixture was stirred overnight at ambient temperature and then worked up by dilution with water and extraction with hexane. The product alcohol was distilled under reduced pressure to give 3.45 g (77.5%): bp 74–79°C/0.05 mm; IR (CCl_4) 3640 cm^{-1}; NMR δ 0.88 (3H, t, CH_3), 1.27 (CH_2 envelope), 2.18 (2H, m, $CH_2\underline{CH_2}C{\equiv}C$), 2.41 (2H, m, $HOCH_2\underline{CH_2}C{\equiv}C$), 3.67 (2H, m, $\underline{CH_2}OH$) ppm.

To synthesize (*Z*)-3-dodecen-1-ol, III, alkynol II (3.25 g, 17.9 mmol) was hydrogenated over 30 mg of Lindlar catalyst prepared according to Mozingo (1955). The reduction was carried out in pentane (35 ml) at atmospheric pressure and ambient temperature. The reduction was monitored by gas chromatography using a 25-m (0.25 mm ID) fused silica capillary column coated with CPS-1 (Quadrex Corp.). After filtration and concentration, the alkenol III was distilled under reduced pressure to give 2.87 g (88.3%): bp 67–70°C/0.05 mm, IR (CCl_4) 3300 broad, 1050 cm^{-1}; NMR δ 0.88 (3H, t, CH_3) 1.27 (CH_2 envelope), 2.05 (2H, m, $\underline{CH_2}C{=}C$), 2.33 (2H, m, $HOCH_2\underline{CH_2}C{=}$), 3.64 (2H, m, $\underline{CH_2}OH$), 5.38, 5.55 (2H, m's, *cis* olefinic H).

To synthesize (*Z*)-3-dodecen-1-ol (*E*)-2-butenoate, I, crotonyl chloride (5.7 ml of 90% technical grade, 59.4 mmol) and alkenol III (8.75 g, 47.5 mmol) were dissolved in methylene chloride (CH_2Cl_2, 90 ml) and cooled in an ice bath. Pyridine (3.8 ml, 47.5 mmol) was added in CH_2Cl_2 (10 ml) dropwise. The resulting mixture was stirred cold for 1 hr and then worked up with water and CH_2Cl_2. The organic phase was dried ($MgSO_4$) and concentrated. The yield of crude ester (12.0 g) was nearly quantitative. Distillation of 6.0 g of crude product under reduced pressure gave 5.1 g (84%) of I containing less than 10% of the isomeric 3-butenoate ester: bp 105–109°C/0.1 mm; IR (CCl_4) 1720 (C = 0), 1660 (C = C) and 970 cm^{-1} (*trans* HC = CH).

The distilled synthetic material was purified on a 5-μm silica gel HPLC column (25 cm × 4.4 mm id) using 2% ether–98% hexane. This HPLC-purified material was then further purified using a 20% $AgNO_3$ silica gel HPLC

column, 25 cm × 1.25 cm OD and was eluted with toluene. This material was analyzed by capillary GC on the same columns mentioned earlier.

RESULTS AND DISCUSSION

Positive laboratory bioaassay responses were obtained only to volatile collections from females; males did not respond to volatile collections from empty containers, containers holding a sweet potato, containers holding male weevils (or males with potato). Females did not respond to any of these preparations. Additional studies showed that more pheromone was obtained from volatile collections from females plus sweet potato than from females only.

The isolation of the SPW female pheromone required a sequence of four chromatographic separations: (1) silica (gravity flow), (2) HPLC, (3) GLC (OV-101), and (4) GLC (Carbowax 20 M).

Beginning with the crude materials, and continuing through the purification, all materials (combined, active and inactive fractions) were bioassayed in serial dilution (6–10 replicates per concentration) in the laboratory (Fig. 2A) to ensure that no loss in activity had occurred. In addition, field bioassays of the crude, HPLC, and the GLC Carbowax samples were conducted to ensure that no loss of activity in the field occurred as a result of the separation performed. Results of these field bioassays are summarized in Figure 2B. The dilution series obtained for the gravity flow silica column and for the micropreparative GLC samples from the OV-101 column and the inactive fractions are omitted from Figure 2 for simplicity. For all comparisons, including the series not shown, there was no indication of loss of biological activity in either field or laboratory bioassays, and in all analyses, the regression data are significant at the 1 or 5% level.

Laboratory bioassays indicated all of the activity of the initial collections was recovered in the 5% ether–hexane eluant (80 ml) from the gravity flow silica column. No increase in activity was observed by the addition of the more polar components that were obtained by further elution with 10 and 20% ether–hexane. The active fractions from several gravity flow runs were combined and subsequently purified by HPLC. The biologically active fraction from HPLC eluted between a column capacity ratio (k') of 3.67 and 4.33. As shown in Figure 2, no loss of activity relative to the crude material was noted over a range of concentrations either in the laboratory or the field bioassay.

The active material that was obtained from the HPLC column was then further purified by GLC on a packed OV-101 column. All biological activity was contained within a 2-min fraction with a Kovats Index (KI) of 1780–1800 (Kovats, 1965). No increase or decrease in biological activity was observed when the active fraction was recombined with other GLC fractions and subsequently bioassayed over a range of 1×10^{-4} to 1×10^{-1} FED in the laboratory.

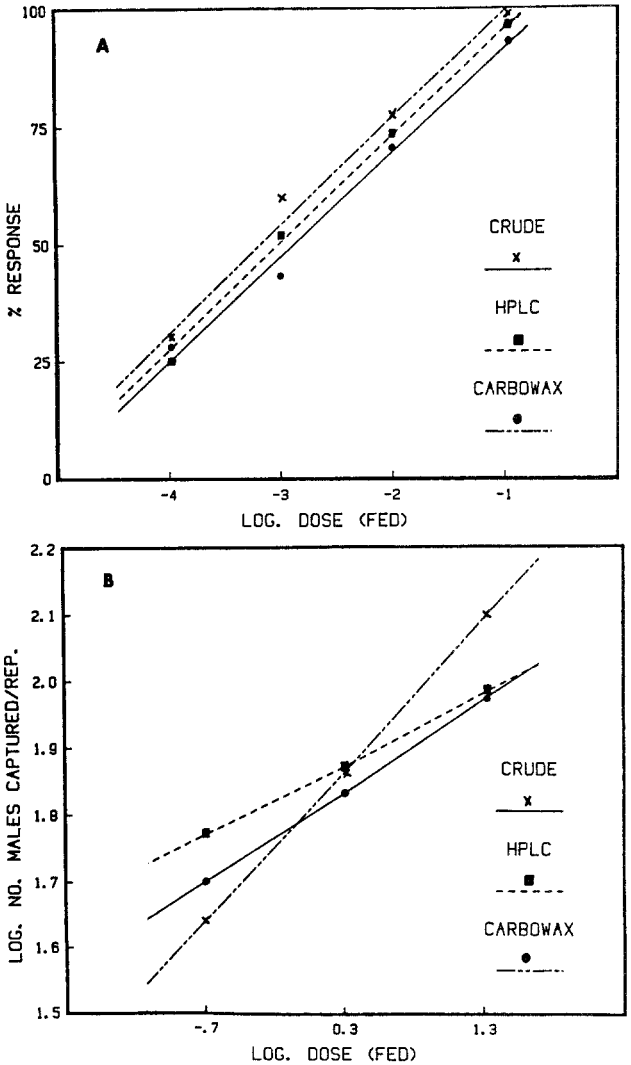

FIG. 2. Laboratory (A) and field (B) responses of *C. f. elegantulus* males to progressive isolates of female sex pheromone. (A) Average of nine replicates/point, (B) average of 6–10 replicates/point, February 1984, St. Croix, U.S. Virgin Islands.

Further purification on a Carbowax 20 M column yielded a single peak with KI = 2200 that produced full activity in laboratory and field bioassays (Figure 2A,B). Quantitative analysis of this material indicated that a FED was equivalent to ca. 4 pg. The active peak from the Carbowax 20 M column was found to be >99.8% pure when analyzed on the CPS-1, Carbowax 20 M, OV-101, and the cholesteryl-*p*-chlorocinnamate capillary columns.

The isobutane CI mass spectrum (Figure 3A) established that the molecular weight of the pheromone was 252 with diagnostic peaks at *m/e* 251 (M − 1), 253 (M + 1), and 254 (M + 2). The fragment ion at *m/e* 167 (M + 1 − 86) suggested the loss of butenoic acid ($C_4H_6O_2$) from the parent molecule. The methane CI mass spectrum (Figure 3B), in addition to showing a peak at (M + 1 − 86), provided a base peak at *m/e* 87 [$C_4H_7O_2^+$] consistent with protonated butenoic acid. Based on the mass spectral data the structure was proposed as a butenoate of a 12-carbon alcohol which contained one degree of unsaturation. Ozonolysis of the pheromone (ca. 50 ng/ozonolysis) produced two major products that were identified as nonanal and decanal (1 : 0.8, respectively) (see below) by comparison of their retention times on a 50-m CW-20M capillary column and their mass spectra with synthetic standards.

High-field 300-MHz PMR with decoupling experiments provided the spectral information needed for structure elucidation of the pheromone. The spectrum (Figure 4) indicated four olefinic protons. The proton giving rise to the signal at δ = 7.03 (1H sextet) was coupled (Table 1) to the olefinic proton at δ = 5.95 (1H, d, J = 15.6) and the methyl protons of δ = 1.43 (3H, d). Thus the crotonate moiety, $0{=}C{-}C\underline{H}{=}C\underline{H}{-}C\underline{H_3}$, was established. The olefinic proton δ = 5.41 (1H m) was coupled to the methylene protons at δ = 2.44 (2H, q), which also were coupled to the methylene protons at δ = 4.22 (2H, t), $CH{=}C\underline{H}{-}CH_2{-}C\underline{H_2}{-}0$. Thus the position of the olefinic bond in the alcohol chain was unequivocally established as being in the three position. The olefinic proton δ = 5.60 (1H, m) was coupled to the methylene protons at δ = 2.12 (2H, m) and the olefinic proton at 5.41 (1H, q, J = 1.01). The methylene envelope δ = 1.40 (12H, m) and the methyl protons δ = 1.05 (3H, t) are consistent with a heptyl moiety. Examination of PMR spectra of synthetic samples of (*Z*)- and (*E*)-3-dodencen-1-ol and the methyl esters of (*Z*) and (*E*)-2-butenoate indicated that the geometry of the olefinic bonds were (*Z*)-3-dodecen-1-ol (*E*)-2-butenoate.

In addition to (*Z*)-3-dodecen-1-ol (*E*)-2-butenoate, the other three possible geometric isomers were synthesized. All four isomeric forms are adequately resolved on a 50-m CPS-1 (Quadrex) capillary column and the isolated natural pheromone had a retention time identical to that of the synthetic (*Z*)-3-dodecen-1-ol (*E*)-2-butenoate. The synthetic (*Z*)-3-dodecen-1-ol (*E*)-2-butenoate was 99+% pure and identical to the natural material by all analytical procedures.

PMR and capillary GC data established that only one compound identical to the synthetic (*Z*)-3-dodecen-1-ol (*E*)-2-butenoate had been isolated as the natural pheromone. Ozonolysis of the synthetic pheromone also produced the same two aldehyde products (nonanal and decanal) that were produced by ozonolysis of the natural pheromone material. We are currently investigating the source of the artifact aldehyde, decanal, that is reproducibly generated in this ozonolysis. Crotonate esters of alkenols in which the double bond is situated more remotely from the alcohol oxygen undergo ozonolysis to give the predicted aldehyde only.

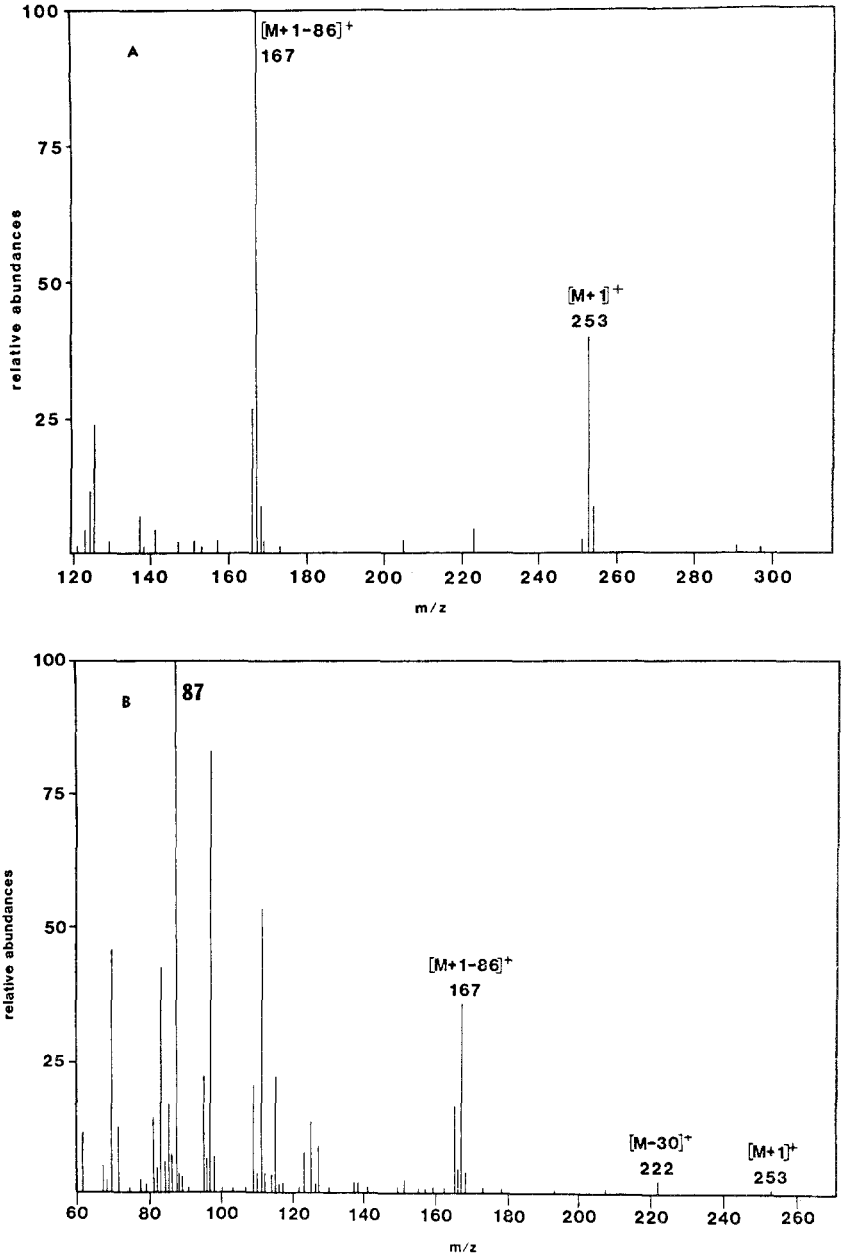

FIG. 3. Isobutane (A) and methane (B) ionization mass spectra of active fraction from *C. f. elegantulus* female volatiles.

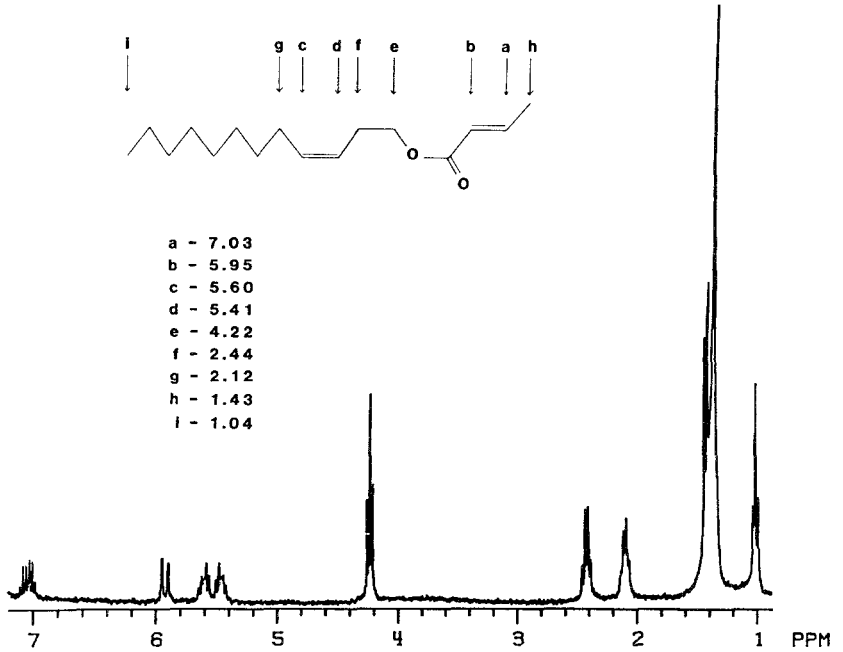

FIG. 4. The 300-MHz PMR of ca. 4 μg of natural pheromone, 2000 transients, benzene d_6 used as solvent.

TABLE 1. RESULTS OBTAINED IN H-PMR[a] DECOUPLING EXPERIMENTS OF PURIFIED NATURAL MATERIAL

Decoupler settings in PPM	PPM of α proton(s)
7.03	5.95 (d → s)[b] + 1.43 (d −> s)
5.93	7.03 (m → q)
5.60	2.12 (m → t) + 5.41 (−)
5.41	2.44 (q → t) + 5.6 (−)
4.22	2.44 (q → d)
2.44	5.41 (m → d) + 4.22 (t −> s)
2.12	5.60 (m → d)

[a] 300 MHz.
[b] s = Singlet, d = doublet, t = triplet, q = quartet, m = multiplet and (−) = not discernable due to decoupler splash.

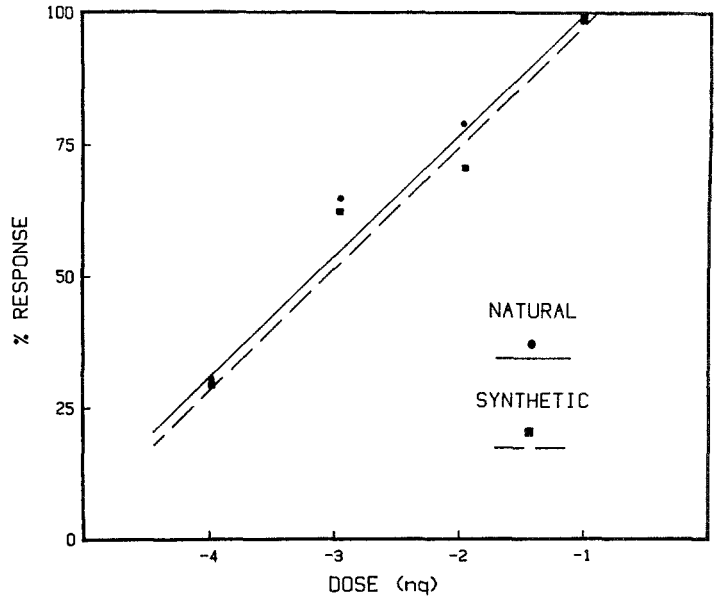

FIG. 5. Laboratory response of *C. f. elegantulus* males to purified natural pheromone and synthetic pheromone (6–10 replicates/point).

Quantitative bioassays comparing the relative activity of the purified natural product and the synthetic material were conducted in the laboratory and in the field. Figure 5 summarizes the results of the laboratory study (6–10 replications at four different dosages for each material). No measurable difference between the two materials was observed. The relative activity, in terms of male attraction in the field, is shown in Figure 6. Two release substrates, glass (Figure 6A) and rubber (Figure 6B), were used, and the similarity in response is shown in the figures. The response of feral males to septa loaded with a higher amount of the synthetic material is shown in Figure 7. Controls did not capture a significant number of weevils in any test.

The biological and chemical data presented in this paper provide evidence that (*Z*)-3-dodecen-1-ol (*E*)-2-butenoate is the major component of the female-produced sex pheromone of *C. f. elegantulus*. Additionally, the similarities in male sweetpotato weevil response in the field to natural crude volatiles and synthetic material, at levels as low as 0.08 ng of this compound, demonstrated that (*Z*)-3-dodecen-1-ol (*E*)-2-butenoate is qualitatively and quantitatively equal to the pheromone volatilized by females. Although Coffelt et al. (1978) reported three areas of activity based on liquid chromatography of extracts of filter paper that had been exposed to female sweetpotato weevils, we found only one active compound in our analysis of volatiles collected from sweetpotato weevil females.

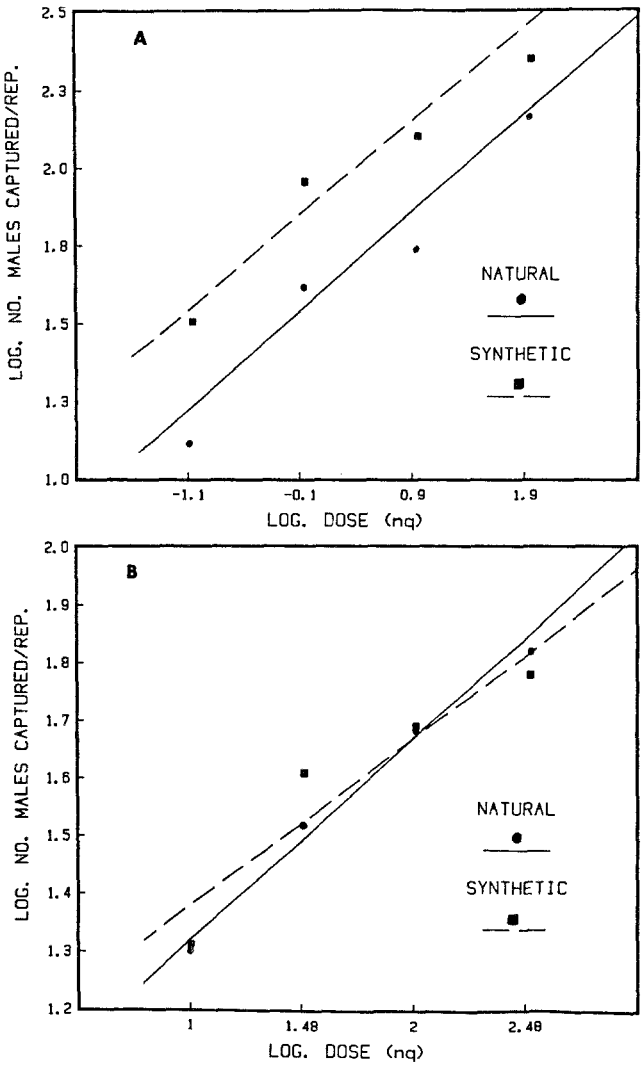

FIG. 6. Field bioassay—isolated natural vs. synthetic pheromone; (A) substrate is glass (nine replicates/point); (B) substrate is rubber septa (eight replicates/point). Tests done in March and September 1984, St. Croix, U.S. Virgin Islands.

Unlike pheromone components that have been described for many Coleoptera species, apparently the material functions as a sex pheromone in that it is produced by one sex and elicits an overt series of behaviors (activation, orientation, homosexual movements) only by the opposite sex. To our knowledge, this appears to be the first case of a crotonate ester being identified as an insect pheromone.

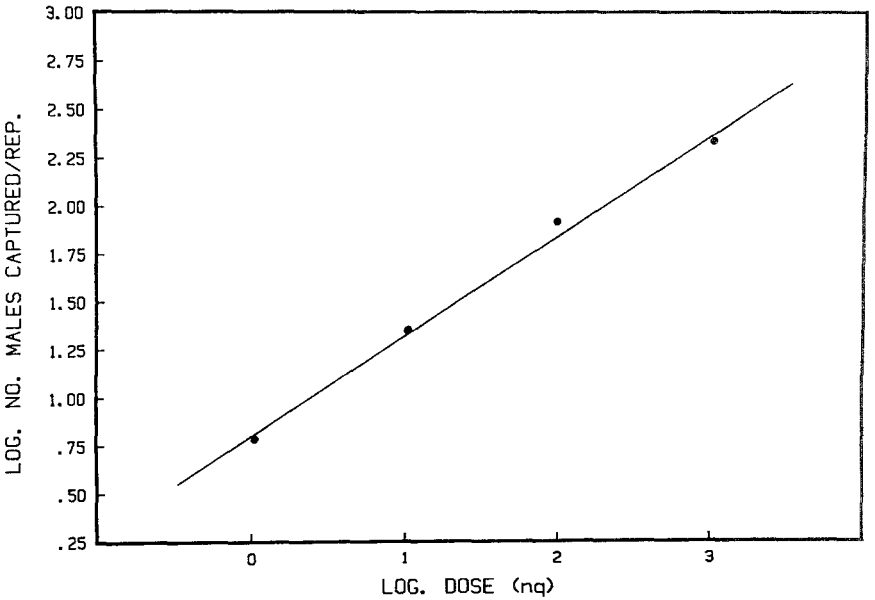

FIG. 7. Dose–response of *C. f. elegantulus* males to (Z)-3-dodecenol-1-ol (*E*)-2-butenoate formulated in rubber septa (eight replicates/point). Tests done in August 1984, St. Croix, U.S. Virgin Islands.

Finally, the availability of this material provides a tool that can be developed to monitor, detect, and perhaps manipulate populations in commercial plantings of sweet potato.

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BEHAVIORAL AND GROWTH RESPONSES OF SPECIALIST HERBIVORE, *Homoeosoma electellum*, TO MAJOR TERPENOID OF ITS HOST, *Helianthus* SPP.

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Abstract—The responses of a sunflower specialist, *Homoeosoma electellum*, the sunflower moth, to the terpenoids produced by its host plant, *Helianthus*, were measured. Larvae were reared on synthetic diet containing one of three concentrations of the dominant sesquiterpene lactone found in glandular trichomes of *H. maximilliani*. Treatments were initiated at each of three larval ages. Pupal weight was significantly reduced, but the effect diminished as the larvae aged. Survival and development time were unaffected by various treatments. In behavioral tests, larvae showed no preference for untreated synthetic diet compared to diet containing the secondary compound at a concentration of 1% by dry weight. When the concentration was raised to 5%, all but last-instar larvae showed a significant preference for the untreated diet. A second behavioral test measured the damage done to whole florets and an array of floral parts offered to larvae of different ages. The damage pattern of floral tissues changed as the larvae grew older, demonstrating that the willingness of larvae to eat tissues contaminated with trichome contents increased with age. The value of the glandular trichome contents as a defense against *H. electellum* is discussed.

Key Words—Chemical defense, feeding behavior, glandular trichomes, *Helianthus*, herbivory, *Homoeosoma electellum*, Lepidoptera, Pyralidae, host-plant resistance, plant–insect interactions, sesquiterpene lactones, terpenoids.

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INTRODUCTION

The ability of specialist herbivores to feed without apparent ill effect on plants containing toxic constituents is often attributed to specific physiological adaptation which enables them to ingest the toxic compounds of their hosts without being poisoned. For example, some specialized herbivores excrete, metabolize, or sequester various plant toxins (Dauterman and Hodgson, 1978; Brattsten, 1979; Duffey, 1980; Blum, 1983; Dowd et al., 1983; Rhoades, 1983), and it is frequently assumed that many other specialist herbivores possess similar abilities. The extent to which these herbivores use behavior rather than physiological mechanisms to avoid deleterious amounts of debilitating compounds has received comparatively less attention. There are a few reports showing that specialists feed preferentially on plant parts with relatively low toxin concentrations (Krischik and Denno, 1983; Schuh and Benjamin, 1984) and that behavior such as petiole or vein cutting prior to feeding on leaf tissue reduces mobilization of secondary compounds (Carroll and Hoffman, 1980; Rhoades, 1983). These reports suggest the need to characterize both behavioral and physiological mechanisms when investigating the relationship between a specialist herbivore and the secondary compounds of its host. Our study highlights the importance of behavior during the larval life of the sunflower moth, *Homoeosoma electellum* (Lepidoptera: Pyralidae), in the face of its changing ability to grow on a diet containing the secondary compounds of its host.

We designed a series of experiments to measure both behavioral and growth responses of *H. electellum* to a major group of secondary metabolites of *Helianthus* in order to discover how this insect is able to feed successfully on sunflowers without being poisoned. The sunflower moth specializes on the inflorescenes of several genera of the Asteraceae, including several species of *Helianthus*, the sunflower (Teetes and Randolph, 1969a; Schulz, 1978). This insect is a significant pest of the cultivated sunflower which is a variety of *H. annuus* (Carlson, 1967; Heiser et al., 1969).

Our experiments were designed to answer these specific questions: (1) Does the concentration of the major secondary compound in the diet have an effect on (a) larval fitness as manifested in survival, development time, and pupal weight or (b) larval behavior as expressed in feeding preference and damage patterns to various floral tissues? (2) Are these effects on fitness and behavior affected by the age at which a larva encounters a particular concentration of its host's secondary compounds? In brief, we found both dose-dependent and age-dependent effects in the larvae on pupal weight, feeding preference, and acceptability of floral tissue.

METHODS AND MATERIALS

Chemical Defenses in Helianthus. Species of *Helianthus* produce a variety of terpenoid secondary metabolites, including high concentrations of sesquiter-

pene lactones and diterpene carboxylic acids (Gershenzon et al., 1981). These compounds are often localized in glandular trichomes which are found on the surfaces of leaves, young stems, and parts of the inflorescence including the phyllaries, the ray corollas, and the terminal appendages of the anthers (Gershenzon, 1984). The florets of both *H. maximiliani* and *H. annuus* (wild), the two species used in our experiments, have a dense trichome area on the terminal appendages of the fused anthers. *H. maximiliani* has a density gradient of trichomes across three quarters of the anthers' length with the greatest density at the top (Figure 1). We did not directly measure the terpenoid concentrations at different points along this gradient. However, we know that in unopened florets (i.e., preanthesis) concentations of the major terpenoid, a sesquiterpene lactone, 8β-sarracinoyloxycumambranolide (8β-SC) (Figure 2a), approaches 10% at the anther tips and averages 0.01–1.0% (dry weight) on the rest of the anther.

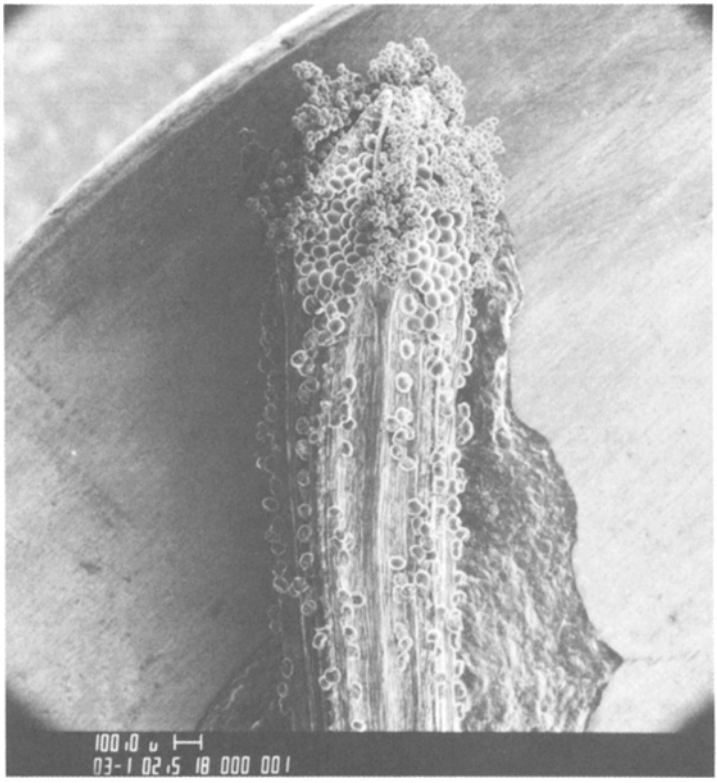

FIG. 1. Fused anther dissected from a preanthesis floret of *H. maximiliani* electron micrograph (SEM) of fresh, unfixed material, taken at 2.5 kV accelerating potential; 30×. Large spherical structures are trichomes, smaller spheres at the anther tip are pollen grains. Courtesy of G. Kreitner.

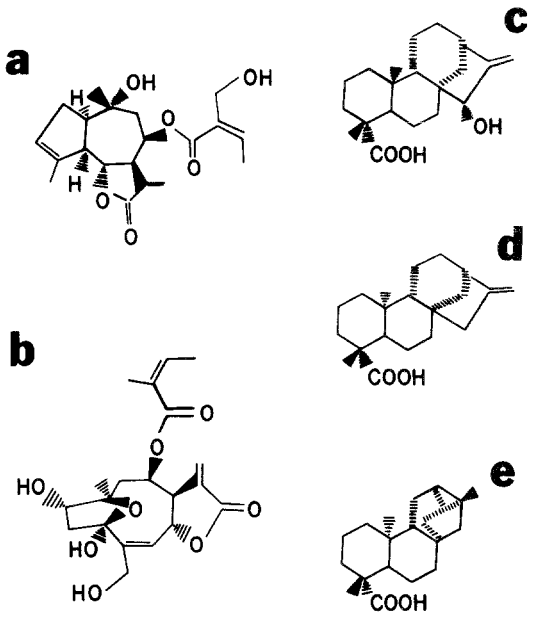

FIG. 2. Some sesquiterpene lactones and diterpene acids of *Helianthus*: (A) 8β-sarracinoyloxycumambranolide, *H. maximiliani*; (B) niveusin A, *H. niveus*; (C) grandifloric acid, *H. annuus*; (D) kaurenoic acid, *H. annuus*; (E) trachylobanic acid, *H. annuus*.

During anthesis, the contents of trichomes can be smeared down the sides of the fused anthers and also contaminate the inner tip of the corolla tube as the anthers push through the top of the corolla tube. This was verified with electron micrographs of florets (G. Kreitner, personal communication). In light of the density pattern of trichomes on the floret and the smearing of trichomes contents during anthesis, we infer that, after anthesis, the upper portion of the fused anthers holds concentration gradients of 8β-SC ranging from 10% at the tip reaching 0.01–1.0% near the bottom.

The glandular trichomes of *H. maximiliani* contain principally sesquiterpene lactones (Gershenzon and Mabry, 1984), while the constituents of *H. annuus* trichomes are principally diterpenoids, with sesquiterpene lactones present in lower quantities (Melek et al., 1985). Sesquiterpene lactones are bitter, colorless compounds found chiefly in members of the Asteraceae (Seaman, 1982). Previous work has shown them to be toxins, growth inhibitors, and feeding deterrents to a number of phytophagous insects (Burnett et al., 1974; Nakajima and Kawazu, 1978; Pettei et al., 1978; Picman et al., 1978; Jones et al., 1979; Doskotch et al., 1980; Ganjian et al., 1983; Nawrot et al., 1983). Diterpene acids, which are widely distributed in conifers, legumes, and composites have also been reported to act as insect toxins and growth inhibitors (Elliger et al.,

1976; Waiss et al., 1977; Singh et al., 1979; Rose et al., 1981; Wagner et al., 1983; Schuh and Benjamin, 1984).

Sunflower Infloresence and Life Cycle of Sunflower Moth. Maturation of a sunflower inflorescence begins at the outer ring of florets and spirals inward. The entire process requires 5–10 days in wild species (Knowles, 1978), and up to 16 days in cultivars depending on environmental conditions and head diameter (Griffiths and Erickson, 1983). The maturation of an individual floret, anthesis, proceeds from the male to female stage (see Figure 3). In the male stage, the fused anthers rise from the corolla, and pollen which has been shed inside the anther tube (formed from the fused anthers) is pushed up and out of the tube by the rising style. In the female stage, the style branches unfold, exposing the now-receptive stigmatic surfaces.

The female sunflower moth deposits one to a few eggs among disk florets after anthesis has begun on the inflorescence. On average, eggs hatch 48 hr later. The developing larva passes through five instars in 18–20 days. When reared on the cultivated sunflower, early second-instar larvae feed principally on pollen, while later second and third instars feed on a variety of floral parts including pollen, corollas, styles, and ovaries. Fourth and fifth instars feed mainly on the developing achenes, although they do considerable damage to other floral parts and the receptacle. Pupation occurs in the soil and lasts either eight days or through the winter, depending on season and latitude (Randolph et al., 1972; Rogers, 1978a; Archer et al., 1981).

Both the herbivore and the two sunflower species used in these experiments

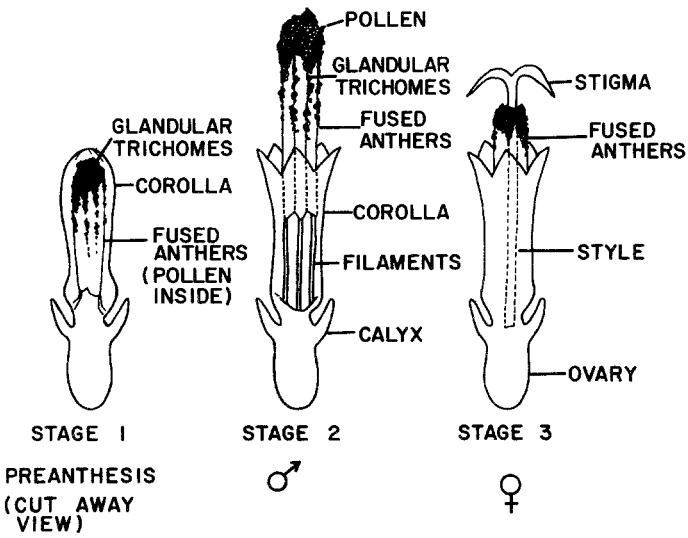

FIG. 3. Floret anatomy for three developmental stages of *Helianthus* species.

are native to North America and have sympatric ranges (Heinrich, 1956; Heiser et al., 1969).

A laboratory colony of *H. electellum* was maintained in a control chamber at 25°C, 70% relative humidity, and a photoperiod of 16 light hours per day. The colony was initiated from pupae obtained from a laboratory culture maintained by C.E. Rogers, USDA, Bushland, Texas. (This culture was originally started from adults collected from cultivated sunflower at Bushland, Texas.) The pupae were placed in screen cages until eclosion. Adults were sexed (Rogers, 1978b) and transferred in groups of 10 pairs to one-gallon jars which served as mating chambers. Jars held a 10% honey solution for adult feeding and cotton balls for ovipositional substrates. First-instar larvae hatched on the cotton balls and were transferred to 2-oz plastic containers (three per container) filled with a wheat germ-based synthetic diet modified from Vanderzant et al. (1962) by Randolph et al. (1972). Larvae were left in these containers until pupation. Young larvae fed primarily on the surface of the diet, while older larvae burrowed and constructed silk-lined tunnels.

Growth Responses. To investigate the tolerance of *H. electellum* larvae to the terpenoids of its host, the principal sesquiterpene lactone of the southern Texas *H. maxmiliani* was extracted as previously described (Gershenzon and Mabry, 1984). This sesquiterpene lactone, 8β-SC, makes up almost 50% of the terpenoid content of the glandular trichomes of *H. maximiliani* and was thus assumed to represent a valid test of the some of the physiological and behavioral capabilities of the herbivore. The use of a purified compound allowed us to interpret the response of the herbivore to a known substance. 8β-SC was incorporated into the synthetic diet and fed to larvae of different ages and at three different concentrations (per dry weight): 0.01, 0.1, and 1%. These levels span the range of concentrations for this sesquiterpene lactone in most plant tissues except the tip of the fused anthers where the concentration approaches 10% (Gershenzon, 1984).

8β-SC, which is highly lipophilic, was evenly distributed in a water-based synthetic diet by dissolving it in reagent-grade acetone and coating the resulting solution onto a cellulose powder (Alphacel, ICN Nutritional Biochemicals) by evaporating the solvent under water pump vacuum. The coated powder was then dried under high vacuum to a constant weight and thoroughly mixed with the agar-containing diet after the diet had cooled to 60°C. Because the acetone impurities could have left a residue on the cellulose powder, we tested two different control diets: one containing cellulose powder which had been soaked in acetone and vacuum dried as above and one containing untreated cellulose powder. The concentration of cellulose in all diets tested was identical.

Larvae of different ages were used in these tests because the feeding preferences of *H. electellum* have been shown to change during development (Rogers, 1978a; Archer et al., 1981). Experiments were carried out with three age

classes: neonates, second instars, and third instars. The latter two groups were reared on plain diet (with untreated cellulose) until tests were initiated. Measurements were made of survival to pupation, development time (hatch to adult emergence), and pupal weight. Indices of feeding efficiency (Waldbauer, 1968) were not calculated because removing *H. electellum* from their silk tunnels for repeated measures would have disrupted their normal behavior. Sample sizes varied between treatment groups as a function of availability of larvae in the appropriate developmental stage. Not all individuals which emerged as adults were weighed as pupae.

Behavioral Responses. The behavioral responses of *H. electellum* larvae to *Helianthus* terpenoids were investigated in two experiments: one using the purified sesquiterpene lactone and one using detached florets and floral parts. In the first experiment, each larva was presented simultaneously with diet containing sesquiterpene lactone and control diet to see which was the preferred food. The diets were prepared as above. Treated diet incorporated cellulose powder coated with 8β-SC to give final concentrations of either 1 or 5%, and control diet contained cellulose powder which had been coated with pure acetone. The 1% concentration was used for comparison with the growth response experiment and the 5% concentration was employed to approximate the high sesquiterpene lactone concentrations close to the tip of the anther appendages. Because preference often changes through development, 30 naive larvae (not previously exposed to sesquiterpene lactones) from each of five instars were tested at each concentration.

A single larva was placed in the center of a Petri dish holding two plugs of treated diet and two plugs of control diet. The configuration of treated and control plugs was alternated between dishes to eliminate any position effect. Larval position was recorded five times over a 24-hr period. Preliminary trials showed that larvae usually made and maintained a food choice within the first 12 hr. After diet selection, a larva spun a silken chamber around its body and affixed the silk to the diet plug. For each succeeding instar tested, the food plugs were spaced slightly further apart, so that the relative distance between them did not change as larval size increased (it approximated $1\frac{1}{2}$ larval lengths), and so that a larva could not affix its silken chamber to different food plugs simultaneously. The experiment was conducted under dim red light because larvae became agitated under white light and tended to locate themselves in a position of minimum exposure.

Our second behavioral experiment measured the damage done to an array of floral parts offered to *H. electellum* larvae of different ages. Wild *H. annuus* collected near Austin, Texas, was used in this experiment. The florets of *H. annuus* have approximately the same abundance and distribution of glandular trichomes as the florets of *H. maximiliani*, but there are differences in trichome terpenoid composition. The use of wild *H. annuus* allowed us to compare our

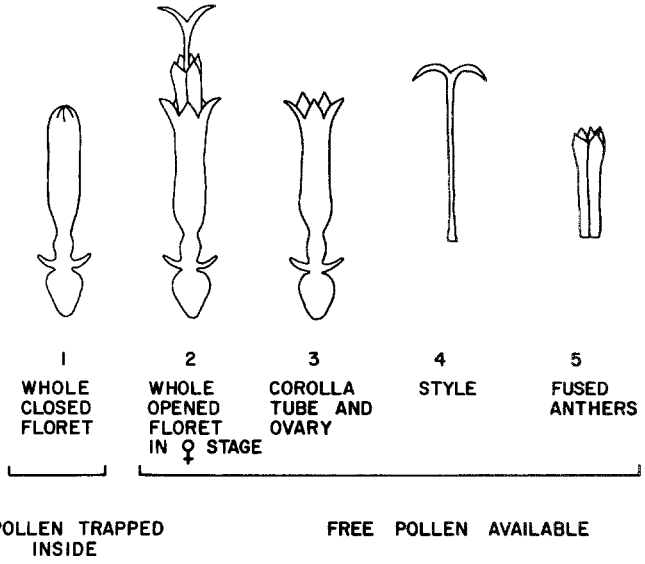

FIG. 4. Five food types, floral tissues of wild *H. annuus*, offered separately to larvae. In all but food type 1, free pollen was available.

results with previous observations on the feeding response of *H. electellum* to the cultivated *H. annuus*. (Rogers, 1978a).

Detached florets and floral parts of wild *H. annuus* were arranged in five treatments: (1) whole closed florets (preanthesis), (2) whole open florets just after style branches had opened, (3) corolla tubes and attached ovaries, (4) styles, and (5) anthers (Figure 4). In all but the first treatment, free pollen was available. Under natural conditions, developing achenes are also available to the larva; achenes are the favored food of fourth and fifth instars (Archer et al., 1981). However, the intent of this experiment was to determine which tissues were acceptable rather than preferable to larvae of different ages. Ten naive first instars and five naive larvae of each additional larval instar (reared on unadulterated synthetic diet) were introduced singly into a Petri dish holding one of five treatments and held under dim red light. Each dish held three representatives of the treatment group. Tissue damage was scored under red light 24 and 48 hr after a larva was placed in a Petri dish.

RESULTS

Growth Responses. Larvae of *H. electellum* reared on artificial diets containing the sesquiterpene lactone 8β-SC suffered significant reduction in pupal weight compared to larvae reared on control diets, but their survivorship and

TABLE 1. PUPAL WEIGHTS FOR DIFFERENT AGED LARVAE REARED ON TREATED AND CONTROL DIET (*N* given parenthetically)

Age at treatment initiation	Mean pupal weight[a,b] (mg)				
	Control diet[b]		Treated diet (% 8β-SC)		
	CC	C	0.01	0.1	1.0
First instar	351 a (150)	344 a (277)	333 a (112)	324 b (121)	315 b (106)
Second instar			341 a (57)	333 a (62)	316 b (57)
Third instar			340 a (43)	357 a (46)	348 a (48)

[a]Means followed by same letters within rows are not significantly different at 5% level (Tukey's studentized test). No interaction between 8β-SC dose and age of treatment initiation ($P = 0.26$).
[b]CC = with plain cellulose, C = with acetone-treated cellulose.

development time were not affected. The reduction in pupal weight was both age-dependent and dose-dependent (Table 1). Larvae that began feeding on 8β-SC treated diet as neonates had reduced pupal weights when 8β-SC concentration in the diet was $\geq 0.1\%$. Larvae that began feeding on treated diet as second instars had reduced pupal weights at 8β-SC concentrations $\geq 1\%$. The pupal weights of larvae that began feeding on treated diet as third instars were unaffected at the concentrations of 8β-SC tested.

The 8β-SC had no significant effect on larval survivorship regardless of concentration or the age at which larvae first encountered treated diet (Table 2). Similarly, 8β-SC had no effect on larval development time (Table 3). There were no significant differences between larvae reared on control diet containing cellulose powder upon which pure acetone had been dried and larvae reared on diet containing untreated cellulose powder. Thus, the solvent used to coat 8β-SC onto the cellulose powder prior to incorporation into the diet did not contribute to the treatment effects measured.

Behavioral Responses. The first through fourth instars showed a significant preference for control diet over a 5% 8β-SC diet; however, the fifth instars showed no preference (Figure 5). At a 1% concentration of 8β-SC there was no demonstrable preference for either control or treated food by larvae of any age class.

The results of the acceptability test based on feeding damage of whole florets and floral organs are illustrated in Figure 6. First instars ate only pollen, scavenging loose pollen from style branches or anthers or crawling down the corolla tube of open florets to graze. When only closed florets were available,

TABLE 2. PERCENT SURVIVAL UNTIL PUPATION FOR DIFFERENT AGED LARVAE ON TREATED AND CONTROL DIET (*N* given parenthetically)

Age at treatment initiation	Survival (%)[a]				
	Control diet[b]		Treated diet (% 8β-SC)		
	CC	C	0.01	0.1	1.0
First instar	84 (224)	82 (225)	83 (75)	89 (75)	77 (75)
Second instar			85 (75)	92 (75)	83 (75)
Third instar			78 (63)	92 (63)	90 (63)

[a] No significant difference in survival between treated and control diets, $G = 1.48$, or between larvae started on treated diet at different ages, $G = 0.82$ (test of independence with G compared to $\chi^2_{(0.05)(1)} = 3.841$.
[b] CC = with plain cellulose; C = with acetone-treated cellulose.

there was no tissue damage and larvae starved to death. Second instars also ate primarily pollen but, in addition, did some minor damage to the ovary. Besides scavenging loose pollen, this age class was also able to make a small entrance hole near the base of the corolla tube of the whole unopened floret to obtain pollen, and thus did not starve when only closed florets were available. Third instars ate pollen, ovaries, parts of the lower corolla and, occasionally, the style

TABLE 3. DEVELOPMENT TIME FOR DIFFERENT AGED LARVAE REARED ON TREATED AND CONTROL DIET (*N* given parenthetically)

Age at treatment initiation	Mean development time (days)[a]			
	Control diet[b]	Treated diet (% 8β-SC)		
		0.01	0.1	1.0
First instar	25.7 (50)	25.6 (55)	25.7 (51)	25.7 (55)
Second instar		25.5 (47)	25.7 (48)	26.3 (47)
Third instar		25.6 (46)	24.9 (54)	25.3 (47)

[a] No significant differences ($P = 0.49$) in development time due to diet (analysis of variance).
[b] Acetone-treated cellulose added.

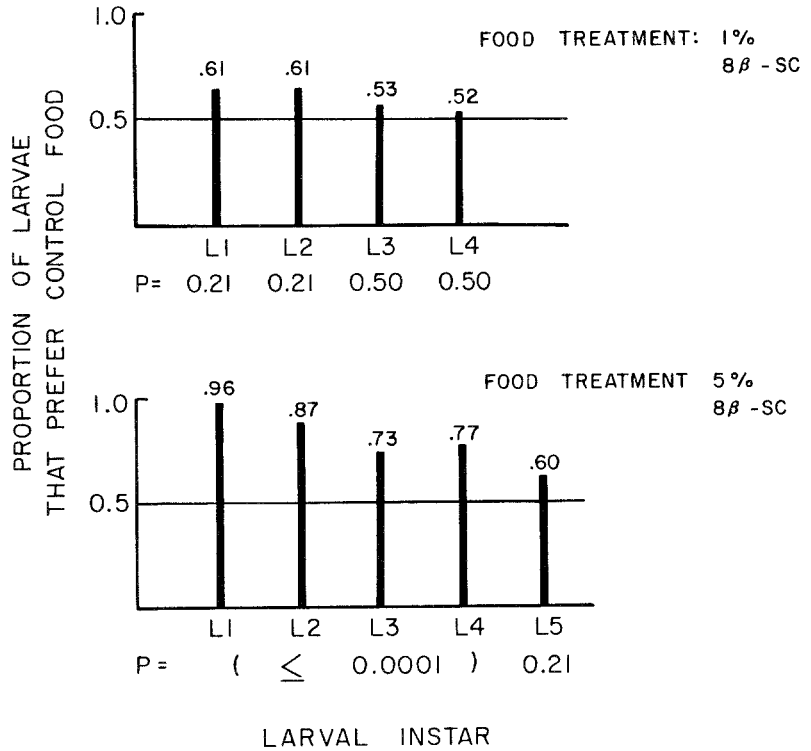

FIG. 5. Larval preference for control diet (with acetone-treated cellulose added) or diet treated with 8β-SC (1 or 5% by dry weight) for different aged larvae. In a binomial test, a P value ≥ 0.05 means that larvae showed a preference for control diet.

tube (but not the rough branches). Fourth instars ate pollen, ovaries, styles, the lower two thirds of the corolla tube, and occasionally the lower edge of the fused anthers. Fifth instars ate everything except the corolla tube tips and terminal appendages of the anthers.

DISCUSSION

Behavioral and Growth Responses. Our results show that the specialist herbivore *H. electellum* is essentially unaffected by the principal terpenoid of its host *H. maximiliani* at the concentrations tested. At concentrations of 1% and below, the sesquiterpene lactone 8β-SC had no influence on larval survivorship or development time and reduced pupal weight only slightly (an average of 5%), and only if consumed during the first two instars. Under natural conditions, *H. electellum* does not consume tissue contaminated with 8β-SC until

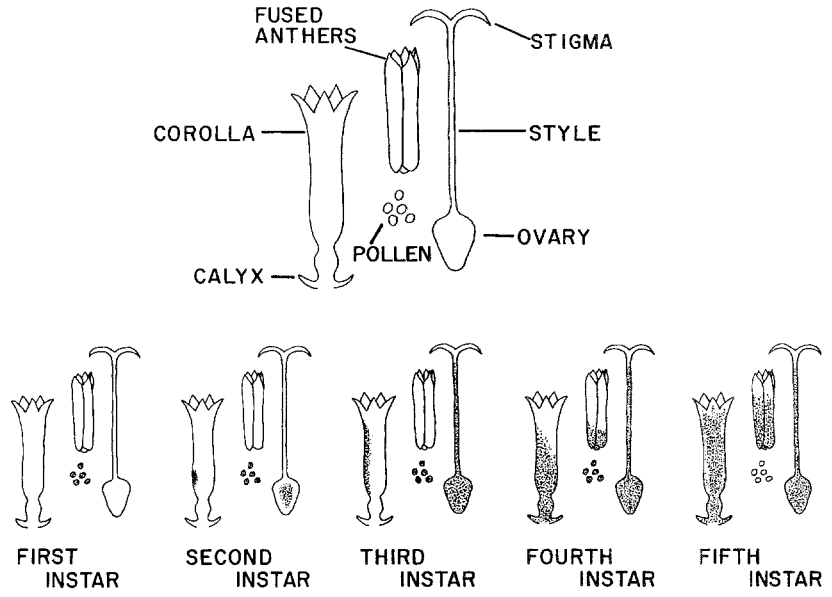

FIG. 6. Schematic diagram of patterns of tissue damage of wild *H. annuus* florets as larvae age. Stippled areas indicate the extent of damage.

the third instar. Therefore, both behavioral and growth responses of this specialist herbivore to the major secondary compound of its host are important in protecting it from ill effects.

8β-SC does have a negative effect on some herbivores. At 1% levels, the southern armyworm, *Spodoptera eridania* (Lepidoptera: Noctuidae), experienced significant depression in growth; when given a choice *S. eridania* also avoided the compound. *Melanoplus sanguinipes* (Orthoptera: Acrididae), occasionally a pest on the cultivated sunflower, also avoided the compound when given a choice (Gershenzon et al., 1985).

The effects on *H. electellum* of 1% concentrations of 8β-SC are comparable to the effects of other *Helianthus* terpenoids on this species. Two compounds, niveusin A, a sesquiterpene lactone from *H. niveus* (Figure 2B), and grandifloric acid, a diterpene from *H. annuus* (Figure 2C), had no significant effects on the growth, survivorship, or development time of *H. electellum* larvae at the 1% level or below (Rogers and Gershenzon, unpublished results). However, two diterpene acids from *H. annuus*, kaurenoic acid (Figure 2D) and trachylobanic acid (Figure 2E) were reported to reduce *H. electellum* growth significantly at concentrations of 0.5–2% (Elliger et al., 1976; Waiss et al., 1977).

Our behavioral experiments showed that *H. electellum* larvae are not significantly deterred from feeding on diet containing 1% 8β-SC. This result might

have been predicted from the growth tests, since *H. electellum* larvae ingest food containing 1% 8β-SC with minimal ill effect. However, higher concentrations of 8β-SC appear to deter feeding significantly. All larval instars, except the fifth, preferred control diet to diet treated with 5% 8β-SC. In addition, no larvae, regardless of age, would feed on the anther appendages, which contain terpenoid concentrations of up to 10%. This suggests that the acceptability of the anther tissue changes as a function of insect age and concentration of 8β-SC. This conclusion is corroborated by the behavior of larvae on wild *H. annuus* where the willingness to eat contaminated tissue increases with age.

In summary, the ability of *H. electellum* to survive on *Helianthus* is due to both behavioral and growth characteristics: mechanisms which allow *H. electellum* to ingest lower levels of terpenoids with impunity and which deter feeding on tissues containing levels of terpenoids that are detrimental. Both physiological and behavioral responses change with larval age.

These results suggest several further investigations. First, it would be valuable to repeat these experiments using a crude extract of the glandular trichome contents of *H. maximiliani* to look for an alteration of response due to other trichome components. Second, since we did not address the physiological mechanism(s) which have an impact on growth, it would be interesting to distinguish sensory responses (e.g., repellency) and digestive responses (e.g., toxicity) to understand why young larvae grow less when fed 1% 8β-SC and avoid diet containing 5% 8β-SC.

The shift in growth potential as a function of age of encounter with the secondary compound should prompt concern about the use of Waldbauer (1968) indices in assessing the nutritional value of a host plant for a particular herbivore during only one instar period. In such studies, a change in physiological capability during development would go undetected.

Sesquiterpene Lactones: Defensive functions? In general, the sesquiterpene lactones of *Helianthus* appear to serve as defenses against some herbivores because of their abundance, their location in glandular trichomes on the surface of the plant, and their toxicity and feeding deterrency to other herbivores (Jones et al., 1979; Gershenzon et al., 1985). Furthermore, the high concentrations of terpenoid-filled trichomes on the anther appendages suggest a role in defense against herbivores that feed on floral parts. The proximity of sesquiterpene lactones to the pollen (Figures 1 and 2) makes it tempting to postulate a role for these constituents in protecting the pollen from predation until the arrival of pollinators. Recently published micrographs show that the activity of some pollinators on *H. annuus* results in the removal of the anther's glandular trichomes (Griffiths and Ericson, 1983).

Our observations of *H. electellum* larval behavior indicate that the anther sesquiterpene lactones, in combination with tissue toughness, negative phototaxis of larvae, and inflorescence phenology, discourage pollen removal before

the arrival of pollinators and discourage severance of the style prior to the opportunity for fertilization.

Toughness and Trichomes. First-instar larvae are unable to reach the pollen in unopened florets because of tissue toughness and the presence of glandular trichomes. Avoidance of tough tissue by entry through the top of the unopened floret through suture lines of the closed corolla and closed anthers would result in contact with easily ruptured glandular trichomes. By the second instar, larvae are able to chew a small hole in the corolla base and crawl up into the anther tube from below, thereby avoiding contact with glandular trichomes or their contents.

Negative Phototaxis. Glandular trichomes do not appear to present a barrier to larval movement (Rossiter, personal observation). Therefore, pollen available at stage two of anthesis (extended above other disk florets by the rising styles) is most likely protected because of the larva's negative phototaxis.

Inflorescence Phenology. The majority of ovipositions on an inflorescence occurs when approximately one quarter of the florets have opened (Teetes and Randolph, 1969b; Depew, 1983). When larvae hatch (two days after oviposition), there is a ready supply of loose pollen which has fallen into the inflorescence due to wind or insect activity. First-instar larvae graze on loose pollen which has fallen onto the silk runways woven between the corolla tubes or into the open corolla tubes (Rogers, 1978a; Rossiter, personal observation). In wild *Helianthus* species, anthesis occurs over a 5- to 10-day period (Knowles, 1978). Oviposition begins one to two days after anthesis begins, and it is another six days before the first eggs laid produce second instars (Randolph et al., 1972), Therefore, by the time any larvae are able to enter unopened florets by chewing a hole in the base of the corolla tube, and remove new pollen or sever styles, most of the florets on the head have had the opportunity to carry out their reproductive function (pollen dispersal and fertilization). Thus, the phenology and habits of this floral specialist do not appear to impede the initial reproductive function of its host. We can speculate that the timing of oviposition has been affected by the necessity for young larvae to forage on pollen and avoid secondary compounds.

Later instars do injure floral organs and developing seeds of wild sunflowers, but the extent of this injury is considerably less than it is in cultivated sunflower (Rogers, 1980). Multiple factors undoubtedly contribute to the greater susceptibility of cultivated sunflower: (1) Cultivated *H. annuus* have lower densities of glandular trichomes on their anthers than either *H. maximiliani* or wild *H. annuus* (Gershenzon, 1984). (2) The period of anthesis, and therefore the opportunity for oviposition, is longer for the cultivar (up to 16 days) due to the increased number of florets on the very enlarged inflorescences (Griffiths and Erickson, 1983). (3) Tissue toughness and glandular trichome contents may differ between cultivated and wild sunflowers. (4) The monoculture habit can

enhance the abundance of an herbivore species (Stanton, 1983). Our data show that the use of floral tissues by larvae of different ages is dependent, at least in part, upon degree of contamination by glandular trichomes contents.

We compared our results for wild *H. annuus* with Rogers' (1978a) data for intact inflorescences of cultivated *H. annuus*. With wild sunflowers, larvae eat only pollen for the first two instars, with slight ovary damage by second instars. With cultivars, however, larvae eat not only pollen but occasionally anthers and styles during the first instar and anthers, pollen, corollas, and styles during the second instar. The type of injury sustained by cultivars is an impediment to the female reproductive function because styles can be damaged before fertilization occurs. Rogers (1978a) has pointed out that the greater the proportion of ovaries fertilized before style damage begins, the greater the seed set despite continued herbivory. The extent to which the abundance of glandular trichomes in wild *Helianthus* inhibits destruction of the style before fertilization is a topic for further investigation.

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BEHAVIOR OF *Heliothis virescens* (F.)[1] IN PRESENCE OF OVIPOSITION DETERRENTS FROM ELDERBERRY[2]

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Abstract—Extracts prepared from elderberry, *Sambucus simpsonii* Rehd., leaves with either acetone, dichloromethane, distilled water, ethanol, hexane, or methanol deterred oviposition by *Heliothis virescens* (F.) on treated substrates in the laboratory. Doses of the aqueous extract equivalent to as little as 0.8 mg of leaves/cm^2 of oviposition substrate were effective in reducing egg deposition. There was no significant difference in the mean number of landings on extract-treated and untreated surfaces. When either the antennae, proboscis, or the metathoracic legs were removed from female moths, there was no significant effect on oviposition on paper towels treated with elderberry leaf–water extract in laboratory bioassays, but in field cages, moths without these appendages deposited significantly fewer eggs on treated leaves of tobacco plants, a preferred host. There was no evidence that elderberry leaf–water extract affected mating by *H. virescens*.

Key Words—*Heliothis virescens*, Lepidoptera, Noctuidae, oviposition behavior, oviposition deterrents, chemoreceptors, *Sambucus simpsonii*.

INTRODUCTION

As scientists become increasingly successful in isolating natural chemicals in plants and animals, interest in the utilization of plants or plant substances as insect oviposition and feeding deterrents in pest management systems has increased (Boddé, 1982). Tingle and Mitchell (1984) found that aqueous extracts from foliage of a variety of nonhost plants deterred oviposition by the tobacco budworm, *Heliothis virescens* (F.). Similarly, cabbage leaves, a host of *Tri-*

[1] Lepidoptera: Noctuidae.
[2] Mention of a commercial or proprietary product does not constitute an endorsement by the USDA.

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choplusia ni (Hübner), produce chemicals that deter oviposition by this species (Renwick and Radke, 1981). Jermy and Szentesi (1978) reported that oviposition by *Acanthoscelides obtectus* Say, *Bruchus pisorum* (L.) and *Pieris brassicae* L. could be inhibited even on the most preferred substrates by substances of very dissimilar chemical structures. Their experiments showed that the acceptability of a given substrate was influenced substantially by the presence of alternative substrates and that both water- and hexane-soluble factors were involved. Mitchell and Heath (1985) determined that chemicals extracted with different solvents from pigweed, *Amaranthus hybridus* L., and from frass of pigweed-fed *Spodoptera exiqua* L. or *S. eridania* (Cramer) larvae deterred oviposition by *S. exigua* when the extracts were sprayed onto pigweed, a preferred host plant.

The objective of the present study was to define and correlate specific reproductive behaviors of *H. virescens* when exposed to extracts of elderberry leaves, *Sambucus simpsonii* Rehd., prepared with various solvents. This plant was selected because aqueous extracts of leaves are an effective oviposition deterrent for *H. virescens* (Tingle and Mitchell, 1984). Adult *H. virescens* females were observed during oviposition to note possible behavioral changes caused by the elderberry extracts. The effect of the extracts on mating and oviposition in both choice and no-choice situations also was determined. Various appendages were removed from moths in an attempt to determine the location of receptors involved in the perception of oviposition deterrent(s).

METHODS AND MATERIALS

Initially, extracts prepared from elderberry leaves with either of six solvents (acetone, dichloromethane, distilled water, ethanol, hexane, or methanol) were evaluated as oviposition deterrents in a Plexiglas assay tunnel in the laboratory (test 1). The holding compartment of the tunnel for the *H. virescens* moths was 25 × 25 × 51 cm long with four ports (7.5 × 20 cm) in the top that were covered with white Viva paper towels used routinely for egg-laying surfaces at this laboratory. Air was pulled through the ports of the compartment from top to bottom (covered with screen wire), at ca. 0.3 m/sec with 55 × 55-cm box fan. The exhaust side of the fan was covered with a fiberglass filter to collect insect scales.

Each extract was prepared by homogenizing 20 g fresh weight of elderberry leaves in a blender with 100 ml of the solvent, which was then filtered through a Viva paper towel. The solvent was removed with a rotary evaporator and the residue was reconstituted with 100 ml of a 3 : 1 acetone–water solution. The extract prepared with distilled water was not evaporated. In each assay, the paper towels were saturated with an extract prepared with one of the solvents at the rate of 6.7 mg equivalent/cm^2, and they were allowed to air dry. Treated and untreated towels were placed alternately on the ports of the assay tunnel.

Before the onset of the scotophase, 20 laboratory-reared 2- to 3-day-old females that had been confined with males for mating were released into the holding compartment and given a choice of laying surfaces. Prior to testing, the moths were held at 27°C on a reversed 14:10 light–dark photoperiod. Temperature in the test area was maintained at ca. 27°C.

Preliminary laboratory tests showed that of the total number of eggs laid by laboratory-reared females in a 10-hr dark period, 32% were deposited during the first hour, 14% during the second hour, and 4–9% during each of the following 8 hr. Therefore, we observed the moths during the first hour of the scotophase using a television monitor equipped with a remotely controlled low-light surveillance camera. The total number of eggs deposited on the two treated towels were counted and compared to the total number of eggs on the untreated towels. Overnight egg counts were made in some tests. All tests were replicated six times.

Next, dose–response experiments were conducted with elderberry leaf extracts prepared with distilled water (test 2). Each dose, ranging from 0.01 to 6.7 mg equivalents of leaf/cm^2 of paper towel oviposition substrate, was replicated six times. Towels saturated with 5 ml each of distilled water and air dried served as controls.

In the third test, extracts prepared from 20g elderberry leaves/100 ml of one of three solvents were tested separately on tobacco leaves (oviposition substrate). The leaves were obtained from mature potted plants and shaped to 7.5 × 20 cm to conform to the size of the ports on the assay tunnel. The extract was brushed onto both sides of the leaves to the point of runoff at the rate of 3.3 mg equivalents/cm^2. Control leaves were treated similarly with 5 ml each of the respective solvent.

In test 4, females were confined for oviposition in eight glass lamp globes (four sets of two) that were positioned above the ports of the assay tunnel so that air would be pulled through them. The globes tapered in diameter from 11.0 cm at the center to 7.5 cm at both ends and were 22.5 cm high. Two no-choice tests were conducted simultaneously. Paper toweling on top of the globes served as the laying surface. Two moths were confined in each globe. The top of one globe in each set was treated with 6.7 mg equivalents/cm^2 of elderberry leaf–acetone extract and air dried; the top of the other globe (control) in the set was treated with 2.5 ml of acetone. The number of eggs deposited on each top was recorded after 1 hr. In two sets of globes, the moths were prevented from contacting either the extract-treated surface or the acetone-treated control surface by separating these surfaces with wire mesh covered with untreated paper towels. Air was pulled over the treated surfaces through the globes. These tests were replicated 12 times. Moths were observed with a low-light television camera in six replicates. The number of landings on the paper towel tops during the first hour of darkness was recorded.

Either the antennae, proboscis, or the pro-, meso-, or metathoracic legs

were amputated from each test female moth in test 5 to determine if the chemoreceptors detecting the plant extract were located on these appendages. The amputations were made at ca. 21°C during the light period while the moths were relatively inactive. The extract used in these tests was prepared with 20 g elderberry leaves/100 ml water. Tests were conducted in both the laboratory and outdoors in Saran field cages (2.9 m diam and 1.2 m high) described by Tingle and Mitchell (1984). In the laboratory, five 3- to 5-day-old *H. virescens* females were confined overnight in glass globes that were positioned on top of the assay tunnel; half of each paper towel top was treated with 6.7 mg equivalents/cm^2 of extract, and half of each top was left untreated for a control (10 replicates).

Potted tobacco plants in the prebutton (prebudding) stage of growth, with all but four leaves removed, were used in the field cages. Using a Badger air brush, two leaves were sprayed on both upper and lower surfaces with extract at the rate of ca. 1.5 mg equivalents/cm^2 of leaf surface at ca. 1500 hr; two control leaves were left untreated. The control leaves were covered with a polyethylene bag to prevent the extract from settling on them during spray applications. Eight plants were spaced equally in a circular pattern in the cages. Twenty-five pairs of laboratory-reared 2- to 3-day-old moths were released and maintained in each cage. Each plant was considered a replicate. The total number of eggs deposited on the treated and untreated leaves were counted the following morning (24 replicates).

Test 6 was set up in two environmental chambers maintained at 27°C to determine if the presence of the oviposition-deterring extract prepared with elderberry leaves and water affected mating by *H. virescens*. Ten pairs of 3- to 5-day-old virgin moths were confined in a 3.8-liter Fonda paper carton that was lined with white paper towels. The linings in some of the cartons were first treated with ca. 7 mg equivalents/cm^2 of the extract (10 replicates). Females were dissected to determine mating status after overnight confinement; eggs deposited also were counted.

Test 7 was conducted to determine if the presence of the extract reduced the total number of eggs laid by an *H. virescens* female in her lifetime. One pair of newly emerged, virgin moths was confined in a 0.47-liter Fonda paper carton until both moths died. The cartons, which were held in an environmental chamber at 27°C, were lined with paper towels treated with ca. 7 mg equivalents/cm^2 of the elderberry leaf–water extract. The linings were changed daily. New towels also were placed in the control cartons daily (15 replicates). Eggs were counted daily, and the mating status was determined after the female died.

RESULTS AND DISCUSSION

In the first experiment, extracts prepared from elderberry leaves with each of the five solvents tested significantly reduced egg deposition by *H. virescens*

($P = 0.01$, paired t test). During the first hour of darkness, reductions in egg deposition ranged from 69% with dichloromethane to 89% with both distilled water and ethanol; the reductions attained with acetone and methanol extracts were 76 and 77%, respectively. Similarly, in overnight tests, significant reductions were attained ($P = 0.01$, paired t test): 90% (distilled water), 81% (acetone), 80% (dichloromethane), 78% (ethanol), 75% (methanol), and 40% (hexane). This suggests that more than one chemical may be involved in oviposition deterrence with extracts from elderberry leaves. From observations made during the first hour of darkness, there was a slight reduction in the number of approaches (within 7.5 cm) and landings on the treated surfaces; however, the differences observed were not significant statistically.

Doses of elderberry aqueous extract from 0.8 mg to 6.7 mg equivalents/cm^2 paper towel (test 2) reduced egg deposition by *H. virescens* significantly ($P = 0.01$, paired t test), while doses of 0.01, 0.1, and 0.4 mg equivalents/cm^2 had no significant effect on oviposition (Figure 1). These results are very similar to those reported by Mitchell and Heath (1985) and Williams et al. (1986) for *S. exigua* and *S. frugiperda* (J.E. Smith), respectively. The primary difference between the dose–response experiments in this study and those involving *Spodoptera* is the source of the oviposition deterrent(s). The deterrent chemicals for *S. exigua* and *S. frugiperda* were obtained from host plants, whereas the deterrent for *H. virescens* was obtained from a nonhost, elderberry.

Significant reductions in egg deposition also were attained (test 3) on to-

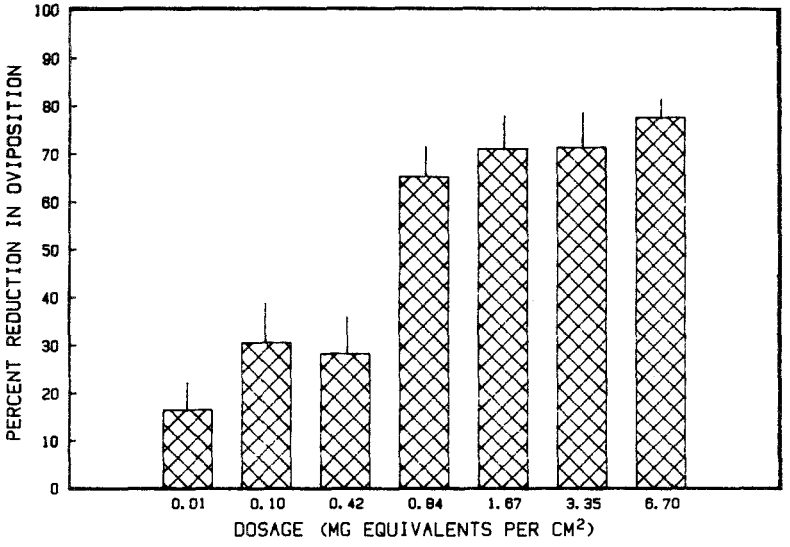

FIG. 1. Relationship between percent reduction of *Heliothis virescens* eggs deposited on extract-treated paper towels and dosage of elderberry leaf aqueous extract. Bars include standard error of means.

bacco leaves treated with extracts (3.3 mg equivalents/cm^2) prepared with either methanol (75% reduction), distilled water (71%), or dichloromethane (54%). Extracts prepared with other solvents were not tested on tobacco leaves.

When *H. virescens* females were confined in glass globes in a no-choice situation (test 4), there was no significant difference (unpaired *t* test) in the number of landings on the extract-treated and untreated surfaces, even when the moths were allowed to contact the treated surface. A significant reduction in egg deposition of 51% ($P = 0.05$, unpaired *t* test) on oviposition substrates was attained when moths were allowed to the contact the treated surface; however, there was no significant reduction in egg deposition when contact with the treated surface was prevented. These results indicate that the insects were not affected by odor from the elderberry extract.

In choice tests in the laboratory (test 5), there was no significant difference in the number of eggs deposited on toweling treated with elderberry leaf–water extract or the control half when moths had either antennae, proboscis, or metathoracic legs removed (Table 1). We did not test the effects of simultaneous amputation of the different appendages. However, in field-cage bioassays, moths with and without antennae, proboscis, or metathoracic legs laid significantly

TABLE 1. EFFECT OF ELDERBERRY LEAF–WATER EXTRACT ON OVIPOSITION BY *Heliothis virescens* WITH VARIOUS APPENDAGES REMOVED IN LABORATORY AND FIELD-CAGE TESTS

	Reduction in oviposition (%) and *t*-values[a]			
	Laboratory[b]		Field cage[c]	
Appendage(s)	With all appendages	Without appendage(s)	With all appendages	Without appendage(s)
Antennae	50.3 2.803*	24.3 1.812 NS	48.3 5.722**	56.3 5.309**
Proboscis	38.2 5.000**	23.0 1.739 NS	31.9 3.397**	59.4 14.532**
Prothoracic legs	32.5 3.596**	68.9 7.230**		
Mesothoracic legs	42.5 3.096*	50.1 4.295**		
Metathoracic legs	48.5 4.665**	21.7 1.772 NS	44.7 7.517**	45.9 4.668**

[a] Calculated by Student's paired *t* test from percent of eggs deposited in treatment and control: NS = nonsignificant; * = significant at $P = 0.05$; ** = significant at $P = 0.01$.
[b] 6.7 mg equivalents/cm^2 of extract on half of 7.5-cm-diam paper towel.
[c] 1.5 mg equivalents/cm^2 of extract on tobacco leaves.

fewer eggs on tobacco leaves treated with elderberry leaf extract than on untreated leaves on the same plant. The use of tobacco plants, a preferred cultivated host that produces an oviposition stimulant for *H. virescens* (Jackson et al., 1983), likely contributed to the difference in results obtained in laboratory and field-cage tests. Although the quantity of extract per cm^2 actually was greater on the towels (6.7 mg equivalents) than on the tobacco leaves (1.2 mg equivalents), moths without antennae, proboscis, or metathoracic legs did not perceive the extract as an oviposition deterrent in the laboratory, but they seemed to avoid it in the cages. Some receptors that were not being utilized in the laboratory may have been responding to the treated and untreated plant surfaces under the more natural conditions in the field cage. Also, if one receptor is missing, the insect may rely on other chemoreceptors. In the laboratory tests, we observed females probing the laying surfaces (i.e., toweling) with their ovipositors, but we did not attempt to correlate this probing with egg deposition.

The amputation of either the proboscis or metathoracic legs from the *H. virescens* females used in test 5 had no significant effect on the total number of eggs deposited (treated and control leaves combined) on tobacco plants in field cages. However, in the cage that contained females without antennae, there was a significant reduction of 76% ($P = 0.01$, unpaired t test) in the combined total number of eggs laid on tobacco plants as compared to the combined total egg counts on tobacco plants in the cage that contained females with antennae intact. Moths in both cages were active, but those without antennae tended to rest on the side of the cage instead of on the plants, as did moths with antennae intact. If the receptors that aid in locating host plants are present on the antennae, then it is reasonable to assume that the moths without antennae would have difficulty in locating the tobacco plants. This difference in behavior did not appear to be due entirely to injury since in the laboratory, females without antennae laid only 26% (not significant, unpaired t-test) fewer eggs than those with antennae when confined in 3.8-liter paper cartons lined with paper towels (Tingle, unpublished data).

Most olfactory and/or contact chemoreceptors of oviposition stimulants have been found on the head appendages and/or tarsi (David and Gardiner, 1962; Yamamoto et al., 1969; Benz, 1969; Yamada, 1971; Städler, 1974). However, Valencia and Rice (1982) found chemosensilla on the ovipositor of the potato moth, *Phthorimaea operculella* (Zell.), which may influence oviposition in this species.

When virgin *H. virescens* moths were confined in 3.8-liter paper cartons lined with paper towels treated with elderberry leaf–water extract (test 6), there was no significant difference in the frequency of mating between pairs of moths confined in treated containers and those in the untreated controls, nor did the leaf extracts have any toxic effect on the moths. However, there were significantly fewer eggs deposited (48% reduction; $P = 0.05$, unpaired t test) by moths confined overnight in the treated containers. Even when laying surfaces

were saturated with the extract and air dried, the females deposited some eggs on the extract-treated surfaces, particularly when they had no choice. Significantly fewer total eggs were laid (36% reduction; $P = 0.05$, unpaired t test) by individual female moths during their lifetimes when they were confined in 0.47-liter paper cartons lined with paper towels treated with elderberry leaf–water extract (test 7). Similarly, Jackson et al. (1983) found that in no-choice tests, *H. virescens* moths deposited less than half as many eggs on an insect-resistant cultivar of primitive tobacco than on a preferred flue-cured tobacco cultivar.

Contact chemoreception has been found to play an important part in the behavioral sequence leading up to oviposition in a number of phytophagous insects (Ma and Schoonhoven, 1973; Städler, 1978; Saxena and Goyal, 1978; Behan and Schoonhoven, 1978; Chadha and Roome, 1980). The presence of multiple receptors on female *P. brassicae* was indicated by Ma and Schoonhoven (1973). Perhaps mechanoreceptors and chemoreceptors (contact and olfactory) of *H. virescens* are utilized in a sequence to select the most suitable surfaces for oviposition. Insects using multiple receptors would be more likely to avoid unsuitable environments. Although the test insects used here responded similarly to extracts prepared with the various solvents, different chemoreceptor mechanisms may have been involved in the response to the chemical substances in each extract because substances of different chemical and physical properties could evoke the same response at the behavioral and presumably also at the receptor level (Jermy and Szentesi, 1978).

Research unraveling the intricate relationships that exist between the insect's ability to perceive the chemistry of a plant and integrate the sensory input into definable behavior could serve as the foundation of future breakthroughs in insect pest management technology. Although the reductions in oviposition on tobacco plants sprayed with elderberry leaf extract were not spectacularly high, the data do demonstrate the feasibility of using oviposition deterring chemicals on preferred hosts even though such plants may have a powerful oviposition stimulant, as does tobacco (Jackson et al., 1983). The extracts used here were in their crudest form. It is not unreasonable to assume, therefore, that the level of oviposition deterrency would improve with further purification of the active chemicals. Even modest reductions in the level of oviposition by *H. virescens* could have a significant impact on the quantity of pesticide used and the frequency of application now required to control this pest on crops such as tobacco and cotton. The combined effects of lower oviposition and less pesticide usage would, in turn, enhance opportunities for utilizing other means of control, especially parasitoids and predators.

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ALLELOPATHIC INFLUENCE OF *Sorghum bicolor* ON WEEDS DURING GERMINATION AND EARLY DEVELOPMENT OF SEEDLINGS

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Abstract—The allelopathic interaction between sorghum [*Sorghum bicolor* (L.) Moench] and 10 species of grass and broadleaf weeds was investigated. Germination of weed seeds was slightly inhibited or stimulated, depending on species, when incubated in closed Petri dishes with germinating sorghum. Subsequent radicle and hypocotyl or coleoptile elongation of weeds was significantly inhibited by the germinating sorghum. For weeds interplanted with sorghum and grown under greenhouse conditions. The inhibitory effect on some weed species was still evident after 2 months of growth. Significant differences were found in the dry matter per weed plant grown in pots in proximity to sorghum vs. weeds grown in monoculture. Aqueous leachates from pots planted with sorghum alone or from a system in which sorghum roots protruded into water had strong allelopathic activity. These results indicate that water-soluble allelochemicals are produced by germinating sorghum seeds and that production of these substances continues during seedling growth.

Key Words—Allelopathy, *Sorghum bicolor*, weeds, weed control, agroecosystems, phytotoxins, seed germination.

INTRODUCTION

Residues of mature sorghum [*Sorghum bicolor* (L.) Moench] contain water-soluble allelochemicals that inhibit seed germination and seedling development of other species of plants (Lawrence and Kilcher, 1961; Guenzi and McCalla,

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1962; Guenzi et al., 1967; Lehle and Putnam, 1982). Johnson grass [*Sorghum halepense* (L.) Pers.] also produces phytotoxic substances (Abdul-Wahab and Rice, 1967; Nicollier et al., 1983). A number of phenolic acids have been implicated as phytotoxins that can be leached from sorghum residues, but there is insufficient evidence that these are the active or sole allelochemicals (Guenzi and McCalla, 1962). Sorghum and Johnson grass also produce two cyanogenic compounds, dhurrin and taxiphyllin, which upon hydrolysis yield HCN and *p*-hydroxybenzaldehyde, but their potential role as allelochemicals is likewise ambiguous (Nicollier et al., 1983).

While the allelopathic effect of residues of mature sorghum on other plants has been described, studies involving the cogermination of sorghum seeds with seeds of other species are lacking. Allelopathic activity expressed by a species in the earliest stages of development could confer a powerful advantage over other plants. The purpose of our investigation was to determine if sorghum exerts an allelopathic influence on selected weeds when cogerminated with them.

METHODS AND MATERIALS

Seeds. Sorghum seed (cv. Bird-a-boo) was obtained from the Taylor Evans Seed Company,[3] Tulia, Texas. Cress [*Lepidium sativum* (L.) cv. Curlycress] was obtained from the Burpee Seed Company, Warminster, Pennsylania. Weed seeds were collected from the following locations in the years indicated: barnyard grass [*Echinochloa crus-galli* (L.) Beavr.] (Arkansas, 1976), curly dock [*Rumex crispus* (L.)] (California, 1975), green foxtail [*Setaria viridis* (L.) Beavr.] (North Dakota, 1976), Johnson grass (Illinois, 1977), bigroot morning glory [*Impomea pandurata* (L.) G.F.W. Meyer] (Illinois, 1978), redroot pigweed [*Amaranthus retroflexus* (L.)] (Maryland, 1976), red sorrel [*Rumex acetosella* (L.)] (Illionis, 1974), velvetleaf [*Abutilon theophrasti* Medic.] (Mississippi, 1974), and wild mustard [*Sinapsis arvensis* (L.)] (North Dakota, 1976).

Cogermination in Petri Dishes. Seeds of sorghum, weeds, and cress (which was treated as a "weed" in this study) were surface treated with an 800 ppm solution of Roccal to minimize mold growth. Tween 20 (0.5%) was added to the solution to serve as a wetting agent. Seeds were agitated for 15 min with the Roccal solution and then rinsed 10 times with sterile water. Seeds were germinated in sterile plastic Petri dishes (9 cm diameter). The bottom of each dish was lined with a sterilized Whatman No. 3 filter paper disk, and 3 ml of sterile distilled water was added. Controls consisted of 20 evenly spaced weed seeds per dish. Treatments consisted of 20 weed seeds and 20 sorghum seeds, evenly and uniformly interspersed, per dish. For each weed tested, data were

[3] Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

collected from five controls and five treatments. Since data was collected at three days and at seven days, the initial number of controls and treatments was doubled because the measurement made at three days was destructive. As a contingency, one additional control and one additional treatment were included for both the three-day and seven-day trials.

Petri dishes were placed on trays, 12 dishes per tray, so that paired dishes for controls and treatments were side by side. Trays were placed in an environmental growth chamber maintained at 25°C. and 80% relative humidity. The photoperiod was 8 hr per day, and the light intensity at the level of the trays was 9500 lux. Trays were rotated daily to compensate for any minor differences in temperature and light intensity at different locations in the growth chamber.

Daily observations were recorded for germination, leaf formation, root formation, and general health of the developing seedlings. Prior to collecting quantitative data at three days and at seven days, one control and one treatment were discarded from each tray. These were selected visually on the basis of being the least typical (low germination, mold growth, etc.) of the six controls and six treatments on the tray. If none was atypical, the paired treatment and control from the lower left-hand corner of the tray were discarded. For the remaining controls and treatments, the number of ungerminated seeds was counted and, for germination seeds, the length of hypocotyls or coleoptiles and radicals (or primary roots) was measured. Data was statistically analyzed by the Waller-Duncan K-ratio t test to determine the effects and interactions of weed variety, presence or absence of sorghum, and length of trial on three responses: length of hypocotyls or coleoptiles, length of radicles, and percent germination.

Sorghum Root Exudates. A 20-cm-diameter desiccator was autoclaved, and 350 ml sterile distilled water was added to bring the water level to the bottom of the perforated porcelain disk that conventionally holds samples above the desiccant. A layer of sterile, moist cheesecloth was placed on top of the porcelain disk, and 30 g (about 1000 seeds) of sorghum seed, which had been surface-treated with Roccal as described earlier, were evenly distributed on top of the cheesecloth. The lid was placed on the desiccator, and it was located under fluorescent light (about 9000 lux for 15 hr/day) at room temperature. As the sorghum seedlings grew to a height of 9–10 cm with roots of 3–5 cm length protruding into the water, the water was withdrawn from the desiccator and replenished at five-day intervals. Water collected from the desiccator was concentrated on a rotary evaporator to a volume of 30 ml, and 3 ml of this concentrate was tested for allelopathic activity against germinating weed seeds in Petri dish assays as previously described. For control assays, 3 ml of distilled water was used in place of the concentrated sample from the desiccator.

Interplantings in Clay Pots. Seeds of sorghum and selected weeds were planted in sand in 12.7-cm clay pots. Pots were maintained in a greenhouse under conditions of summer sunlight and were watered daily by immersing the

base of the pots in water. Plants were fertilized twice a week by immersing pots in Hoagland and Arnon (1950) nutrient solution with iron content increased to 1.6 times that of the standard solution. Control pots containing only sorghum or a weed and pots interplanted with varying ratios of sorghum and a weed were maintained for periods of up to 60 days. In some cases, leachate was obtained by watering pots planted with 25 sorghum seeds and collecting the runoff. The unconcentrated leachate was used to water an equivalent number of pots planted with weeds or interplanted with sorghum and weeds.

RESULTS

The data on the influence of sorghum on weeds cogerminated in closed Petri dishes is summarized in Table 1. Minor differences in percent germination of weed seeds between weeds germinated alone or in the presence of germinating sorghum seeds were noted. The greatest differences were in the growth and development of seedlings over a period of three or seven days.

Of the 10 species of weeds investigated, statistical analysis of data revealed that cogerminating sorghum in the Petri dish significantly influenced the development of all, except green foxtail and velvetleaf, at the 5% probability level by the seventh day. In several cases, statistically significant differences were noted by the third day. Cogermination with sorghum suppressed the elongation and development of hypocotyls or coleoptiles of weeds with one exception: green foxtail showed a slight but statistically significant increase in average length of coleoptiles. Morning glory, barnyard grass, and velvetleaf exhibited the greatest percent reductions in hypocotyl or coleoptile elongation in the presence of germinating sorghum. Cogermination with sorghum produced significant reduction in the average radicle length of all species tested.

In addition to effects on elongation, weeds germinated in the presence of sorghum generally were less healthy in appearance with higher incidences of necrosis, chlorosis, and retardation of development. Visual observations of differences other than measured growth are presented in Table 2.

Concentrated water samples obtained from desiccators in which the roots of young sorghum seedlings protruded into the water had general effects on the development of selected sprouting weed seeds that were comparable to the effects of proximity to germinating sorghum seeds. In three-day Petri dish assays, the average lengths of barnyard grass coleoptiles and radicles were reduced 30% and 20%, respectively, in comparison with controls. Pigweed hypocotyls and radicles were, on the average, shorter by 20% and 6%, respectively, at three days, and 25% and 30% shorter at seven days. Coleoptile development of green foxtail, as in other tests, was stimulated slightly rather than inhibited and showed an average increase of 11% over controls with a reduction of 8% in radicle length at seven days. The sorghum root leachate reduced the germination of

TABLE 1. INFLUENCE OF GERMINATING SORGHUM SEEDS ON DEVELOPMENT OF GERMINATING WEED SEEDS

	3 Days				7 Days			
	Control		Cogerminated with sorghum		Control		Cogerminated with sorghum	
Weed	Germ[a] (%)	Average length (cm)[b]	Germ[a] (%)	Average length (cm)[b, c]	Germ[a] (%)	Average length (cm)[b]	Germ[a] (%)	Average length (cm)[b, c]
Barnyard grass	76a	1.3 ± 0.5	66b	0.9 ± 0.5†	73a	2.0 ± 0.6	68b	1.4 ± 0.7†
		1.9 ± 1.0		0.9 ± 0.7†		4.5 ± 1.5		1.9 ± 1.2†
Curly dock	74a	0.3 ± 0.1	73a	0.2 ± 0.1†	78b	0.5 ± 0.2	71a	0.4 ± 0.2†
		0.5 ± 0.4		0.4 ± 0.2		1.8 ± 1.0		1.1 ± 0.5†
Green foxtail	83a	1.0 ± 0.6	70b	1.0 ± 0.5	81a	1.5 ± 0.7	74b	1.7 ± 0.8
		2.0 ± 1.0		1.9 ± 0.8		4.2 ± 1.2		3.4 ± 1.4†
Johnson grass	0	0	0	0	47a	2.1 ± 1.0	47a	1.5 ± 1.0†
		0		0		3.2 ± 1.8		2.5 ± 1.6†
Morning glory	74a	1.5 ± 0.8	91b	0.9 ± 0.4†	83c	1.7 ± 1.0	85c	1.2 ± 0.7†
		2.1 ± 0.7		2.1 ± 0.8		2.5 ± 1.0		1.9 ± 1.0†
Pigweed	95ab	0.5 ± 0.1	92b	0.5 ± 0.1	97a	0.7 ± 0.2	92b	0.5 ± 0.2†
		1.1 ± 0.2		0.8 + 0.3†		2.1 ± 0.5		1.3 ± 0.4†
Red sorrel	60a	0.1 ± 0.1	59a	0.1 ± 0.2	72b	0.5 ± 0.1	85c	0.4 ± 0.1†
		0.4 ± 0.2		0.4 ± 0.2		2.0 ± 0.7		1.4 ± 0.5†
Velvetleaf	91a	1.1 ± 0.3	83b	0.7 ± 0.2†	90a	1.2 ± 0.4	92a	0.7 ± 0.2†
		1.6 ± 0.5		1.1 ± 0.5†		1.9 ± 0.8		1.7 ± 0.7
Wild mustard	37a	0.9 ± 0.5	45b	0.9 ± 0.3	32c	1.2 ± 0.4	36ac	0.8 ± 0.3†
		3.6 ± 1.8		2.2 ± 1.0†		4.2 ± 1.7		3.0 ± 1.5†
Cress[d]	100a	1.2 ± 0.3	100a	0.9 ± 0.3†	98a	1.4 ± 0.4	99a	1.1 ± 0.3†
		0.9 ± 0.8		0.8 ± 0.3		1.3 ± 1.2		1.0 ± 0.7†

[a] Two germination means in the same line with a letter in common are not significantly ($P < 0.05$) different.

[b] First value in column is average length of hypocotyl (for dicots) or coleoptile (for monocots). Second value is average length of radicle. Standard deviation is indicated by ± value. Seeds that did not germinate were not included in calculations of average length and standard deviation. The number of measurements was equal to the figure given under % germination, since the beginning number of seeds was 100.

[c] Statistically significant difference between control and treatment indicated by † (5% level).

[d] Not a weed, cultivar Curlycress.

TABLE 2. VISIBLE DIFFERENCES BETWEEN GERMINATING WEED SEEDS IN PRESENCE AND ABSENCE OF GERMINATING SORGHUM SEEDS

Weed	Control	Cogerminated with sorghum
Barnyard grass	At 3 days, radicles appeared healthy with no necrosis. At 7 days, plumules were uncurled and seedlings appeared vigorous with strong, branching roots.	At 3 days, necrosis of some radicles was evident. At 7 days, plumules were not uncurled and most roots had no branching with some necrosis.
Curly dock	At 4 days, radicles had abundant root hair development and 48 green, emerging hypocotyls were visible. At 7 days, roots were hairy and branching and 41 sets of cotyledons were developed with 28 fully open. Cotyledons were deep green in color.	At 4 days, only some radicles had root hairs and only 21 green, emerging hypocotyls were visible. At 7 days, roots had little branching and only 14 sets of cotyledons were developed with only four fully open. Cotyledons were much smaller and less green than controls.
Green foxtail	Overall appearance was healthy.	Overall appearance was healthy. Other than size, not visibly different from controls.
Johnson grass	At 7 days, seedlings were healthy in appearance with branching roots and two developed leaves.	At 7 days, roots were thinner than those of the controls and only five seedlings had developed two leaves.
Morning glory	At 3 days, radicles were branched and cotyledons were open. The general appearance was healthy with no necrosis. At 7 days, seedlings had overall healthy appearance with no necrosis.	At 3 days, radicles had fewer and much smaller side branches than the controls and some were necrotic. Only two seedlings had open, fully developed cotyledons. At 7 days, considerably more necrosis than control including both roots and hypocotyls.

Pigweed	At 3 days, all seedlings appeared healthy. At 7 days, lateral root development was apparent. Roots had no necrosis and 76 sets of cotyledons were fully developed and open.	At 3 days, necrosis of some radicles was evident. At 7 days, roots were brown and necrotic with almost no lateral root development. Only 33 sets of cotyledons were opened.
Red sorrel	At 3 days, seeds were just visibly germinated. At 7 days, seedlings had 56 sets of open cotyledons and all roots were healthy with hairs.	At 3 days, not visibly different from controls. At 7 days, seedlings had 50 sets of open cotyledons but their roots were thinner and less vigorous than controls, and only some roots had hairs.
Velvetleaf	At 3 days, radicles were long and healthy in appearance and seven hypocotyls were formed. At 7 days, 48 sets of cotyledons were fully developed and some roots were branched.	At 3 days, there was pronounced necrosis of radicles and no cotyledons were formed. At 7 days, hypocotyls were thinner than controls and only six sets of cotyledons were developed. Roots were necrotic.
Wild mustard	At 3 days, 32 open, developed sets of cotyledons were visible, radicles were sturdy with some side branches and no necrosis. At 7 days, seedlings were sturdy and healthy with only one underdeveloped set of cotyledons; roots were well developed with much branching.	At 3 days, 40 open, developed hypocotyls were visible. Radicles were less healthy in appearance than controls and some were necrotic. At 7 days, many seedlings were pale and underdeveloped with necrosis of roots and necrosis at juncture of radicles and hypocotyls.
Cress	At 3 days, seedlings were healthy in appearance with large, green cotyledons. At 7 days, some necrosis of radicles and cotyledons was visible.	At 3 days, seedlings were less developed than controls. At 7 days, seedlings had much more necrosis than controls and were, in general, unhealthy in appearance.

barnyard grass by 20% and pigweed by 40%, but increased the germination of green foxtail by as much as 17%. The effect on weed germination was more pronounced for the concentrated root exudate than for cogermination with sorghum seed.

Growth of interplanted sorghum and selected weeds in clay pots for periods of up to two months demonstrated that the inhibitory influence of sorghum extends at least into early stages of sorghum growth. The suppression of weed growth by sorghum was clearly more than competition for light and nutrients as demonstrated by the data presented in Figure 1. As the ratio of barnyard grass seeds to sorghum seeds per pot was decreased, the dry weight per plant of the 5-week-old leaves and roots of barnyard grass decreased (white bars of Figure 1). Alone, this could be interpreted as competition, but when identical interplantings were watered not with tap water, but with the leachate collected from pots planted with 25 sorghum seeds, the dry weight per plant of barnyard grass leaves and roots was reduced greatly in all cases (black bars of Figure 1).

The data presented in Figure 2 show that pigweed also was affected adversely in interplantings with sorghum. The harvested leaves and stems of pigweed plants grown in pots with sorghum for two months contained less than 5% of the amount of dry matter in the controls in the most extreme case where five pigweed seeds were interplanted with 20 sorghum seeds. Root development

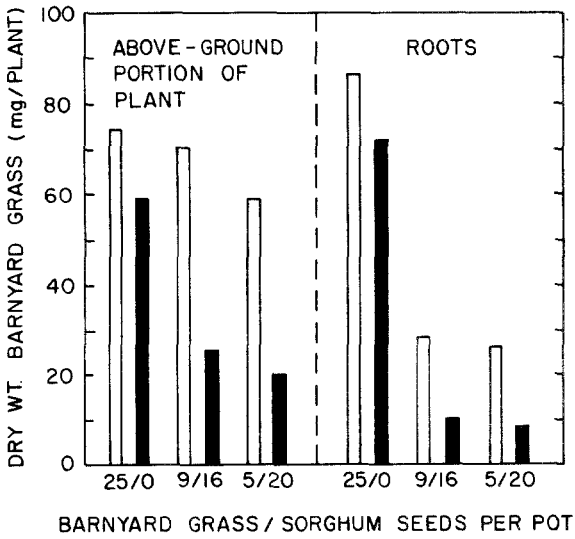

FIG. 1. Dry weights of barnyard grass five weeks after planting in pots by itself or interplanted in two different ratios with sorghum. White bars indicate average weights obtained from pots watered with tap water. Black bars represent average weights from pots watered with leachate obtained from watering pots planted with 25 sorghum seeds.

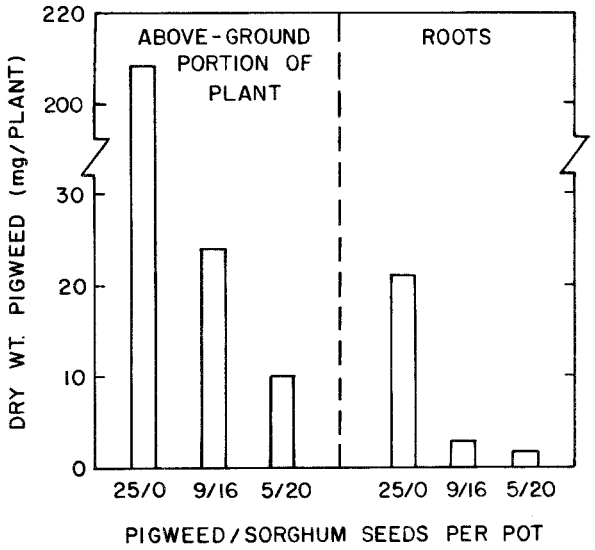

FIG. 2. Average dry weights of pigweed two months after planting in pots by itself or interplanted in two different ratios with sorghum.

of pigweed was suppressed to less than 10% of the growth of controls in the most extreme case.

The growth of red sorrel interplanted with sorghum in pots was inhibited greatly. Two months after planting, the average dry weights of controls (25 red sorrel seeds per pot) was 49.4 and 44.9 mg/plant for the above-ground portion of the red sorrel plants and the roots, respectively. For pots interplanted with 12 red sorrel seeds and 13 sorghum seeds, the harvested average dry weights of the above-ground portions and roots of red sorrel were 23.4 and 15.2 mg/plant (reductions of 53% and 66%), respectively.

In visual observation for periods of up to two months of Jimsonweed and velvetleaf interplanted with sorghum in pots revealed that growth of these weeds also was suppressed greatly by sorghum in comparison to control plantings of the weeds alone. Sorghum had no visible effect on interplanted wild mustard, morning glory, and green foxtail.

DISCUSSION

Our results suggest that young sorghum seedlings produce allelochemicals that can influence the growth and development of a number of weeds. The allelopathic influence of sorghum on some weeds appears to extend from the early stages of germination through at least two months of growth. Other pre-

viously cited investigators have demonstrated that mature sorghum and residues of sorghum plants exert allelopathic effects on other plants, so it is likely that this phenomenon persists throughout the entire growth cycle of sorghum. Under some circumstances, sorghum exerts a pronounced detrimental effect on subsequent planting of sorghum in the same plot (Burgos-Leon et al., 1980), again suggesting the production of allelochemicals that can persist until the next growing season.

In a preliminary experiment, we arranged cress seeds in concentric circles around sorghum seeds germinating on moist filter paper in the center of large, 12-cm Petri dishes. In every trial, the cress seedlings farther removed from sorghum were visibly larger and healthier as they developed. When sorghum was germinated on one side of a divided Petri dish and cress or weeds on the other side, no effect was observed even though the cress or weeds were in close proximity and shared the atmospheric environment of the Petri dish. Taken together, these observations suggested that sorghum produces allelochemicals that are nonvolatile and are water-soluble to the extent that they were able to migrate from sprouting sorghum seeds to nearby locations on a moist filter paper. The reduction in the detrimental effect on cress with increasing distance from the germinating sorghum suggested that the allelochemicals are produced in limited amounts and diffuse slowly from the origin.

Later experiments with root exudates obtained from sorghum potted in sand or grown so that the roots protruded into the water gave further proof that the active allelochemicals are water-soluble. These root exudates suppressed the development of sprouting weed seeds, and the activity could be increased by concentrating the aqueous leachate.

While the allelopathic potential of young sorghum seedlings grown under the laboratory conditions described in this report seems evident, extrapolation of these findings to field conditions is not possible. Our laboratory systems were free of all but a few adventitious microorganisms. In the field, allelochemicals exuded by germinating sorghum seeds or sorghum roots would be subject to possible alteration by soil microorganisms, although the allelopathic effect of mature sorghum residues has been shown to persist from one growing season to the next. Furthermore, our experiments were carried out on moist filter paper, in sand, or with sorghum roots in water while certain soils probably have the capacity to bind and immobilize some allelochemicals. The growth of sorghum following sorghum is markedly decreased in sandy soil, but not at all in soils high in montmorillonite (Burgos-Leon et al., 1980), which supports the contention that allelochemicals may be absorbed on certain types of soil particles, such as clay. Extensive testing would be necessary to document the influence of young sorghum on weeds under various field conditions.

Phenolic compounds, such as *p*-coumaric, *m*-hydroxybenzoic, and protocatechuic acids, have been implicated as the principal allelochemicals produced

by mature sorghum roots (Burgos-Leon et al., 1980). It is likely that these or related phenolics are responsible for the very early allelopathic influence of sorghum on weeds that we have observed. Work is in progress to isolate and identify the active allelochemicals that are produced by sorghum from the time of germination through the first few weeks of growth.

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STUDY OF FEMALE SEX PHEROMONE OF LEOPARD MOTH, *Zeuzera pyrina L.* Isolation and Identification of Three Components.

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Abstract—Three compounds have been identified in the abdominal tip extracts from the female leopard moth, *Zeuzera pyrina L.* Gas–liquid chromatography and mass spectroscopy data showed that (*E*, *Z*)-2, 13-octadecadien-1-ol acetate was the main component and that (*Z*)-13-octadecen-1-ol acetate and octadecan-1-ol acetate were secondary components. The electroantennographic responses of male *Z. pyrina* to nanogram amounts of all four 2, 13-octadecadien-1-ol acetate isomers indicated that the *E*, Z isomer had the maximum activity. A strong EAG response was also recorded for (*Z*)-7-dodecen-1-ol acetate, which was not detected in the female extracts.

Key Words—Sex pheromone, leopard moth, *Zeuzera pyrina*, Lepidoptera, Cossidae, (*E*, *Z*)-2, 13-octadecadien-1-ol acetate, (*Z*)-13-octadecen-1-ol acetate.

INTRODUCTION

The leopard moth *Zeuzera pyrina L.* (Lepidoptera: Cossidae) is a wood borer of worldwide distribution. In Italy the larvae inflict serious damage on pear, apple and olive trees. When newly hatched, the larvae wander for some time and then enter and feed in upper twigs; as they develop, they move to larger branches and increase the damage. Pupation and adult emergence take place one or two years later.

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The control of this pest is difficult because of the inaccessibility of the older larvae to insecticides. The availability of the *Zeuzera pyrina* sex pheromone would then be very useful for the timing of insecticide applications during the migration of the young larvae.

METHODS AND MATERIALS

The larvae and pupae used were collected from apple trees in the Mantova and Ravenna areas and were fed on an artificial diet (Navon, 1977). Pupae were segregated by sex and kept at a 18–25°C and 75 ± 5% relative humidity on a 9-hr dark–15-hr light regime. Each pupa was rolled up in a wet filter paper tube (ID 1 cm, length 6 cm) to aid emergence. Two weeks after pupation, adults emerged over a period of 20 days. Crude extracts from calling females were obtained as previously described (Capizzi et al., 1983).

Observation of the insects' behavior and gas–liquid chromatography (GLC) analyses of extracts of single female abdominal tips showed that the highest emission of pheromone occurred during the first day and two hours into the scotophase. GLC was done on Carlo Erba gas chromatographs (models HRGC 5300 or HRGC 4160), equipped with an automatic splitless injector system and flame ionization detector. OV-1 glass capillary column (15m, 0.3 mm ID, 0.15 μm coating, H_2 carrier, flow 28 cm/sec), OV-101 glass capillary column (30 m, 0.25 mm ID, 0.2 μm coating, H_2 carrier, flow 32 cm/sec), Carbowax 20 M fused silica capillary column (30 m, 0.25 mm ID, 0.25 μm coating, H_2 carrier, flow 34 cm/sec), or SP-2340 fused silica capillary column (30 m, 0.32 mm ID, 0.25 μm coating, He carrier flow 30 cm/sec) were used.

Mass spectra (MS) were determined on a Varian Mat 112 S instrument with electron impact source, on-column injector, and a Mat 200 data system, that was interfaced with a Varian 3700 capillary GLC. An OV-1 glass capillary column was used (25 m, 0.3 mm ID, 0.15 μm coating, He carrier flow 30 cm/sec).

The technique for recording male moth electroantennographic responses (EAGs) was described elsewhere (Tonini et al., 1982). Antennal responses to GLC fractions of female extracts or to standards were determined and normalized against a reference compound.

Precoated silica gel TLC plates were used to separate active components in the crude extracts. The plates were developed with hexane–ether (98:2, v/v) and detected by UV (254 nm).

Microepoxidation was done on less than 300 ng of purified active component in hexane by adding 20 μg of *m*-chloroperbenzoic acid and allowing the mixture to stand for 3 hr at room temperature. The crude reaction mixture was analyzed by GLC and GLC-MS.

Microozonolysis was done on hexane solutions of the purified active compounds at −78°C as previously described (Capizzi et al., 1983)

Derivatization with dimethyl disulfide (DMDS) was a modification of the method described by Francis and Veland (1981), by Buser et al. (1983), and by Leonhardt and DeVilbiss (1985) for diunsaturated acetates. The results are described elsewhere (Vincenti et al. 1985).

The route used to prepare all the isomers of the natural pheromone is outlined in Figure 6.

Field tests were done in apple orchards of the Ravenna area using Traptest (Farmoplant) traps (Arsura et al., 1977) during the 1983 and 1984 *Zeuzera pyrina* flight seasons. All the traps were suspended from branches 1.5 m above the ground and positioned 300 m apart.

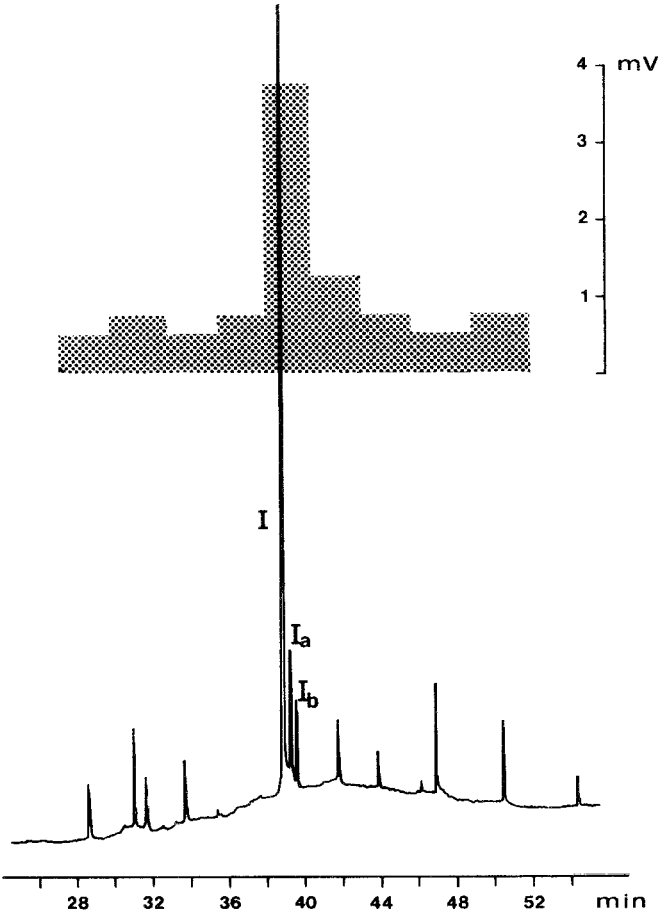
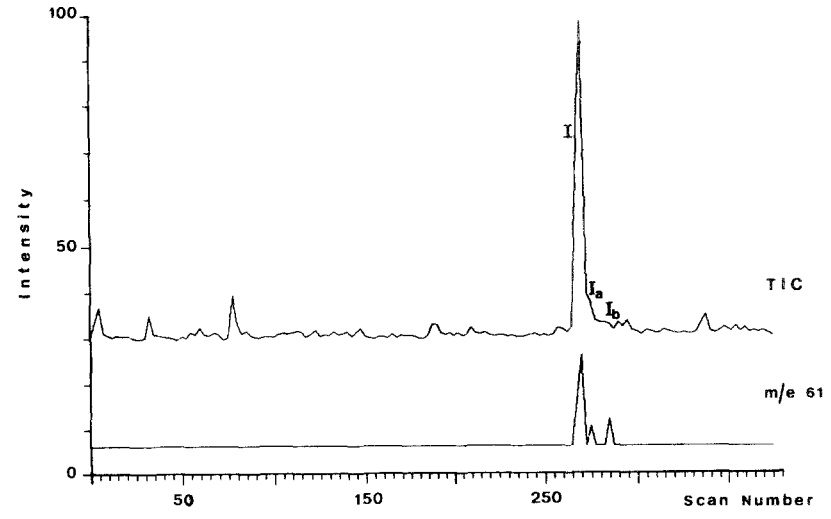
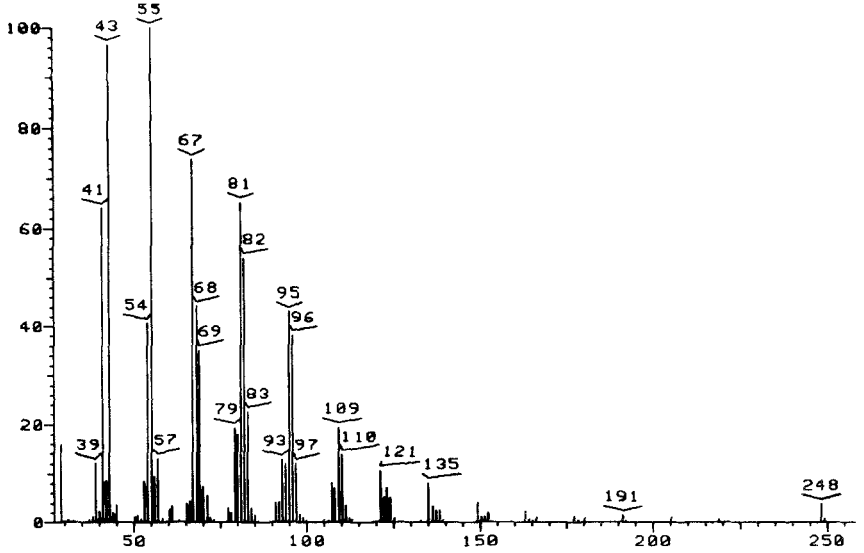


FIG. 1 . *Zeuzera pyrina* female ovipositor wash: EAG responses of timed collections and GLC tracing. OV-101 GOTC column, 30 m, 0.21 mm ID, splitless injection 5 min 50°C, 50–100°C at 20°C/min, 100–260°C at 5°C/min and then isothermal, H_2 carrier gas flow 32 cm/sec.



(A)



(B)

FIG. 2. (A) Mass chromatogram with electron ionization (70 eV) showing the total ion current (TIC) and the selected ion *m/e* 61 of the TLC-purified *Zeuzera pyrina* female ovipositor wash. OV-1 GOTC column, 25 m, 0.3 mm ID, He carrier, on-column injection, 80°C, 7°C/min to 300°C, 1 scan/sec (*m/e* 29–500); ion source temperature 260°C, filament current 1.5 mA. (B) MS data for I: *m/e* (relative intensity) [assignment]: 248(4)[M^+-60], 219(0.6)[$M^+-(60+29)$], 205(1)[$M^+-(60+43)$], 192(0.4), 191(1.5), 61(3.5), 55(100), 43(97).

Synthetic chemicals were dissolved in methylene chloride and the appropriate amount of solution soaked into rubber septa (5 × 9-mm rubber stoppers, sleeve type), then used as baits.

RESULTS

Seventy females tips were extracted in hexane. A two-females equivalent was injected into the OV-101 column. Fractions were collected at 2-min intervals up to 60 min and were assayed by EAG. As shown in Figure 1, EAG activity was recorded for the fraction collected at 38–40 min. The main peak I in Figure 1 was followed by two minor peaks, Ia and Ib, and all the retention times corresponded to those of linear 18-carbon acetates (18:Acs).

Samples for GLC-MS analysis and microreactions were purified by TLC and were a mixture of three components (Figure 2A). From the MS data (Figure 2B), the significant ions at *m/e* 248(M^+-60, 4%) and 61 [$(CH_3CO_2H_2)^+$, 3%], diagnostic for diunsaturated 18:acetates, were assigned to peak I. The significant ions at *m/e* 250(M^+-60, 8%) and 61 (15%), diagnostic for monounsaturated 18:acetates, were assigned to peak Ia. The significant ions at *m/e* 252(M^+-60, 0.5%) and 61 (53%), diagnostic for octadecan-1-ol acetate, were assigned to peak Ib. The GLC of standards on OV-1 and Carbowax 20 M columns confirmed these data.

Microepoxidation of I gave only one mono epoxide ($M^+ = 324$; $M^+-60 = 264$ *m/e*). In conjunction with the MS data, this suggested that I had one of the double bonds in a poorly reactive position. Microozonolysis of I produced 1,11-undecanendial which was identified by comparison of the GLC-MS data and of the GLC retention times with those obtained for a standard. These results suggested that there was a nine-carbon-atom interval between the two double bonds. No other oxy compound was identified in the reaction mixture by GLC, probably because the other two products were eluted with the solvent.

The assignment of the position of double bonds was done by preparing the DMDS adducts of I, as outlined in Figure 3. Five hundred nanograms of I were

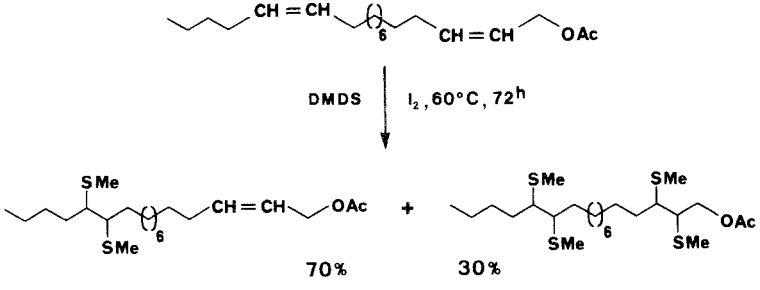
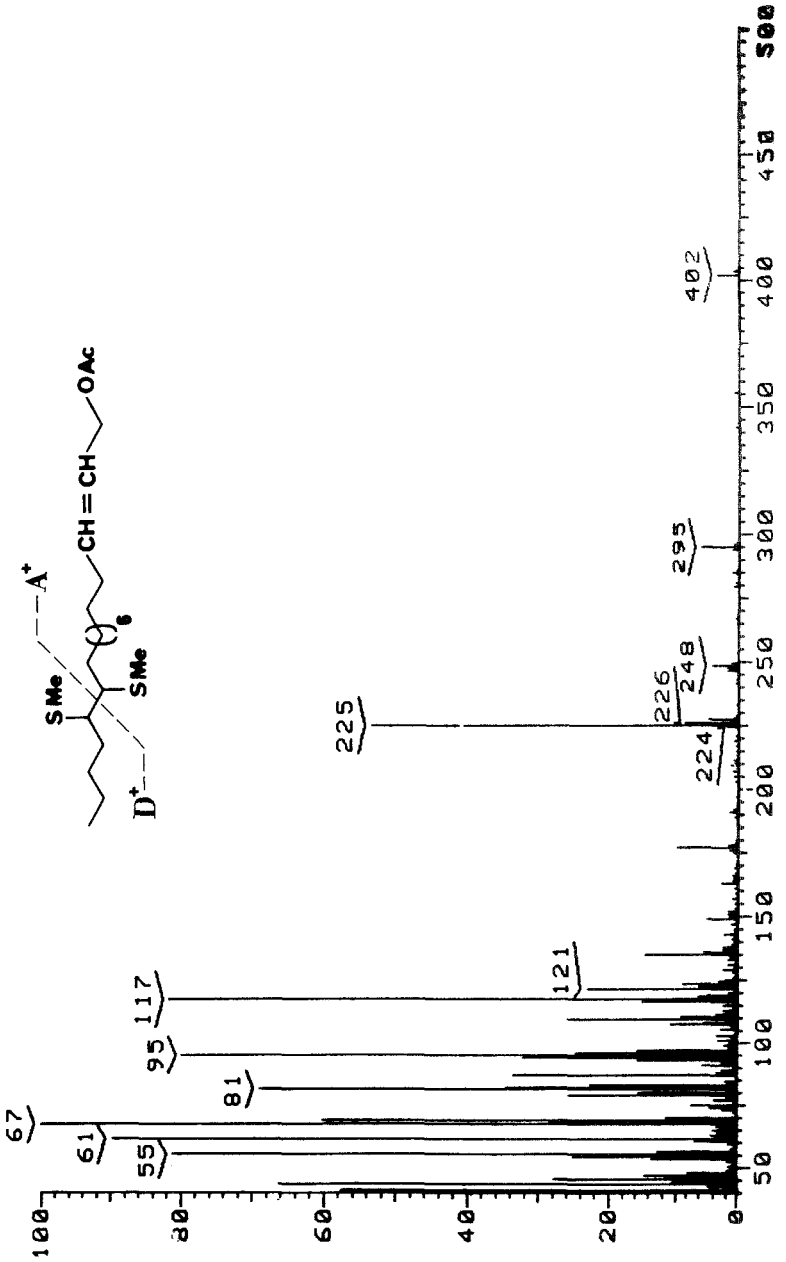


FIG. 3. Scheme of the derivatization of I: mono- and di-DMDS adducts.


SMe
A+
CH=CH
OAc
6
SMe
D+
67
61
55
81
95
117
121
225
224
226
248
295
402
100
80
60
40
20
0
50
100
150
200
250
300
350
400
450
500

(A)

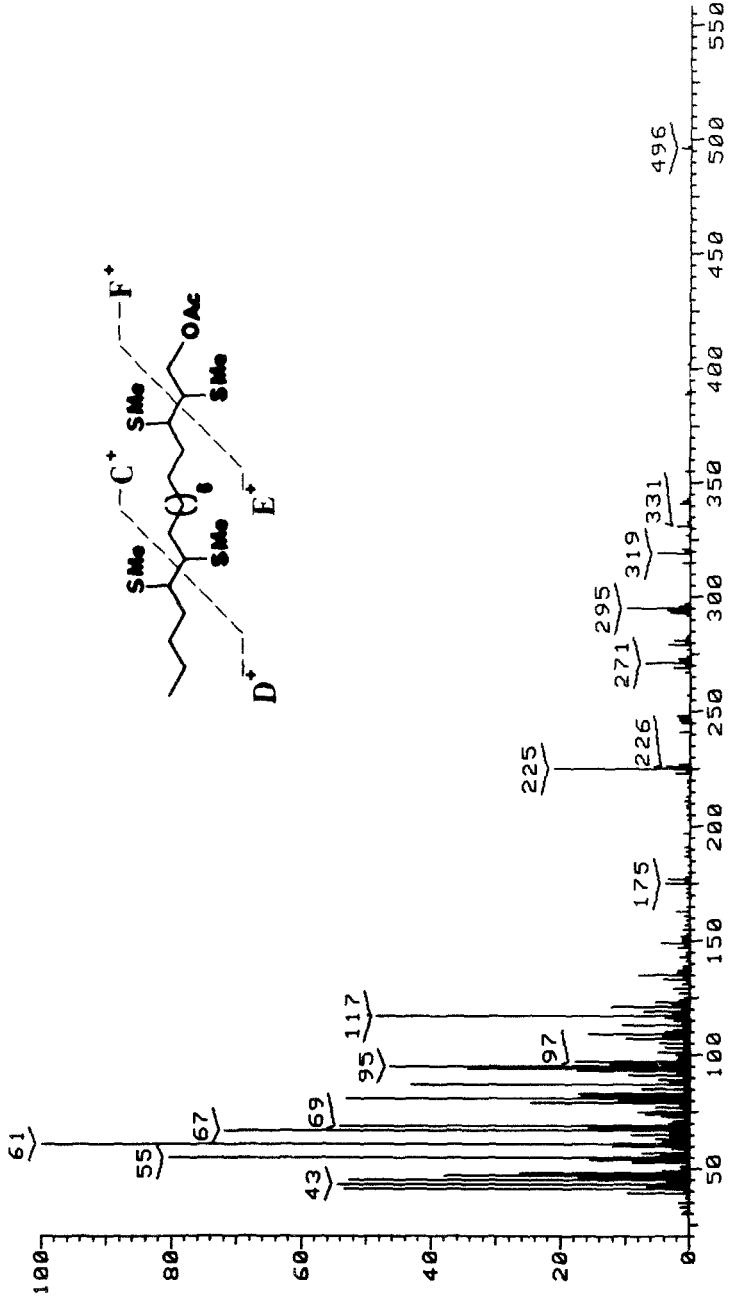


FIG. 4. (A) EI mass spectrum of the mono-DMDS adduct of I: *m/e* (relative intensity) [assignment]: 402(4)[M^+], 285(1)[A^+], 225(53)[$A^+ - 60$], 117(81)[D^+]. (B) EI mass spectrum of the di-DMDS adduct of I: *m/e* (relative intensity) [assignment]: 496(1.5)[M^+], 331(2)[$C^+ - 48$], 319(5)[$C^+ - 60$], 315(1.2)[$E^+ - 48$], 269(2.6)[$E^+ - 47 - 47$], 225(21)[$C^+ - 47 - 47 - 61$], 133(4)[F^+], 117(48)[D^+].

reacted with DMDS for three days and the adducts were analyzed by GLC-MS. The reaction mixture contained only two adducts: the di-DMDS derivative (30%) and one mono-DMDS derivative (70%) (Figure 3). The other possible mono-derivative was not detected. The MS (EI, 70 eV) showed recognizable ions and key fragment ions that indicated the positions of the CH_3S groups. The mono-DMDS adduct (Figure 4A) showed unequivocally that one double bond was in the 13 position. The di-DMDS adduct (Figure 4B) confirmed the same position and showed the 2 position for the other double bond. The spectrum of the di-DMDS adduct of I was identical with that of the corresponding derivative of the synthetic material. Thus I was identified as a 2, 13-octadecadien-1-ol acetate.

A more precise analysis of the DMDS adducts mixture allowed us to find the double bond in Ia (Figure 5). In spite of the very low amount available, we identified the double bond in the 13 position; all the data corresponded to those of a standard sample of *Z*13–18 : Ac.

The preparation of the four isomers of 2, 13–18 : Ac was done by the route reported in Figure 6. All the compounds were analyzed by GLC on the Carbowax 20 M column, and the retention time of the *E*, *Z* isomer coincided with the one of the TLC purified I (Figure 7). This result was confirmed by an independent experiment on the SP-2340 capillary column.

The results of male EAGs to the most active compounds are shown in Figure 8. The four isomers of 2, 13–18 : Ac, *Z*13–18 : Ac, the four isomers of 3, 13–18 : Ac, and *Z*7–12 : Ac were the only standards showing an activity higher than 1 mV. No other active compound was found among most of the known sex attractants and pheromones of Lepidoptera, all available in this laboratory.

*Z*7–12 : Ac was not in the female extracts but its EAG activity (Figure 8) was in agreement with the hypothesis of the relevance of the apolar alkyl end of pheromones (Priesner, 1979; this laboratory, unpublished results). *Z*9–14 : Ac and *Z*11–16 : Ac were slightly active (responses lower than 1 mV).

Preliminary field tests were done in the Ravenna area. The traps were baited with either single compounds or various binary mixtures of all the 2, 13–18 : Ac, 2–18 : Ac, and 13–18 : Ac isomers. *Z*7–12 : Ac was tested as a possible synergist (Table 1). None of the compounds or mixtures was found to be attractive. A trap baited with two virgin females caught five males over a period of two weeks.

DISCUSSION

The data indicated that the ovipositor extracts from *Zeuzera pyrina* females contained at least three compounds that are structurally similar to the known pheromone components of Lepidoptera. The main component of the extracts was characterized as *E*,*Z*2, 13–18 : Ac. This compound is present as a sex at-

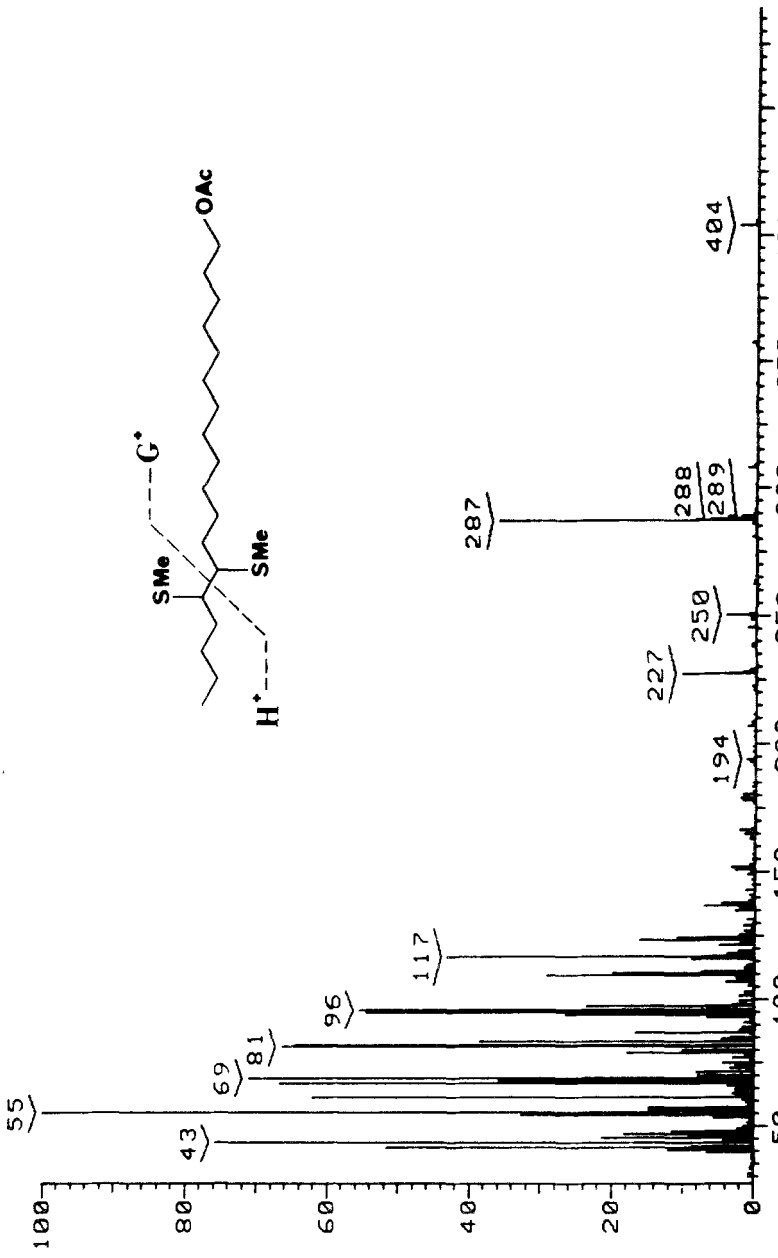


FIG. 5. EI mass spectrum of the DMDS-adduct of Ia: *m/e* (relative intensity) [assignment]: 404(2.5)[M^+], 287(36)[G^+], 227(10.5)[$G^+ - 60$] and 117(43)[H^+].

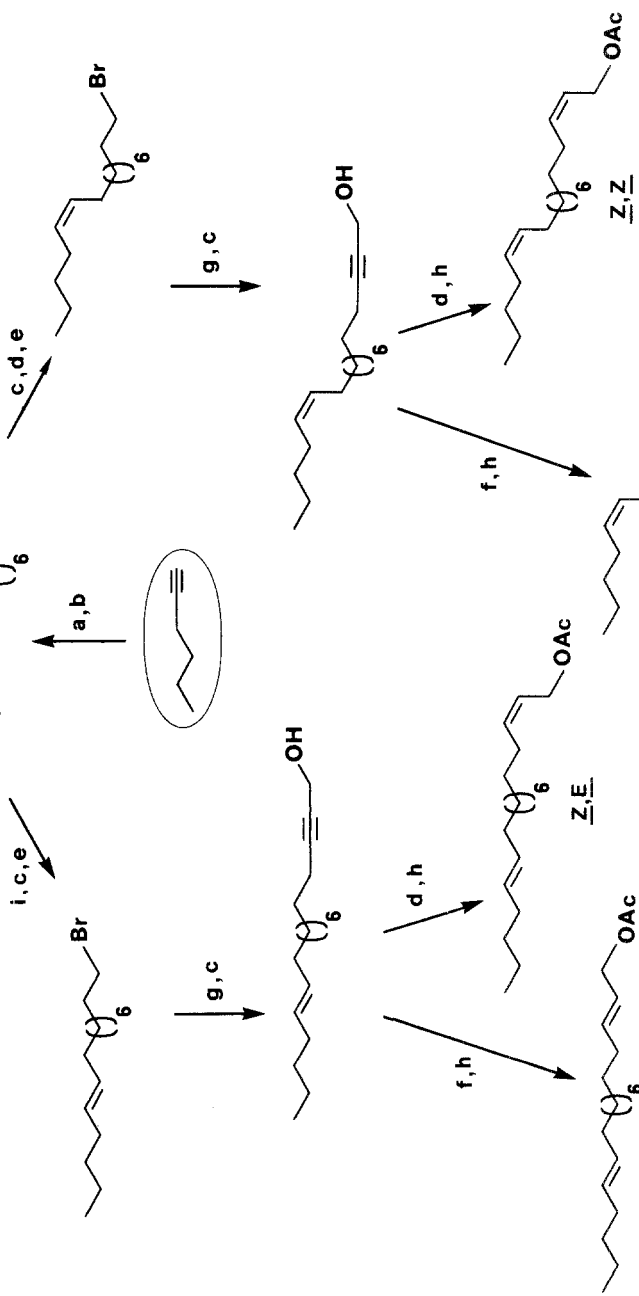


FIG. 6. Synthetic route to prepare the four isomers of 2,13–18 : acetate: (a) $LiNH_2$, liq NH_3; (b) $_{Br}$⟋$_6$⟍⟋^{OTy}(c)H_3O^+; (d) H_2, P_2Ni cat, MeOH (Brown, 1973); (e) PBr_3; (f) LAH, THF (Attenburrow, 1952); (g) Li≡⟍OTy, THF/HMPT; (h) Ac_2O, Pyr; (i) Na/NH_3.

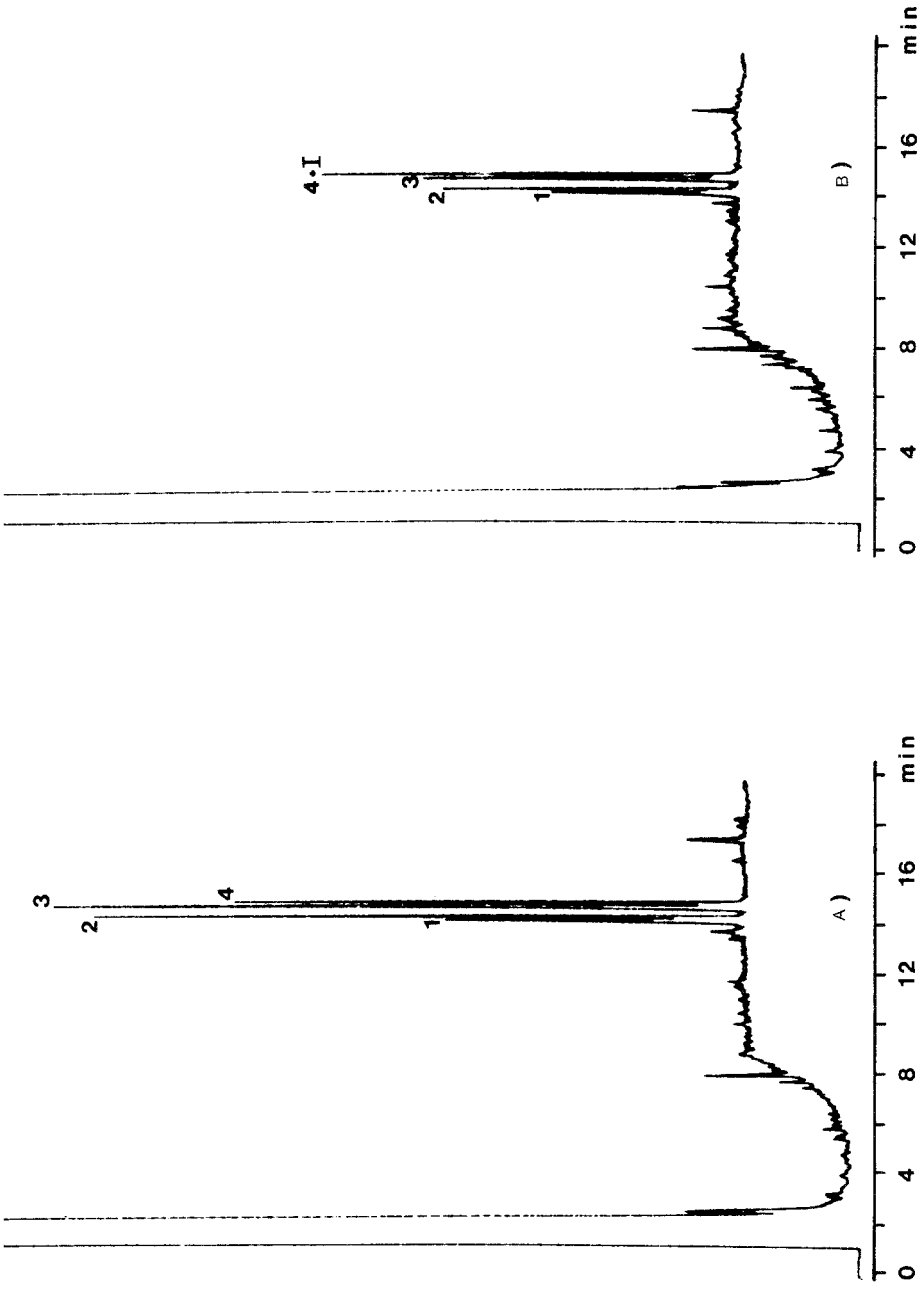


FIG. 7. Carbowax 20 M fused silica column, 25 m, 0.32 mm ID, splitless injection, 1 min 80°C, 80–200°C at 15°C/min and then isothermal, H_2 carrier gas at 34 cm/sec. (A) Mixture of the four isomers of 2,13-18:Ac:(1) = *Z,E*, (2) = *Z,Z*, (3) = *E,E*, (4) = *E,Z*. (B) Cochromatography of the mixture of (A) and TLC-purified I.

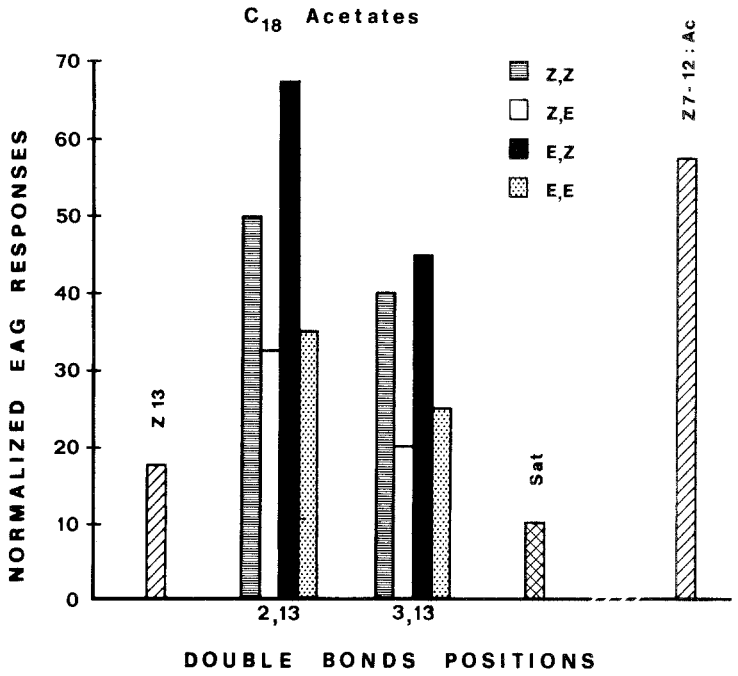


FIG. 8. EAG profiles of male *Zeuzera pyrina* antennal responses to the most active compounds. Responses to 10 μg of chemicals; mean of five replicates. Responses to sat = 18 : Ac was measured before each chemical and the responses were compared to its value converted to 10.

tractant in two species of Sesiidae: *Vitacea polistiformis* (Schwarz et al., 1983) and *Synanthedon tipuliformis* (Voerman et al., 1984). A second component we identified as Z13–18 : acetate is also found as main attractant in the *Chilo saccariphagus* sex pheromone (Nesbitt et al., 1980).

Our EAG results have shown that Z7–12 : Ac also had some activity; however, this compound was not detected in the female gland. Among the four isomers of 2,13–18 : acetate, the natural compound *E*, *Z* showed greater activity than the others.

Although the field results were negative, we believe that this cannot be accounted for by a lack in the design of the trap or by inactivity of the products. Our results show that using the virgin female as a bait, the trap is effective. And the EAG activities of the single products were highly positive. It is possible that other components were not detected in the female extracts and that they are essential for the attraction. It is also possible that the release rate of the chemicals was the critical factor in the field tests. Thus we are now working on these two lines with the aim of clarifying these crucial points.

TABLE 1. BAITS FOR FIELD TESTING ON MALE *Z. pyrina*
(Four replicates, rerandomized 8 times)

Treatment and chemical components (mg)
1. *E,E*2,13-18:Ac (1)
2. *E,Z*2,13-18:Ac (1)
3. *Z,E*2,13-18:Ac (1)
4. *Z,Z*2,13-18:Ac (1)
5. *E,E*2,13-18:Ac (0.95) + *E*13-18:Ac (0.05)
6. *E,Z*2,13-18:Ac (0.95) + *Z*13-18:Ac (0.05)
7. *Z,E*2,13-18:Ac (0.95) + *E*13-18:Ac (0.05)
8. *Z,Z*2,13-18:Ac (0.95) + *Z*13-18:Ac (0.05)
9. *E,Z*2,13-18:Ac (0.95) + *E*13-18:Ac (0.05)
10. *E,E*2,13-18:Ac (0.95) + *E*2-18:Ac (0.05)
11. *E,Z*2,13-18:Ac (0.95) + *E*2-18:Ac (0.05)
12. *Z,E*2,13-18:Ac (0.95) + *Z*2-18:Ac (0.05)
13. *Z,Z*2,13-18:Ac (0.95) + *Z*2-18:Ac (0.05)
14. *E,Z*2,13-18:Ac (0.95) + *Z*2-18:Ac (0.05)
15. *E*13-18:Ac (1)
16. *Z*13-18:Ac (1)
17. *E*2-18:Ac (1)
18. *Z*2-18:Ac (1)
19. *E,E*2,13-18:Ac (0.95) + *Z*7-12:Ac (0.05)
20. *E,Z*2,13-18:Ac (0.95) + *Z*7-12:Ac (0.05)
21. *Z,E*2,13-18:Ac (0.95) + *Z*7-12:Ac (0.05)
22. *Z,Z*2,13-18:Ac (0.95) + *Z*7-12:Ac (0.05)
23. *Z*7-12:Ac (1)

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SEX-SPECIFIC ACTIVITY OF (*R*)-(−)- AND (*S*)- (+)-1,7-DIOXASPIRO[5.5]UNDECANE, THE MAJOR PHEROMONE OF *Dacus Oleae*[1]

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Abstract—1,7-Dioxaspiro[5.5]undecane (olean), the major component of the female sex attractant pheromone blend of the olive fruit fly *Dacus oleae* (Gmelin) was shown to be released as a racemate. The response of males and females to pure (*R*)-(−) and (*S*)-(+)- enantiomers was tested under laboratory and field conditions. Males in laboratory and field tests responded only to (*R*)-(−)-olean, which functions as a sex attractant. Females responded only to (*S*)-(+)-olean in laboratory tests but not in the field. There are indications that the latter enantiomer fuctions as a short-range arrestant throughout the day and as an aphrodisiac in the process of mating.

Key Words—*Dacus oleae*, olive fruit fly, Diptera, Tephritidae, pheromones, enantiomers, sex attractants, sex-specific enantiomers.

INTRODUCTION

Female *Dacus oleae* flies release a sex pheromone which functions as a potent long-range male attractant (Haniotakis, 1974; Haniotakis et al., 1977; Delrio et al., 1982). This attractant has been found to be a mixture of the following four substances: 1,7-dioxaspiro[5.5]undecane (I), α-pinene (II), *n*-nonanal (III), and ethyl dodecanoate (IV) (Mazomenos et al., 1981). We suggest "olean" as the name for 1,7-dioxaspiro[5.5]undecane and will use this term in this paper. Baker et al. (1980) identified olean from female *D. oleae* rectal glands. Ma-

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zomenos and Haniotakis (1981) found that the natural olean is more attractive than any of the other three alone, but a mixture of all four is more attractive than pure olean. Similar results were obtained with the four synthetic compounds (Mazomenos and Haniotakis, 1984).

The presence of a male sex pheromone has also been reported; De Marzo et al. (1978) observed female attraction by males in laboratory bioassays. Mazomenos and Pomonis (1983) reported the isolation of olean from rectal gland extracts of wild males which is as attractive to males as olean isolated from females. The same authors observed no female attraction to olean of either male or female origin. EAG studies of the racemic mixture of synthetic olean showed similar response of male and female antennae (van der Pers et al., 1984). These findings raised doubts as to the exact function of olean as a sex pheromone. Could the same compound be utilized by both sexes for inter- or intra-sex communication?

The purpose of the present work was to clarify this as well as the following questions: (1) Do the enantiomers of olean show different biological activities against males and females of the olive fly? (2) Which enantiomeric composition does the naturally occurring olean show?

METHODS AND MATERIALS

Test Compounds and Chemical Analysis. Samples of (*R*)-(−)- and (*S*)-(+)-enantiomers of 1,7-dioxaspiro[5.5]undecane (olean) were available through the syntheses of Redlich and Francke (1984) and Mori et al. (1984). Optical purity of the compounds was 96–99% e e as determined by complexation gas chromatography (Weber and Schurig, 1984).

Volatile compounds from female and male olive flies were obtained by pentane extraction of excised rectal glands, cold trap condensation of head space components (Haniotakis et al. 1977), and "closed loop stripping" of living insects with a modified Grob apparatus (Grob and Zurcher, 1976: Boland et al., 1984). Gas chromatographic separation of enantiomers of natural olean was carried out by the complexation method.

Lab Bioassays. The biological activities of (*R*)-(−)- and (*S*)-(+)-olean were tested on both sexes in two types of olfactometers.

Olfactometer type A has been described in detail by Haniotakis (1974). It consisted of a wooden 1.38 × 0.96 × 0.38-m box with a clear Plexiglas top divided into two sections by a partition along its long axis so that two components can be tested simultaneously. A regulated air stream inside each section carried the chemical stimulus, dispensed from 1-ml polyethylene vials loaded with 1 mg of the compounds under test. Responding insects entered a glass cage upwind where they were counted. Insect response was observed for 24 hr and recorded 3 hr before the end and at the end of the photoperiod.

Olfactometer type B, consisting of a 95 × 70 × 40-cm screen cage, has been described by Mazomenos and Haniotakis (1981). A regulated air stream along the long axis of the cage carried the chemical stimulus, evaporated from a triangular 7.5-cm^2 Whatman No. 1 filter paper placed close to the cage ceiling. Concentrations of 1 or 10 g of the compounds under test were used on a different piece of filter paper for each test. The number of insects landing on the paper was counted for 10 min and recorded every 5 min. Tests were conducted during the last 2 hr of the photophase.

Lab-cultured *D. oleae* flies (Tsitsipis, 1977) were used for the tests. Flies 1–2 days old (unmated) were immobilized by chilling at 2–4°C for 1–2 hr and separated according to sex. They were kept in screen cages 270 cm^3 in artificial light (3000 lux intensity) and 10:14 hr dark–light regime. Temperature was 25 ± 2°C and relative humidity 65 ± 5%.

Field Tests. Plywood rectangles 15 × 20 cm coated with sticky substances were used as traps. One-milliliter polyethylene vials loaded with 1 mg of the compound to be tested in 100 μl of pentane containing traces of triethylamine, which served as a stabilizer, were used as dispensers. Traps were checked and rotated once per week.

RESULTS AND DISCUSSION

Chemical Analysis. In a nonacidic medium 1,7-dioxaspiro[5.5]undecane (olean) proved to be rather stable. In contrast to the isomeric bark beetle pheromone, 2-ethyl-1,6-dioxaspiro[4.4]nonane, chalcogran (Francke et al., 1977), pure enantiometers of olean do not tend to racemize easily.

Complexation gas chromatography of gland extracts and cold-trap condensation from both sexes showed the obtained natural olean to be a racemate. A pentane solution of the charcoal absorbed volatiles from a "stripping experiment" with 4000 living females was cooled on Dry Ice and analyzed within 30 hr after collection; the sample contained large amounts of racemic olean.

Tests with Olfactometer Type A. Table 1 shows the response of *D. oleae* males and females to both enantiomers of olean in olfactometer type A. Significantly more males were attracted to (*R*)-(−)-olean (62.2) than to (*R*)-(+)-olean (24.6). Male responses to pentane was 22.5 ± 8.8%. Male response to (*R*)-(−)-olean coincided with the mating period of this insect.

More females on average responded to (*S*)-(+)-olean (9.6) than to the (*R*)-(−)-enantiomer (5.6), but the difference is not significant. Female response to pentane was 4.7 ± 3.3%. Observation of female behavior during testing hours suggested that female response was not the result of a chemical attractant but rather to an arrestant or tranquilizer. Since such pheromones usually have short-range activity, it was decided to repeat the same bioassays but with a shorter distance between dispenser and insect cage. This distance was set at 10 cm,

TABLE 1. RESPONSE OF *Dacus oleae* MALES AND FEMALES TO 1 mg OF (*R*)-(−)- AND (S)-(+)-ENANTIOMERS OF 1,7-DIOXASPIRO[5.5]UNDECANE DISPENSED FROM POLYETHYLENE VIALS 30 cm FROM INSECT CAGE[a]

Males				Females			
Dispenser Insect age (days)	Insect age (days)	Response (%) to (*R*)-(−)-	Response (%) to (*S*)-(+)-	Dispenser age (days)	Insect Age (days)	Response (%) to (*R*)-(−)-	Response (%) to (*S*)-(+)-
1	8	80.0	19.0	0	3	8.0	12.0
2	9	80.0	14.0	1	4	10.0	13.0
5	10	61.0	18.0	2	5	11.0	22.0
6	11	65.0	52.0	8	11	7.5	6.3
7	12	52.0	17.0	9	12	10.0	11.3
8	13	53.0	24.0	10	6	4.0	6.0
9	14	48.0	20.0	14	10	3.0	4.0
1	8	60.8	55.8	15	11	5.0	5.0
3	4	36.0	11.3	17	13	5.0	8.8
4	5	41.7	36.7	21	17	2.5	10.0
5	6	70.0	12.5	22	18	2.5	2.5
8	9	62.5	26.7	23	6	6.0	8.0
9	10	74.8	32.0	24	7	3.3	11.3
11	4	73.0	12.3	28	11	7.3	8.0
12	5	75.0	18.3	29	4	6.0	9.3
—	—	—	—	30	5	3.3	6.6
—	—	—	—	31	6	3.3	13.3
—	—	—	—	35	10	6.7	8.0
—	—	—	—	36	11	4.0	16.0
—	—	—	—	38	6	3.4	11.3
Means		62.2*[b]	24.6*			5.6 NS[b]	9.6 NS

[a] Laboratory bioassays with lab-cultured flies. Olfactometer type A, one test per day from May 24 to August 17 for males and June 5 to July 13, 1984, for females. Ambient temperature during tests 25 C ± 1°C.

[b] Significant difference, Student's *t* test, $P = 0.001$; NS = nonsignificant.

and the results of these tests are shown on Table 2. The difference in female response between (*R*)-(−)- (13.8) and (*S*)-(+)- (4.0) enantiomers is highly significant. Contrary to that of males, female response was not restricted to the mating period. It was expressed throughout the light period.

Tests with Olfactometer Type B. Table 3 shows male response in olfactometer typr B. Significantly more males responded tp (*R*)-(−)- (47.0 total) than to S-(+)-olean (21.3 total). Response to (*S*)-(+)-olean or to the racemic mixture of the two enantiomers (41.6 total) does not differ from that to the control (30.1 total). The behavior of responding males called to mind typical positive

TABLE 2. RESPONSE OF *Dacus oleae* FEMALES TO 1 mg OF (*R*)-(−)- AND (*S*)-(+)-ENANTIOMERS OF 1,7-DIOXASPIRO[5.5]UNDECANE DISPENSED FROM POLYETHYLENE VIALS 10 cm FROM INSECT CAGE[a]

Dispenser Age, Days	Insect Age, Days	% Response to	
		R-(−)-	*S*-(+)-
2	10	4.7	16.0
3	11	1.3	19.3
4	5	4.7	10.0
5	6	2.7	18.7
9	10	3.3	8.0
10	11	6.0	12.6
11	5	4.7	8.0
12	6	4.0	9.0
16	10	7.0	14.0
17	12	4.0	9.0
18	13	2.0	27.0
Means		4.0*[b]	13.8*

[a] Laboratory bioassays with lab-cultured insects. Olfactometer type A. One test per day from July 17 to August 3, 1984. Ambient temperature 25 ± 1°C.
[b] *Significant difference, Student's test, $P = 0.001$.

olfactory attraction, namely zig-zag upwind flying inside the plume toward the source and landing on it. The same tests were repeated with female insects. In this case no response was observed to either enantiomer, while the behavior of females showed no signs of olfactory attraction. The lack of demonstration of female response, such as appeared in tests with olfactometer type A, may be attributed to the restricted time of each test (10 min).

Field Tests. Table 4 shows the number of insects captured by both enantiomers in field tests. Significantly more males were captured by (*R*)-(−)- (5.3) than (*S*)-(+)- (0.4) olean. No females were captured by either enantiomer. It should by pointed out that *D. oleae* populations at the site of these experiments, as is known from many years of observations, decline about mid-May and increase again in late August or early September. The low captures from May 17 to August 23 must therefore be attributed to that reason.

The data presented up to this point show that (*R*)-(−)-olean is a male attractant and male response coincides with the mating period of this species. It is therefore clear that it functions as a sex attractant. In addition, as known from previous work (Haniotakis, 1979), female *D. oleae* flies release this compound throughout the light period at a very low rate, and its release increases greatly during the mating period. It is also known from previous work that olean is a

TABLE 3. RESPONSE OF *Dacus oleae* MALES TO 10 μg OF (*R*)-(−)- AND (*S*)-(+)- ENANTIOMERS OF 1,7-DIOXASPIRO[5.5]UNDECANE DISPENSED FROM FILTER PAPER.

Date & insect age (days)	Test sequence	Response (%) to											
		Check			(*R*)-(−)-			(*S*)-(+)-			Racemic		
		0–5′	6–10′	Total	0–5′	6–10′	Total	0–5′	6–10′	Total	0–5′	6–10′	Total
February 24	1							2	6	8			
(6)	2				9	13	22						
	3										9	13	22
	4	1	2	3									
February 27	1	10	11	21									
(3)	2							9	4	13			
	3				13	12	25						
	4										9	12	21
	5	8	14	22									
	6							6	11	17			
	7				11	12	23						
February 28	1	10	10	20									
(4)	2	7	12	19									
	3				18	22	40						
	4	6	10	16									
	5							5	8	13			

	6	8	6	14									
	7				16	27	43						
	8	8	7	15									
	9							10	14	24			
	10										3	10	13
February 29	1	32	25	57									
(5)	2	18	33	51									
	3	19	38	57									
	4							37	17	54			
	5				51	61	102						
	6	15	18	33									
	7										52	21	73
	8	15	13	28									
	9				25	16	41						
March 1	1	14	29	43									
(6)	2										40	39	79
	3	19	28	47									
	4				44	36	80						
	5	15	12	27									
	6							11	9	20			
Means		13.0	17.1	30.1a	23.4	24.9	47.0b	14.4	9.9	21.3a	22.6	19.0	41.6a

[a] Laboratory bioassays with lab-cultured insects. Olfactometer type B. Five min recordings for 10 min 1984.
[b] Means of totals were compared only after transformation of data to log x. Means followed by same letter no significantly different, Student's t test, $P = 0.05$.

TABLE 4. NUMBERS OF *Dacus oleae* FLIES CAPTURED PER TRAP PER WEEK ON STICKY BOARDS BAITED WITH1 ml POLYETHYLENE VIALS CONTAINING 1 mg OF (*R*)-(−)- OR (*S*)-(+)- ENANTIOMERS OF 1,7-DIOXOSPIRO[5-5]UNDECANE[a]

Date	Dispenser age (days)	Insects captured by			
		(*R*)-(−)-		(*S*)-(+)-	
		♂♂	♀♀	♂♂	♀♀
March 12[b]	7	2.0	0.0	1.0	0.0
March 29	24	8.3	0.0	1.8	0.0
April 5	31	3.0	0.0	0.5	0.0
April 12	38	3.0	0.0	1.5	0.0
April 19	45	3.5	0.0	0.5	0.0
May 3	59	1.0	0.0	0.0	0.0
May 10	66	5.5	0.5	0.5	0.0
May 17	7	1.7	0.0	0.0	0.0
May 24	14	0.3	0.0	0.0	0.0
July 5	43	1.3	0.0	0.0	0.0
July 12	50	0.0	0.0	0.0	0.0
July 19	57	0.7	0.0	0.3	0.0
Aug. 16	85	0.3	0.0	0.0	0.0
Aug. 23	7	1.7	0.0	0.0	0.0
Aug. 30	14	3.0	0.0	0.0	0.0
Sept. 6	21	2.3	0.0	0.0	0.0
Sept 13	28	4.3	0.0	0.0	0.0
Sept. 20	35	1.3	0.0	0.3	0.0
Sept. 27	42	4.3	0.0	0.0	0.0
Oct. 4	49	8.3	0.0	0.3	0.0
Oct. 11	7	43.7	0.0	1.7	0.0
Oct. 18	14	15.0	0.0	0.0	0.0
Oct. 25	21	6.7	0.0	0.0	0.0
Means		5.3[c]	0.0	0.4[c]	0.0

[a] Field experiments with wild insects, 1984. Means of 3 replicates. Traps used: March 12–May 10, white poster board; May 10–Oct. 4, plywood natural color.
[b] Trap checks were made weekly. Missing dates mean zero captures.
[c] Significant difference, Student's t test, $P = 0.01$. Data were transformed to $\sqrt{x + 0.5}$ prior to statistical analysis.

long-range attractant which does not primarily influence the males' ability to mate after visual contact has been established with receptive females (Delrio et al., 1982, and upublished data).

Furthermore, females respond to (*S*)-(+)-olean, but its exact function is not clear. The mating behavior of this species has not been studied under field conditions, and it is difficult to even make assumptions of its role. Observations of the mating behavior between receptive, nonreceptive , and antennectomized

olive flies in the various combinations of sexes (unpublished data) strongly indicated the requirement of a chemical stimulus on the female for successful mating. This stimulus must play the role of female aphrodisiac or tranquilizer in the absence of which females strongly reject males. It is possible that this role may be undertaken by (*S*)-(+)-olean. In other fruit fly species which form leks during mating, there are indications that, besides visual and olfactory cues emanating from the host, pheromones seem also to be involved in lek formation (Prokopy and Raitberg, 1984, and cited references). A female population increase has indeed been observed in individual trees or orchards in which dispensers of racemic olean were used (Haniotakis et al., 1983, and unpublished data). Further work is required to verify all the above indications.

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SUSCEPTIBILITY TO VOLE ATTACKS DUE TO BARK PHENOLS AND TERPENES IN *Pinus contorta* PROVENANCES INTRODUCED INTO SWEDEN

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Abstract—Seedlings of North American *Pinus contorta* introduced to Sweden and Finland are severely gnawed by voles, e.g., *Microtus agrestis*. The level of damage varies between provenances. Chemical analyses of various phenolic compounds, monoterpenes, and resin acids of different provenances and of damaged and undamaged stems showed that some phenolic substances in the bark increased after damage without deterring the animals, that monoterpene differences between provenances were not related to vole damage, and that certain resin acids occurred in larger amounts in lightly than in severely damaged provenances. Levopimaric and neoabietic acid, and possibly abietic acid, may be important for a partial resistance to vole browsing.

Key Words—*Pinus contorta*, vole, damage, *microtus agrestis*, defense, phenols, monoterpenes, resin acids.

INTRODUCTION

Pinus contorta has been introduced in Swedish forestry as a fast-growing pine species. However, it has suffered serious damage from bark consumption by voles, chiefly *Microtus agrestis* (Hansson and Lavsund, 1982). Seeds or seedlings have been transferred from several localities in western Canada and north-

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western United States, constituting distinct provenances. These provenances have been tested for growth and survival in field trials on reforestation areas all over Sweden (Lindgren, 1983). Clear differences in bark attacks by voles have been found between the provenances (Hansson, 1983, 1985).

Genetic differences in resistance to animal consumption are usually related to chemical defense systems, be they constant or induced (Haukioja, 1980), although mechanical defense or nutritional value may also vary. Two different chemical systems seem to be operating (cf. Rhoades and Cates, 1976), one with digestion-reducing substances (quantitative defense) and one with acutely toxic substances (qualitative defense). The former is assumed to be more important for long-lived and common plants such as pines and is suggested to be based on phenolic or terpenoid compounds, while the latter, based on, e.g., alkaloids, is known mainly in the case of rare or fugitive species. Phenolics have played an important role in the discussion of induced defense of deciduous trees and bushes (Bryant and Kuropat, 1980), while terpenes seem more important in conifers (e.g., Sturgeon, 1979, for bark beetles, and Radwan et al., 1982, regarding rodents). Therefore in the present study, bark of *Pinus contorta* was analyzed for various phenolic compounds, monoterpenes, and resin acids (diterpenes) in order to reveal any defense systems related to genotypes or provenances.

METHODS AND MATERIALS

Samples were taken from a field trial at Skalet, Strömsund (64°N), established in 1971 (IUFRO 1970/71), in northern Sweden on September 23, 1983, after vole damage inflicted during the winters of 1980–1981 and 1981–1982. Frost and snow appear usually in early October in this area, where indigenous pines (*Pinus silvestris*) are in the winter physiological state by late September. One square decimeter of bark was cut at 0.5 m height, i.e., at places usually attacked by the voles, from six different provenances. Three of them had been damaged severely by voles in various trials in Sweden (the conditions at Strömsund, with very high level of damage, were not completely representative) and the other three only slightly (Hansson, 1983). From the severely damaged provenances, both damaged and undamaged stems were sampled, but only undamaged stems were taken from the slightly damaged provenances. It was planned to analyze four samples in each series, but in two of the severely damaged provenances only three samples of undamaged stems could be examined.

With the material obtained, differences could be estimated between all the provenances, between severely and slightly damaged provenances, and between damaged and undamaged stems. All these examinations of differences in chemical contents were statistically tested with one-way analysis of variance. In this

way provenance variation independent of vole resistance could be separated from true defense varying between provenances or individuals. An induced defense should be manifested in a higher level of defense chemicals in damaged plants.

The bark was kept deep-frozen until chemical analyses were performed. The methods used for the various substances are described below.

Phenolics. The bark, cut into small pieces, (5 g), was refluxed with 2 × 10 ml acetone for 2 × 30 min. The extract was filtered and evaporated at reduced pressure, and the evaporated residue was fractionated between water (10 ml) and hexane (2 × 10 ml). Glycosides in the water phase were hydrolyzed with pectinase (Sigma, No. P-4625 from *Aspergillus niger*) in the dark at room temperature for 24 hr. The hydrolysate was acidified with one drop of concentrated hydrochloric acid and extracted with 3 × 10 ml ethyl acetate. The organic phase was dried with anhydrous sodium sulfate, filtered, and evaporated. The sample was dissolved in 10 ml methanol and analyzed with a Waters high-performance liquid chromatography system on a Radia-PAK C18, 5 μm column (Waters). The mobile phase consisted of a linear gradient of water–methanol–formic acid 80:20:1 to 30:70:1 for 50 min at a flow rate of 1.5 ml/min. UV absorbance at 280 nm was measured.

Monoterpenes. These were analyzed in *n*-pentane extracts of fresh bark samples by gas chromatography (GC), and absolute amounts of monoterpenes were determined by reference to *p*-cymene (1-isopropyl-4-methyl-benzene), used as an internal standard. Pentane extracts were analyzed using GC on a 50-m × 0.3-mm WCOT column with SP-1000 stationary phase inserted into a glass precolumn of 3% SP-1000 on Chromasorb W, an inlet splitter, flame ionization detector, and chromatographic data system. Operating conditions were: injector 200°C, detector 230°C, carrier gas N_2 at 1.5 ml/min, split ratio 2:55, sample vol 1–2 μl, temperature programmed from 50° to 110° at 4°/min then isothermally at 150°C.

Resin Acids. The bark samples were crushed in a mortar with liquid nitrogen and thereafter freeze-dried for 24 hr. A 100-mg sample of the freeze-dried bark meal was extracted with 2 ml of petroleum ether–diethyl ether (1:1) containing heptadecanoic acid as an internal standard in an ultrasonic bath for 2 hr. The sample was centrifuged and the extractive solution was transferred to a screw cap test tube. The residue was washed twice with 1 ml of diethyl ether, and the combined extracts were evaporated to dryness under a stream of nitrogen. The dried extract was redissolved in 1 ml of diethyl ether methanol (9:1) and methylated with freshly prepared diazomethane prior to the GC analysis. The samples were analyzed on a Varian 2700 GC equipped with a glass wall, coated capillary column with SE-30 as a stationary phase and equipped with a flame ionization detector (FID). The chromatograph was operated isothermally at 240°C. Other operation parameters were as follows; injector and detector temperatures 280°C, carrier gas hydrogen at 2.0 ml/min, and a split ratio of

1:50. Individual resin acids were identified by comparing their relative retention times to published data and with GC–mass spectrometry (Holmbom, 1977; Foster and Zinkel, 1982).

RESULTS

Vole Damage. During 1980–1982, 60% of the still-living *P. contorta* stems were damaged by voles (Table 1). Damaged appeared often repeatedly on the same stems during the two winters. Earlier, almost half the plants had died, many of them due to vole damage. Examinations of provenances with more than 12 still-living seedlings showed also in this trial significant differences between provenances as regards damage with complete stem girdling ($\chi^2 = 40.00$, $df = 22$, $P < 0.05$). This difference was still more pronounced in trials with a lower total frequency of vole damage (Hansson, 1983).

Provenances with IUFRO Nos. 2003, 2019, and 2027 were used as examples of severely damaged provenances; and Nos. 2017, 2022, and 2034 as lightly damaged ones. Differences between these provenances were greater in other less damaged trials.

Phenols. The types of phenolic compounds were generally identified with regard to the retention time in HPLC chromatograms (but see below for specific cases) and the amounts as the height (mm) of the peaks or, in the case of large amounts, in a relative sense as the integrated area below the peaks (Table 2). Analyses of variance showed significant differences for certain compounds in

TABLE 1. VOLE DAMAGE AT SKALET PROVENANCE TRIAL (IUFRO 70/71 SERIES), NEAR STRÖMSUND IN NORTHERN SWEDEN[a]

	Stems		
Provenance (IUFRO-No.)	Vole attacked 1980–1982	Undamaged 1982	Dead earlier
2003	13	7	13
2019	12	4	8
2027	6	4	13
2017	9	9	6
2022	9	9	4
2034	8	10	2
Remaining 77 provenances	433	298	612

[a] Seedlings of 83 North American *Pinus contorta* provenances were planted as random single-tree plots.

TABLE 2. STATISTICAL ANALYSES OF HOMOGENEITY IN PHENOLIC CONTENTS BETWEEN PROVENANCES AND STEMS[a]

Substances (retention time, min)	Probability of homogeneity between		
	Six provenances	Severely and lightly damaged provenances	Total damaged and undamaged stems
3.40	0.18	0.29	0.27
~5	0.15	0.45	0.24
5.20	0.02	0.14	0.008
6.89	0.18	0.45	0.002
8.07	0.88	0.23	0.36
9.27	0.95	0.53	0.39
9.62	0.02	0.89	0.64
9.93	0.02	0.95	0.57
11.61	0.05	0.03	0.01
12.52	0.31	0.05	0.11
15.63	0.20	0.03	0.08
17.93	0.12	0.08	0.86
18.55	0.15	0.04	0.01
19.44	0.0001	0.01	0.006
20.63	0.33	0.19	0.31
23.38	0.20	0.74	0.35
27.95	0.35	0.14	0.89
33.63	0.15	0.08	0.30

[a] Significant deviations ($P \leq 0.05$) are underlined. The various phenolic substances were distinguished by chromatographic retention times.

at least some of the combinations of damaged/undamaged seedlings and provenances.

Peaks with retention times of 9.62 and 9.93 min showed only differences between the six provenances and were thus not related to the vole damage. Peaks at 5.20, 6.89, 11.61, 18.55, and 19.44 showed a lower probability of being samples from the same population for separate plants than for severely and lightly damaged provenances and thus there was no evidence of a provenance effect in those cases. Only the differences for the peaks at 12.52 and 15.63 min were significant between severely and lightly damaged provenances, and there were no separate stem effects. However, these latter probabilities were just at the border of significance.

The means amounts, as defined above, showed a consistently higher level among vole-damaged stems or provenances than among undamaged stems or lightly damaged provenances (Table 3). This demonstrates that either the voles were attracted by high contents of phenolics or that the vole damage had in-

TABLE 3. AMOUNTS OF VARIOUS PHENOLIC SUBSTANCES, DIFFERING BETWEEN PROVENANCES OR STEMS[a]

Substance (retention time, min)	Measurement	Between provenances		Between stems	
		Severely damaged provenances (N = 22)	Lightly damaged provenances (N = 12)	Damaged stems (N = 12)	Undamaged stems (N = 22)
5.20	h	25 ± 6	21 ± 8	27 ± 6	21 ± 7
6.89	h	18 ± 8	16 ± 6	22 ± 7	15 ± 6
11.61	a	445 ± 174	322 ± 115	491 ± 170	354 ± 142
12.52	h	41 ± 20	28 ± 16	44 ± 23	28 ± 12
15.63	h	38 ± 24	21 ± 8	41 ± 31	27 ± 12
18.55	a	264 ± 110	172 ± 92	318 ± 92	193 ± 93
19.44	a	904 ± 118	745 ± 238	959 ± 118	786 ± 186

[a]The values refer to either chromatographic peak height (h) or integrated areas under peaks (a) and are given as means ± standard deviation.

duced an increase in the amounts of several phenolic compounds. This higher level in damaged stems was especially pronounced at the retention times of 19.44 (identified as *trans-p*-coumaric acid), 11.61, and 18.55 min (the flavonoid taxifolin).

Monoterpenes. There were significant differences in the percentages of certain monoterpenes (retention times 14.14, 15.30, and 18.41 min) between the six provenances but not between severely and lightly damaged provenances nor between damaged and undamaged stems (Table 4). Thus, the provenance variations were not related to any resistance against vole attacks.

Resin Acids. Significant differences appeared between severely and lightly damaged provenances as regards certain resin acids (levopimaric and neoabietic acids, and less clearly so for abietic acid) and for total resin acids (Table 5). No differences appeared when only undamaged and damaged stems were compared, so individual differences were smaller than those between provenances. The amounts of the resin acids mentioned were consistently higher in the slightly damaged provenances (Table 6), which might be of importance in browsing repellence.

TABLE 4. STATISTICAL ANALYSES OF HOMOGENEITY IN MONOTERPENE CONTENTS BETWEEN PROVENANCES AND STEMS[a]

Substances (retention time, min)	Probability of homogeneity between		
	Six provenances	Severely and lightly damaged provenances	Total damaged and undamaged stems
7.86	0.58	0.28	0.53
10.08	0.25	0.55	0.95
11.23	0.50	0.38	0.36
12.41	0.28	0.38	0.61
12.70	0.10	0.27	0.80
12.93	0.28	0.10	0.82
13.19	0.47	0.21	0.79
13.39	0.38	0.11	0.79
13.61	0.58	0.23	0.74
13.81	0.50	0.09	0.75
14.14	0.04	0.66	0.72
15.30	0.03	0.66	0.58
15.71	0.28	0.44	0.40
18.41	0.04	0.31	0.90

[a] Significant deviations ($P \leq 0.05$) are underlined. The various monoterpene substances were distinguished by chromatographic retention times.

TABLE 5. STATISTICAL ANALYSES OF HOMOGENEITY IN RESIN ACID CONTENTS BETWEEN PROVENANCES AND STEMS[a]

Resin acid	Probability of homogeneity between: Six provenances	Severely and lightly damaged provenances	Total damaged and undamaged stems
Pimaric	0.98	0.80	0.85
Isopimaric	0.97	0.35	0.95
Levopimaric	0.33	0.02	0.23
Dehydroabietic	0.81	0.28	0.73
Abietic	0.67	0.08	0.31
Neoabietic	0.39	0.03	0.16
Total	0.56	0.05	0.37

[a] Significant deviations ($P \leq 0.05$) are underlined.

TABLE 6. CONCENTRATIONS OF RESIN ACIDS DIFFERING SIGNIFICANTLY IN AMOUNT BETWEEN SEVERELY AND LIGHTLY DAMAGED PROVENANCES

Resin acid	Dry matter (% $\bar{X} \pm$ SD): Severely damaged provenances ($N = 22$)	Lightly damaged provenances ($N = 12$)
Levopimaric	1.31 ± 0.73	1.90 ± 0.64
Abietic	0.92 ± 0.46	1.19 ± 0.35
Neoabietic	0.66 ± 0.68	1.23 ± 0.51
Total resin acids	5.65 ± 2.86	7.56 ± 2.06

DISCUSSION

The severely damaged experimental field at Strömsund was chosen for study partly because it would be possible to find induced defense in such an area, if anywhere. The increase observed in several phenolic components in attacked stems demonstrated a metabolic response of the pines, but it is not clear that it is a defense mechanism towards browsers. In spite of this reaction, the vole damage was extremely high during the whole 10-year span of this field trial and, furthermore, stems once attacked were often damaged again, usually at the bark edge of the earlier damage. The alternative, that voles selected stems

high in certain phenolic compounds, is very unlikely; phenolics are considered as generally deterring substances due to their complex formation with proteins (Whittaker and Feeny, 1971). Possibly the increase in phenolics was not directed towards the injurious agent but was just a consequence of tissue damage or repair. Davies et al. (1964) found that a high content of polyphenolic substances was associated with lack of nitrogen (cf. also McKey, 1979).

Rousi and Häggman (1984) measured the total phenol contents both of whole small *P. silvestris* seedlings (excluding roots) and of bark of *P. silvestris* grafts of various provenances and condition in northern Finland. The variation between the grafts of the same clone was so large that it seemed the phenol content in trees was not strictly controlled by genotype. However, grafts and seedlings with high contents of phenol were less damaged by hares (*Lepus timidus*).

Introduced *P. contorta* has also been severely damaged in northern Finland (Rousi, 1983). There, as in Sweden (Hansson, 1983), southern provenances showed higher frequencies of damage from voles than northern ones. Measurements of the total phenol content of *P. contorta* seedlings in Finland (Rousi, personal communication) did not show any significant difference between provenances nor any clear correlation with the frequencies of vole damage.

Rousi and Häggman (unpublished) did not find any seasonal variation of total phenols of *P. silvaticus* (graft) bark but an early autumn peak in whole shoots of one-year seedlings. The Strömsund *P. contorta* samples were taken just before the time for first snowfall and may well represent conditions during winter, when the vole attacks appear. Possibly there are provenance differences, e.g., as observed for phenols at the retention times of 9.62 and 9.93 min, due to the geographical origin as it has been observed that phenols often increase at the time of winter hardening of the trees (Alden and Herman, 1971).

A genetically determined antibrowsing resistance against mammals has been clearly demonstrated in Douglas fir (Dimock et al., 1976). Less is known about effects of plant terpenes than of phenolics on grazing or browsing animals. However, Radwan et al. (1982) demonstrated differences in pocket gopher damage to *Pinus ponderosa* seedlings differing in terpene contents.

There was no completely undamaged provenance at the Strömsund trial. This does not exclude the possibility of finding resistant genotypes, as provenances are still genetically heterogeneous. However, the main impression is that any natural defense will work for this pine species only at fairly low vole browsing pressure.

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SEX-SPECIFIC PRODUCTION OF IPSDIENOL AND MYRCENOL BY *Dendroctonus ponderosae* (COLEOPTERA: SCOLYTIDAE) EXPOSED TO MYRCENE VAPORS[1]

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Abstract—Male mountain pine beetles, *Dendroctonus ponderosae* Hopkins, produced ipsdienol [97.0% ± 0.3 *S*-(+)] and myrcenol (90.3% ± 4.0 *E*) when exposed to myrcene vapors. Females which were exposed to myrcene vapors did not produce any ipsdienol, but did produce low levels of myrcenol (98.0% ± 0.7 *E*). Neither sex produced detectable levels of ipsdienol or myrcenol when fed for 24 hr on lodgepole pine, *Pinus contorta* var. *latifolia* Engelmann. The sex-specific conversion of myrcene to ipsdienol and myrcenol suggests that these compounds may have behavioral significance within the species. In addition, the *S*-(+)-ipsdienol produced by male *D. ponderosae* probably functions as a repellent allomone against *Ips pini* (Say).

Key Words—*Dendroctonus ponderosae*, bark beetle, Coleoptera, Scolytidae, myrcene, aggregation pheromones, ipsdienol, myrcenol.

INTRODUCTION

Although males of *Ips* spp. produce the terpene alcohol pheromones ipsdienol [2-methyl-6-methylene-2,7-octadien-4-ol] and/or ipsenol [2-methyl-6-methylene-7-octen-4-ol] when exposed to the host tree (*Pinus* spp.) monoterpene, myrcene (Hughes, 1974), these compounds have been found only rarely in *Den-*

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droctonus spp. Hughes (1973) reported that males of *D. brevicomis* LeConte, *D. valens* LeConte, *D. ponderosae* Hopkins, and *D. pseudotsugae* Hopkins produced ipsdienol when exposed to myrcene vapors, but females were apparently not exposed to myrcene, and the behavioral significance of ipsdienol was not investigated. Renwick et al. (1976) and Byers (1982) found that only male *D. brevicomis* produce ipsdienol when exposed to myrcene vapors. Byers (1982) also discovered that this ipsdienol was predominantly the *S*-(+) enantiomer, and that, in the field, racemic ipsdienol significantly reduced the response by *D. brevicomis* of both sexes to a mixture of myrcene, *exo*-brevicomin, and frontalin.

The terpene alcohol myrcenol (2-methyl-6-methylene-octa-2,7-dien-1-ol] was also found in *Dendroctonus* spp. after beetles were exposed to myrcene. *D. brevicomis* of both sexes contained significant amounts of myrcenol after exposure to myrcene vapors (Renwick et al., 1976), and female *D. ponderosae* produced small quantities of myrcenol when fed on pine logs (Conn, 1981); neither study reported whether the compound was *E*- or *Z*-myrcenol or a mixture of both isomers. Conn (1981) found myrcenol to be attractive to *D. ponderosae* of both sexes in laboratory bioassays, but in the field, response to other attractants was slightly inhibited by the presence of *E*-myrcenol (Conn et al., 1983).

Our objectives were to confirm that *D. ponderosae* can produce ipsdienol and myrcenol, to determine which sex(es) produce the compounds, and to identify which geometric isomers of myrcenol or enantiomers of ipsdienol are produced.

METHODS AND MATERIALS

Logs of lodgepole pine, *Pinus contorta* var. *latifolia* Engelmann, infested with the mountain pine beetle were obtained near Princeton in the southwestern interior of British Columbia. The cut ends of the logs were sealed with hot paraffin wax, and the logs were stored at 4°C. To obtain adult *D. ponderosae*, logs containing brood were placed in cages at 27°C. Emergent beetles were collected daily and were stored for a maximum of two weeks on moistened paper toweling at 2–4°C in loosely capped, screw-top jars. When beetles were needed for experiments, the jars were rewarmed to room temperature, and active individuals were selected and their sex determined using the method of Lyon (1958).

D. ponderosae of both sexes were exposed to myrcene by allowing the beetles to bore in fresh lodgepole pine logs for 24 hr or by exposing them to the vapors from a 2-ml vial containing 24 μl of myrcene (>99% pure) in a 500-ml jar for 24 hr. Abdomens from individual insects were excised and extracted in twice distilled pentane. The amounts of ipsdienol and myrcenol pre-

sent in these abdominal extracts were analyzed by gas–liquid chromatography (Hewlett Packard HP5880 A) in the direct injection mode on a glass capillary column (30 m × 0.50 mm ID) coated with SP-1000 (Supelco, Inc., Bellafonte, Pennsylvania). Racemic ipsdienol was obtained from Borregaard, A.S., Sarpsborg, Norway. *E*-Myrcenol was synthesized by the selenium dioxide oxidation of myrcene (Büchi and Wüest, 1967). Analysis of the distilled product (Hewlett Packard 5985B GC-MS) revealed an *E/Z* ratio of 97:3.

The chirality of the ipsdienol produced by individual beetles was determined by the technique developed by Slessor et al. (1986). To a pentane solution (10–30 μl), containing both racemic 3-octanol (4.1 ng/μl) as an internal standard and the insect extract containing >40 ng of ipsdienol, was added a solution of pyridine (50 mg/ml) in ether (15 μl), followed by 25 μl of acetyl-*S*-lactyl chloride reagent (25 mg/ml) in methylene chloride (30 μl) prepared from chirally pure *S*-(+)-lactic acid. The components were mixed, cooled to −20°C, and sealed in a glass ampoule. Ampoules were kept at room temperature overnight. The samples were then diluted with hexane (50 μl), washed by agitation with water (50 μl), and the aqueous phase removed. The organic phase was further washed with aqueous 5% sodium bicarbonate (3 × 50 μl) and once more with water (50 μl). The samples were analyzed by splitless capillary gas chromatography on a Hewlett Packard HP5890 using a 30-m × 0.2-mm ID methyl silicone DB-1 (J&W Scientific, Inc., Rancho Cordova, California) programmed at 60°C for 2 min, 7°C/min to 130°C, 2°C/min to 240°C. Helium carrier gas was used at a flow rate of 1 ml/min, with an injector temperature of 200°C and detector temperature of 250°C. Retention times for the free alcohols, 3-octanol and ipsdienol, were 5.80 and 8.92 min, respectively. The acetyl *S*-(+)-lactic derivatives exhibited retention times of 16.21 and 16.39 min for *R*-(−)- and *S*-(+)-3-octanol, and 19.23 and 19.45 min for *S*-(+)- and *R*-(−)-ipsdienol.

RESULTS

Only male *D. ponderosae* contained detectable levels of ipsdienol (>10 ng/beetle) after exposure to myrcene vapors (Table 1). This ipsdienol was found to be predominantly the *S*-(+) enantiomer (Table 1), with relatively little variation between individuals (Figure 1). However, there was considerable variability in the amount of ipsdienol produced by individual beetles; some produced none at all, while one male contained over 7 μg of the compound (Figure 2). Neither male nor female beetles contained detectable levels of ipsdienol after feeding on *P. contorta*, either alone or with an individual of the other sex. Ipsenol was not produced at any time by either sex.

D. ponderosae of both sexes contained myrcenol, predominantly the *E*-

TABLE 1. QUANTITIES AND ISOMERIC COMPOSITION OF IPSDIENOL AND MYRCENOL PRODUCED BY MALE AND FEMALE *D. ponderosae* WHEN EXPOSED TO MYRCENE THROUGH FEEDING OR VAPORS

Method of exposure to myrcene	Sex	Ipsdienol			Myrcenol		
		No. exposed	ng/beetle $\bar{X} \pm$ SE	% (+) $\bar{X} \pm$ SE	No. exposed	ng/beetle $\bar{X} \pm$ SE	% E $\bar{X} \pm$ SE
Vapors, 24 hr	Male	20	1946 ± 497	97.0 ± 0.3	9	1497 ± 215[a]	90.3 ± 4.0
	Female	17	< 10	—	9	427 ± 119[a]	98.0 ± 0.7
Fed alone for 24 hr on lodgepole pine logs	Male	12	< 10	—	12	< 10	—
	Female	12	< 10	—	12	< 10	—
Fed for 24 hr with an individual of the other sex	Male	12	< 10	—	12	< 10	—
	Female	12	< 10	—	12	< 10	—
No exposure; held for 24 hr at room temperature	Male	12	< 10	—	12	< 10	—
	Female	12	< 10	—	12	< 10	—

[a]Myrcenol content significantly different between sexes, Mann-Whitney U test, $P < 0.01$.

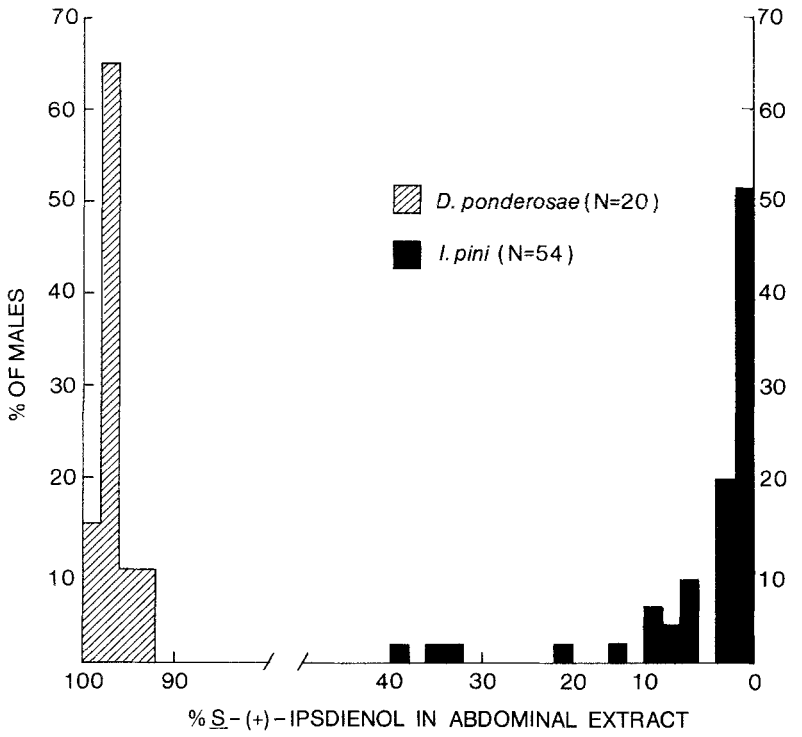

FIG. 1. Chirality of ipsdienol produced by individual male *D. ponderosae* exposed to myrcene vapors and *I. pini* fed on *Pinus ponderosa*. *I. pini* data adapted from Slessor et al. (1986).

isomer, after exposure to myrcene vapors, although males contained significantly more than females (Table 1). Neither males nor females contained detectable levels of myrcenol after feeding on *P. contorta*.

DISCUSSION

In most bark beetles, myrcene is converted to ipsdienol more efficiently if the exposure is through feeding, while α-pinene is converted to *cis*- and *trans*-verbenol more efficiently through exposure to vapors. As a result, ipsdienol has been termed a "frass" pheromone, while *cis*- and *trans*-verbenol are termed "contact" pheromones (Vité et al., 1972). Our data, as well as those presented by Byers (1982) and Hughes (1974), indicate that in *Dendroctonus* spp. myrcene is actually oxidized much more efficiently with vapor exposure than with feeding. Apparently this oxidation is not performed in the same way as in *Ips* spp. This conclusion is in agreement with the hypothesis presented by Vité et

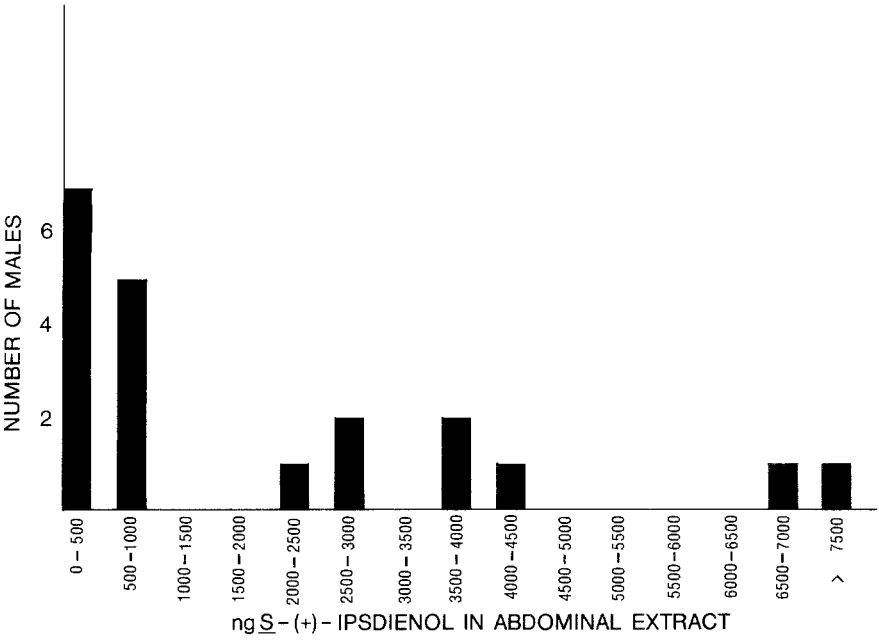

FIG. 2. Variation in *S*-(+)-ipsdienol production by 20 individual male *D. ponderosae*.

al. (1972), suggesting that aggressive bark beetles, such as many *Dendroctonus* spp., begin oxidizing monoterpenes upon initial contact with a new host, while less aggressive species, such as many *Ips* spp., depend on feeding for the conversion of monoterpenes.

The variation in chirality of *S*-(+)-ipsdienol produced by individual male *D. ponderosae* is similar to but not as extensive as the variation in the enantiomeric composition of *R*-(−)-ipsdienol in *Ips pini* (Say) from southeastern British Columbia (Figure 1) (Slessor et al., 1986). Therefore, if ipsdienol proves to be of behavioral significance to *D. ponderosae*, there seems to be less potential than in *I. pini* for natural or artificial selection pressures, such as pheromone-based trapping programs, to select for individuals that utilize different enantiomeric mixtures. The high degree of variation in total ipsdienol content (Figure 2) is in agreement with data on *trans*-verbenol in *D. ponderosae* (Borden et al., 1985), ipsdienol in *I. pini* (Borden et al., 1985) and *cis*-verbenol in *Ips typographus* Linnaeus (Birgersson et al., 1984). Borden et al. (1985) speculated that the few beetles with a capacity for producing very large quantities of terpene alcohol pheromones may be those that are successful pioneer beetles.

There is no overlap in the range of chirality of ipsdienol produced by individual *D. ponderosae* and *I. pini* (Figure 1). Birch et al. (1980) have shown

that *I. pini* from California are attracted to *R*-(−)-ipsdienol, the naturally predominating enantiomer, while *S*-(+)-ipsdienol interrupted the response of *I. pini* to an attractive source in field tests. Therefore, as in the hypothesized interaction between *D. brevicomis* and *I. pini* (Byers, 1982), the *S*-(+)-ipsdienol produced by *D. ponderosae* may function as a repellent allomone, inhibiting the orientation of *I. pini* and thus reducing interspecific competition for the same host. Moreover, the inhibitory effect of racemic ipsdienol on pheromone-positive orientation by *D. brevicomis* (Byers, 1982) suggests that the *R*-(−)-ipsdienol produced by *I. pini* may function reciprocally as a repellent allomone for *D. brevicomis*.

The sex-specific conversion of myrcene vapors to ipsdienol and myrcenol by *D. ponderosae* suggests that either or both products may have behavioral significance within the species. However, with the exception of the work by Byers (1982) on *D. brevicomis*, and Conn (1981) and Conn et al. (1983) on *D. ponderosae*, the oxidation of myrcene by *Dendroctonus* spp., and its possible biological roles, have not been studied. This omission is likely due to the fact that in most pheromone isolation studies beetles are induced to produce pheromones by allowing them to bore in host phloem tissue in cut logs that no longer produce copious amounts of monoterpene-rich resin. Thus, it is not surprising that oxidation products of monoterpene vapors have been largely overlooked. In addition, female *Dendroctonus* spp., which have been studied more intensively than males because they initiate attacks on new host trees, do not appear to oxidize myrcene to the same extent as males. As a result the possible behavioral significance of ipsdienol and myrcenol for *Dendroctonus* spp. has been largely ignored. This topic is presently under study.

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INTERSPECIFIC ACTIVITY OF SEMIOCHEMICALS AMONG SIBLING SPECIES OF *Pissodes* (COLEOPTERA: CURCULIONIDAE)

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Abstract—*Pissodes strobi*, *P. approximatus*, and *P. nemorensis* are sibling species of pine weevils that can hybridize in the laboratory but are presumed to be reproductively isolated in nature. Males of all three species produce the terpenoids grandisol and grandisal; these compounds serve as an aggregation pheromone for *P. approximatus* and *P. nemorensis* when deployed with odors from pine bolts. A series of field experiments examined the possibility of cross-attraction among the three species. Tests in New York and Florida found that parapatrically distributed *P. approximatus* and *P. nemorensis* were cross-attractive, but different photoperiodic conditioning was required for pheromone production in males of the two species. Long-day pheromone production (*P. approximatus*-type) was inherited in interspecific hybrids. Other tests showed that *P. strobi* males, or hybrid males from crosses of *P. strobi* with *P. approximatus*, were not attractive to sympatric *P. approximatus*. When the response of *P. strobi* was assessed to males of either *P. strobi* or *P. approximatus* confined on white pine leaders (the breeding site of *P. strobi*), no evidence of cross-attraction or pheromone activity was found; *P. strobi* were caught in equal numbers on *P. strobi*-baited leaders, *P. approximatus*-baited leaders, and unbaited leaders. Tests of interspecific interactions found that male *P. strobi* produce an allelochemical signal that interrupts the response of *P. approximatus* to its natural or synthetic aggregation pheromone. This interspecific response is apparently adaptive for members of both species (classified as an allomone-kairomone or synomone) because it may ultimately serve to prevent interspecific matings that would lower the fitness of the parents.

Key Words—*Pissodes*, Coleoptera, Curculionidae, aggregation pheromone, cross-attraction, synomone, reproductive isolation, grandisol, grandisal.

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INTRODUCTION

Aggregation pheromones in *Pissodes* weevils were first reported by Booth and Lanier (1974), who found that males of *P. approximatus* Hopkins, the northern pine weevil, feeding on pine bolts would attract large numbers of conspecific males and females, while females feeding on pine bolts and pine bolts alone were not attractive. They also postulated that *P. strobi* (Peck), the white pine weevil, uses a male-produced aggregation pheromone. Booth et al. (1983) reported that males of *P. approximatus* produce two terpenoid compounds, grandisol (*cis*-2-isopropenyl-1-methylcyclobutaneethanol) and its corresponding aldehyde, grandisal, that, together with odor from cut pine logs, serve as an aggregation pheromone. They also found that males of *P. strobi* produce grandisol and grandisal, but repeated field tests have not convincingly documented attraction of *P. strobi* to these compounds (Booth, 1978; Phillips, 1981; Booth et al., 1983). Phillips and coworkers (1984) reported that male Deodar weevils, *P. nemorensis* Germar, also produce grandisol and grandisal and use them with host odors as an aggregation pheromone (cf. Fontaine and Foltz, 1982). No substantial quantitative differences in the production of these compounds among the three species, either in absolute amounts or relative concentrations, have been found (Silverstein and West,[2] unpublished data). Possible differences in enantiomeric composition of grandisol and grandisal released by these species have not been determined.

We consider *P. strobi*, *P. approximatus*, and *P. nemorensis* to be sibling species because they are very similar morphologically but are presumed to be reproductively isolated in nature. These species are genetically very similar (Phillips, 1984), can hybridize with each other in the laboratory (Godwin and ODell, 1967; Smith, 1973), but differ ecologically and behaviorally. *P. strobi* breeds in one-year-old leaders of vigorous young pines, *Pinus* spp., and spruces, *Picea* spp., across North America (MacAloney, 1930; Smith and Sugden, 1969). *P. approximatus* is sympatric with *P. strobi* in northeastern North America, but breeds in the boles, branches, and root collars of weakened or recently cut pines and spruces (Finnegan, 1958). Breeding site separation between *P. strobi* and *P. approximatus* is strong; artificially produced hybrids display reduced reproductive fitness (Phillps and Lanier, 1983). If *P. strobi* and *P. approximatus* use aggregation pheromones that incorporate grandisol and grandisal, we predict that qualitative differences would prevent cross-attraction between the species.

P. nemorensis also breeds in weakened or cut pines, but is distributed in southeastern North America and is presumed to be parapatric with *P. approximatus* (Baker, 1972; Hopkins, 1911). *P. approximatus* and *P. nemorensis* respond to the same synthetic pheromone (Booth et al., 1983; Phillips et al.,

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1984), and it is quite probable that they could be cross-attractive in nature. However, *P. approximatus* and *P. nemorensis* are apparently reproductively active in different seasons (Finnegan, 1958; Fontaine et al., 1983), so cross-attraction in nature may never occur.

Here we report a series of experiments in which we investigated the activity of pheromones and allelochemics among *P. strobi*, *P. approximatus*, and *P. nemorensis*. Using field tests with live weevils, we examined cross-attraction among species, the attractiveness of interspecific hybrids, and the interspecific interruption of pheromone activity. We will assess the importance of pheromones and allelochemics in reproductive isolation and host location, and we will discuss the evolution of semichemicals in these species.

METHODS AND MATERIALS

Field experiments, described in detail below, were conducted in 1982, 1983, and 1985. Traps for assessing the response of either *P. approximatus* or *P. nemorensis* were sticky hardware-cloth cylinders placed directly on the forest floor (design of Booth and Lanier, 1974). Freshly cut pine bolts, about 15 × 25 cm, were enclosed in 30 × 45-cm fiberglass screen bags with or without male weevils and were placed inside the traps. Traps for *P. strobi* (one experiment) were 20 × 91-cm sticky hardware-cloth cylinders with the top ends covered by 20 × 20-cm sticky hardware-cloth squares. These traps were placed on poles supported by metal stakes and suspended over screen-enclosed (15 × 100-cm fiberglass screen sleeves) one-year-old leaders of 3- to 5-m tall eastern white pine trees, *Pinus strobus* L. Male *Pissodes* were then confined on leaders as attractive sources (Phillips, 1981).

Because there are no diagnostic characters for adequately separating specimens of *P. strobi*, *P. approximatus*, and *P. nemorensis*, we initially employed three working hypotheses for the identification of trapped weevils based on geographic locality or ecological qualities of the field site and experiment. Weevils trapped in Florida (experiment 2, see below) were *P. nemorensis* based on distribution. Weevils flying to traps on white pine leaders (experiment 3) in open stands were considered *P. strobi*, while those caught in New York on ground traps containing cut logs (experiments 1, 4–7) were deemed *P. approximatus*. These assumptions are drawn from accumulated information on *Pissodes* behavior, ecology, karyology, and geographic distribution; unfortunately, there is no guarantee that they are true. Therefore, we challenged our designations by performing the discriminant analysis technique of Godwin et al. (1982) on subsamples of the trap-caught specimens (Phillips, 1984). Discriminant analysis corroborated the identification of *P. strobi* and *P. approximatus* by our a priori assumptions, but of weevils that could only be *P. nemorensis* based on geographic definition (experiment 2), a majority were identified

as *P. approximatus*. The utility of the discriminant analysis technique is discussed elsewhere (Phillips, 1984; Phillips et al., 1986).

Weevils used as attractive sources were either laboratory-reared or field-collected; unless otherwise indicated, all were reproductively mature. Females of the three species have certain environmental requirements for reproductive maturity and oviposition (ODell et al., unpublished; Atkinson, 1979; Fontaine et al., 1983). Although males of all three species will produce sperm within a few days of eclosion, Booth et al. (1983) indicated that males of *P. strobi* and *P. approximatus* will not produce pheromone unless they have experienced the same maturation period and conditioning required by females for reproduction. Lab-reared insects were manipulated to ensure reproductive and pheromonal activity (Phillips, 1981), while feral insects were collected on host material during the breeding season and were considered mature and pheromonally active.

RESULTS

Experiment 1. Attraction of wild *P. approximatus* to males of *P. approximatus*, *P. nemorensis*, and *P. strobi* was assessed in a mature red pine, *Pinus resinosa* Ait., stand at Heiberg Memorial Forest (Cortland County, New York) between May 11, and June 18, 1982. This was chosen as the initial test because of the well documented pheromone behavior of *P. approximatus* (Phillips, 1981; Booth et al., 1983). All male weevils were laboratory-reared and conditioned prior to the test. *P. approximatus* and *P. strobi* were fed fresh cuttings of red and white pine, respectively, and held under ambient lighting in an insectary during April and May (increasing from 12 to 15 hr of light per day) at 25–28°C. *P. nemorensis* were fed red pine under 12:12 (light–dark) lighting at 23°C in a growth chamber for at least one month before the test. Experimental treatments consisted of five males of one species confined on a red pine bolt that was then placed in a trap; control traps included a bolt on which no weevils were caged. The four treatments were deployed in five completely randomized blocks of traps, with at least 15 m between traps and 20 m between blocks. Traps were checked weekly and live weevils or fresh bolts were replaced as needed.

Traps baited with *P. approximatus* and *P. nemorensis* males caught significantly more weevils than those baited with *P. strobi* males, which caught no more weevils than uninfested bolts (Figure 1; analysis of variance (ANOVA) followed by Student-Newman-Keuls test, $P < 0.05$). There were no differences in the number of each sex responding to any treatment (t test, $P < 0.05$). There was no marked variation in weekly responses of weevils to *P. approximatus* and *P. nemorensis* traps (Figure 2), which indicates that *P. nemorensis* males continued to produce pheromone after five weeks away from their presumed reproductive photoperiod (12:12, light–dark).

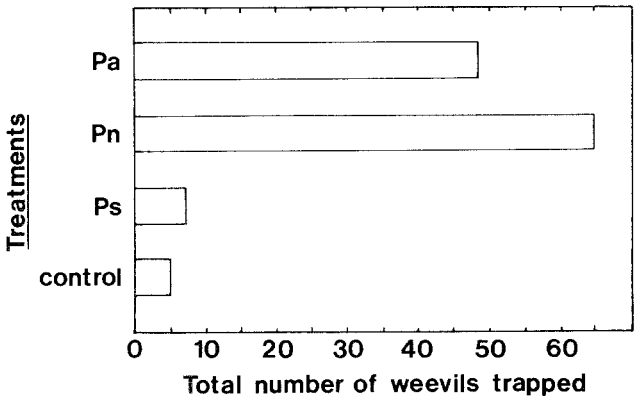

FIG. 1. Total numbers of *P. approximatus* trapped in experiment 1, five traps per treatment. All traps include bolts of red pine. Treatment designations: Pa = five male *P. approximatus*, Pn = five male *P. nemorensis*, Ps = five male *P. strobi*, control = bolt with no weevils.

Experiment 2. A second experiment was conducted in northern Florida from November 12, to December 8, 1982, in which the response of *P. nemorensis* to males of *P. approximatus* and *P. nemorensis* was examined. *P. approximatus* males were laboratory-reared and conditioned under a 16:8 (light–dark) photoperiod at 23°C for one month prior to the test; *P. nemorensis* males were field-collected from split pine billet traps (Fontaine, 1981) and kept in an insectary no more than one week before the test. Five males of each species were caged on freshly cut bolts of slash pine, *Pinus elliottii* Engelm., and bolts

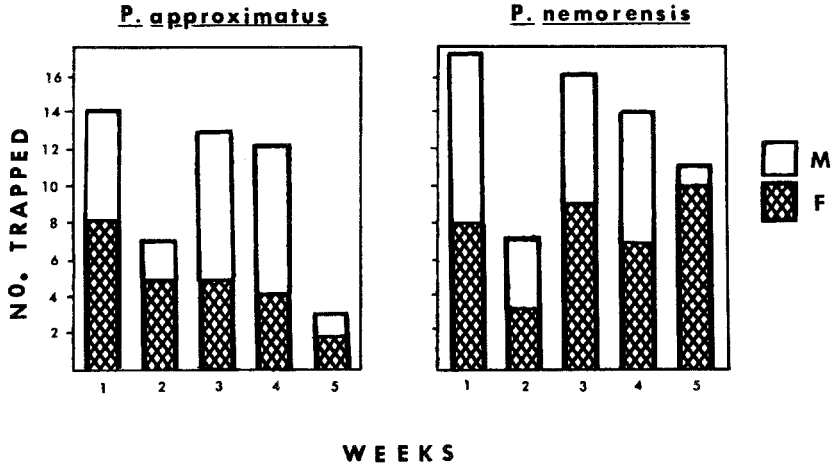

FIG. 2. Total numbers of weevils caught weekly on traps baited with either male *P. approximatus* or male *P. nemorensis* in experiment 1; M = males, F = females.

without weevils served as controls. Five completely randomized blocks, each containing the three treatments, were deployed in a young slash pine plantation west of Newberry, Florida, in Gilchrist County.

The highest responses were to traps baited with males of *P. approximatus* and *P. nemorensis* (ANOVA, $P < 0.10$); only one weevil was caught on a control trap during the three-week period (Figure 3). These results complement those of experiment 1 and lead us to conclude that *P. approximatus* and *P. nemorensis* are cross-attractive and utilize functionally identical aggregation pheromones.

Experiment 3. To further investigate the possibility of cross-attraction between *P. approximatus* and *P. strobi*, sticky traps on eastern white pine leaders were used to determine the attractiveness of males of each species to *P. strobi*. Weevils used as pheromone sources for this test were field-collected from their natural breeding sites in May 1983; *P. strobi* from white pine leaders, and *P. approximatus* from red pine logs. Leaders on randomly selected 3- to 5-m tall white pines at a plantation near Harford, Cortland County, New York, were covered with screen sleeves in April to prevent attacks by native *P. strobi*. Five replicates each of the two male treatments and the blank leader control were distributed in a totally randomized design over the 2-hectare test area; five males per leader were used for the respective baited treatments.

After a three-week period (May 23, to June 13, 1983) all traps had caught weevils, and there were no significant differences among treatments (ANOVA, $P > 0.05$; Figure 4). Although traps baited with males of either *P. approximatus* or *P. strobi* caught equal numbers of weevils, we cannot make any conclusions about cross-attraction. The large number of weevils on control traps

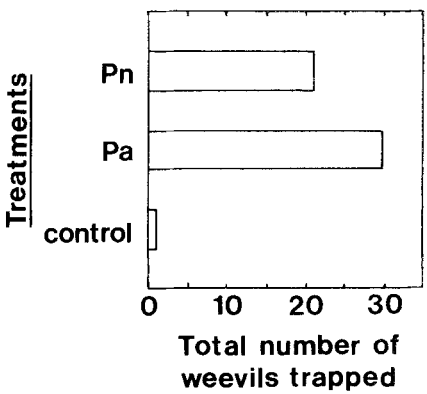

FIG. 3. Total number of *P. nemorensis* trapped in experiment 2, five traps per treatment. All traps included bolts of slash pine. Treatment designations: Pn = five male *P. nemorensis*, Pa = five male *P. approximatus*, control = bolt with no weevils.

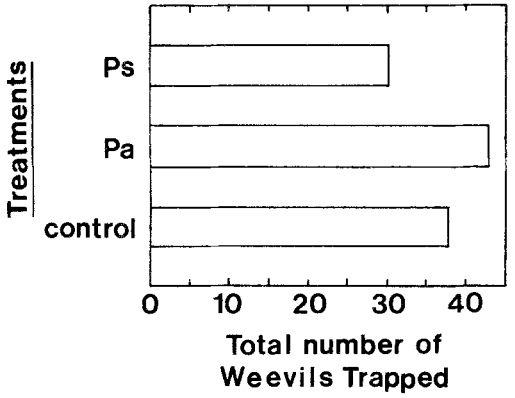

FIG. 4. Total numbers of *P. strobi* trapped in experiment 3, five traps per treatment. All traps included intact leaders of living white pines. Treatment designations: Ps = five male *P. strobi*, Pa = five male *P. approximatus*, control = leader with no weevils.

precludes any discussion of possible pheromone activity for either of the male-baited treatments.

Experiment 4. This test examined the effects of site exposure and host tree species on the attractiveness of male *P. strobi* and *P. approximatus* to feral *P. approximatus*. Bolts of eastern white pine, rather than red pine, were used as host material. Sites were chosen at Heiberg Forest so that traps could be placed in a shaded, closed-canopy stand and at a nearby sunny, open canopy or open field area. Ground traps were deployed at five sun/shade sites as paired randomized blocks in an unbalanced design. Preferred host trees for both weevil species, i.e., red pine, white pine, and Norway spruce, *Picea abies* (Karst), were available at all sites (see Smith and Sugden, 1969). Site exposure was varied because each species is known to prefer certain types of sites: *P. strobi* predominantly attacks trees in sunny, open-growth stands (MacAloney, 1930), and *P. approximatus* oviposits on the undersides of logs, generally in shaded conditions (Finnegan, 1958; Hard, 1962). Weevils used as baits (five males per bolt) were field-collected; limited numbers of male *P. approximatus* prevented equal replication of treatments.

The results after a four-week period (May 25, to June 27, 1983) indicated that the *P. approximatus* males were attractive when feeding on white pine bolts in either sunny or shaded conditions (Figure 5); traps baited with *P. strobi* males or bolts alone caught lower numbers of weevils in both shaded and sunny sites (ANOVA performed separately on sun and shade blocks, $P < 0.05$). These data are similar to those in experiment 1 in which *P. strobi* was on red pine bolts in a shaded site. *T*-test comparisons between the same treatments in sun and shade found no significant differences ($P > 0.05$).

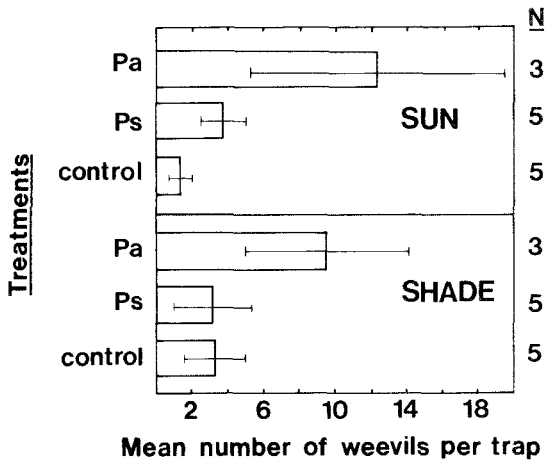

FIG. 5. Mean numbers of *P. approximatus* caught on traps in adjacent sun and shade blocks in experiment 4; N = number of traps per treatment. All traps included bolts of white pine. Treatment designations: Pa = five male *P. approximatus*, Ps = five male *P. strobi*, control = bolt with no weevils. Horizontal lines indicate standard errors of the means.

Experiment 5. The heritability of *P. approximatus* pheromone production in various types of hybrid males was examined in this experiment. Laboratory hybrids were obtained from the reciprocal crosses of *P. approximatus* with *P. nemorensis* and of *P. approximatus* with *P. strobi*. Pure laboratory strains of *P. approximatus* and *P. nemorensis* were also used; lab-reared *P. strobi* were not available for this test. Six different male-baited treatments and a control were deployed in an incomplete randomized block design at Heiberg Forest from June 6 to 28, 1983; red pine bolts were the host material, and five males were caged with each bolt. Limited numbers of progeny from various crosses prevented us from having equal sample sizes. All treatment weevils, including *P. nemorensis* and hybrids, were preconditioned in an insectary at least one month under the naturally occurring increasing spring photoperiod (14–15 hr of light) at 25–28°C. Therefore, *P. nemorensis* males were not exposed to conditions for reproductive maturation, but rather to the conditions for reproductive maturation of *P. approximatus* and *P. strobi*.

The largest catches were on *P. approximatus*-baited traps and the two *P. approximatus* × *P. nemorensis* hybrid treatments (Figure 6). *P. nemorensis*, both *P. approximatus* × *P. strobi* hybrids, and a blank bolt caught lower numbers of weevils. We presume that *P. nemorensis* males were not attractive because they were not conditioned for pheromone production. Like host selection behavior (Phillips and Lanier, 1983), pheromone production apparently breaks down in hybrids of *P. approximatus* and *P. strobi*. Significant differences oc-

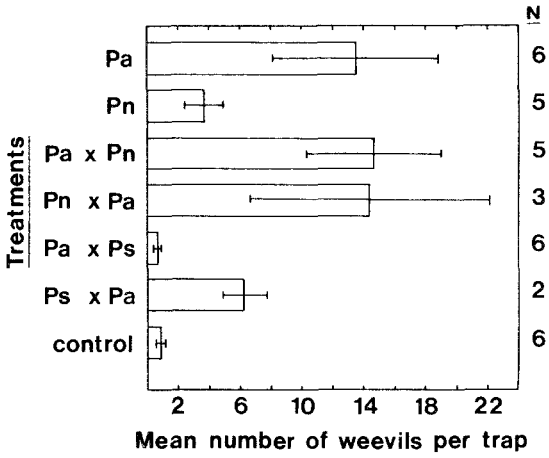

FIG. 6. Mean numbers of *P. approximatus* caught on traps in experiment 5; N = number of traps per treatment. All traps included bolts of red pine. Treatment designations: Pa = five male *P. approximatus*, Pn = five male *P. nemorensis*, Pa × Pn = five male progeny from the cross of female Pa with male Pn, Pn × Pa = five male progeny from the cross of female Pn with male Pa, Pa × Ps = five male progeny from the cross of female Pa with male *P. strobi* (Ps), Ps × Pa = five male progeny from the cross of female Ps with male Pa, control = bolt with no weevils. Horizontal lines indicate standard errors of the means.

curred among treatment means (ANOVA, $P < 0.05$), but any direct comparison of means is unreliable because of the unbalanced design of the experiment.

Experiment 6. In this experiment we investigated the possibility that, in addition to simply being unattractive to *P. approximatus*, *P. strobi* males may actively prohibit the flight of *P. approximatus* to suitable host material. Freshly cut white pine bolts and field-collected male weevils (five per bolt) were used in ground traps in a mature red pine stand at Heiberg Memorial Forest. The response of *P. approximatus* to the following treatments, arranged in five completely randomized blocks, was assessed from May 10 to 22, 1985: five male *P. approximatus* on a bolt, five male *P. strobi* on a bolt, five male *P. approximatus* together with five male *P. strobi* on a bolt, and a bolt alone as a control.

The highest responses were to traps baited with male *P. approximatus*, while the other three treatments caught significantly lower numbers of weevils (ANOVA followed by Student-Newman-Keuls test, $P < 0.05$; Figure 7). As in experiments 1 and 4, male *P. strobi* were not attractive to *P. approximatus*. However, it is quite clear from this experiment that male *P. approximatus* do not attract conspecifics when male *P. strobi* are confined with them on the same pine bolt. It is possible that male *P. strobi* produce a chemical signal that interrupts the response of *P. approximatus* to its natural pheromone, or that the

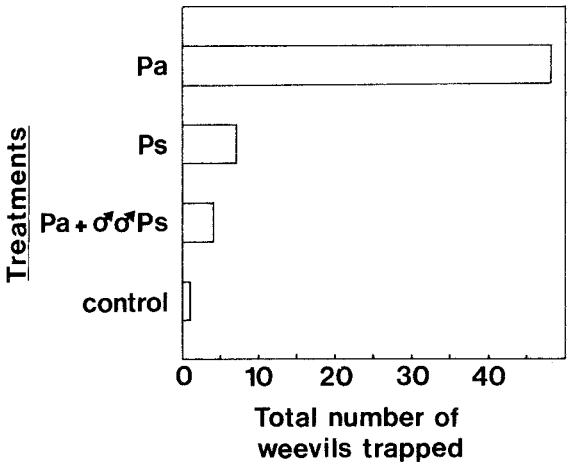

FIG. 7. Total number of *P. approximatus* trapped in experiment 6, five traps per treatment. All traps contained bolts of white pine. Treatment designations: Pa = five male *P. approximatus*, Ps = five male *P. strobi*, Pa + ♂♂ Ps = five male *P. approximatus* with five male *P. strobi*, control = bolt with no weevils.

presence of male *P. strobi* on the same bolt somehow suppresses pheromone production in male *P. approximatus*.

Experiment 7. Our last field experiment examined the possible interruption of synthetic *P. approximatus* pheromone by male *P. strobi*. Ground traps with fresh white pine bolts were used in a mature red pine stand at Heiberg Memorial Forest from May 22 to June 21, 1985. Three treatments, deployed in five completely randomized blocks, were compared for their attractiveness to *P. approximatus*: synthetic racemic grandisol and grandisal with a pine bolt (*P. approximatus* pheromone), grandisol and grandisal with seven field-collected male *P. strobi* feeding on a pine bolt, and an unbaited pine bolt as a control. Racemic grandisol was obtained commercially and grandisal was synthesized from it (method of Booth et al., 1983). Grandisol (5 mg/bait) and grandisal (10 mg/bait) were evaporated from rubber septa at the levels reported by Phillips and coworkers (1984).

The addition of male *P. strobi* to traps baited with the aggregation pheromone of *P. approximatus* depressed the response of *P. approximatus* (Figure 8). The highest response was to traps baited with grandisol and grandisal, but this was reduced by almost half (ANOVA, Student-Newman-Keuls test, $P < 0.10$) in traps containing grandisol, grandisal, and male *P. strobi*; both these treatments caught significantly more weevils ($P < 0.05$) than the control. These data suggest that pheromone production by *P. approximatus* was not suppressed in experiment 6, but confirm that *P. strobi* males produce an allelochemical signal that blocks the pheromone response of *P. approximatus*. We believe that

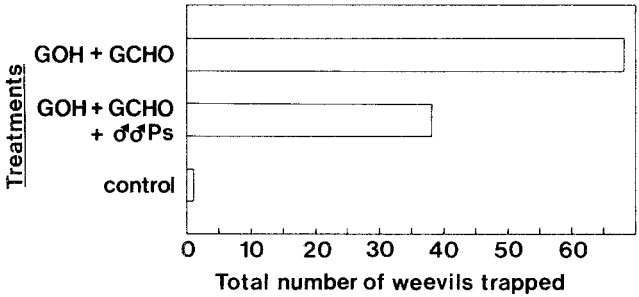

FIG. 8. Total numbers of *P. approximatus* trapped in experiment 7, five traps per treatment. All traps contained bolts of white pine. Treatment designations: GOH + GCHO = pheromone bait of grandisol and grandisal, GOH + GCHO + ♂♂ Ps = pheromone bait of grandisol and grandisal with seven male *P. strobi*, control = bolt with no weevils or bait.

male *P. strobi* could not totally interrupt the response of *P. approximatus* in this experiment because the high levels of synthetic pheromone release (about 1.0 mg/day), compared to natural release rates from male weevils (about 20 ng/day; Booth, et al., 1983), probably overwhelmed the blocking odor produced by *P. strobi*.

DISCUSSION

We can draw four conclusions about weevil-produced semiochemicals in these sibling species: (1) *P. approximatus* and *P. nemorensis* are cross-attractive and apparently use functionally identical aggregation pheromones. (2) Different environmental stimuli (e.g., photoperiod) are prerequisite for pheromone production in *P. approximatus* and *P. nemorensis*; long-day pheromone production (*P. approximatus*-type) is heritable in interspecific hybrids. (3) *P. strobi* males are not attractive to *P. approximatus*, the reciprocal relationship is not clearly supported. (4) *P. strobi* males produce an allelochemical signal that can interrupt the response of *P. approximatus* to its natural or synthetic aggregation pheromone.

If *P. approximatus* and *P. nemorensis* are biologically distinct species (i.e., no gene flow between them) that are geographically contiguous but potentially cross-attractive, there must be some mechanism preventing introgression at the zone of parapatry. The distributions of the two species are not well defined, and their designation as separate species has more of an historical rather than a biological meaning (see species descriptions by Germar, 1824; and Hopkins, 1911). There are no geological barriers that might separate their distributions, and suitable host conifers for both species are continuously distributed from

north to south in the eastern United States. Seasonal isolation between *P. approximatus* and *P. nemorensis* may prevent hybridization in nature, despite the potential for cross-attraction. *P. nemorensis* is flying and reproductively active during the fall and winter months (Atkinson, 1979; Fontaine et al., 1983), while *P. approximatus* is active in the spring and summer. In another study (Phillips et al., 1986), we found that weevils responded to synthetic pheromone in both spring and fall seasons at one site in Virginia, which led us to question the validity of *P. approximatus* and *P. nemorensis* as separate species. As with reproductive development, pheromone activity is apparently dependent on environmental stimuli like photoperiod (experiment 5; Booth et al., 1983). Once the threshold level of conditioning for pheromone production has been crossed (e.g., about 30 days of short-day conditioning for *P. nemorensis*), the phenomenon cannot be reversed by a drastic change in conditions (experiment 1, Figure 2). This threshold phenomenon is similar to that found in studies of diapause with various insect species (Tauber and Tauber, 1976). Our finding that hybrid males of *P. approximatus* and *P. nemorensis* produce pheromone after long-day conditioning (Figure 6) allows for a model of conspecificity that entails successful introgession of southern with northern populations.

P. strobi males or interspecific hybrids are not attractive to *P. approximatus* (experiments 1, 4–6), despite the fact that males of both species produce the pheromone components grandisol and grandisal (Booth et al., 1983). The basis for this lack of cross-attraction lies in the fact that *P. strobi* males apparently produce one or more chemicals that block the response of *P. approximatus* to its pheromone (experiments 6 and 7); the ability to produce this blocking odor is probably inherited in hybrids (experiment 5). Intra- and interspecific interactions in several species of insects are known to be mediated by enantiomeric specificity of semiochemicals (Silverstein, 1979). Since grandisol and grandisal are chiral, we might presume that the blocking phenomenon is imparted by species differences in enantiomeric blends of these compounds. However, we do not believe that the blocking phenomenon results from enantiomers of grandisol and grandisal because *P. approximatus* responds optimally (i.e., greater than or equal to the response to conspecific males) to racemic mixtures of these compounds. Theoretically, *P. approximatus* males may produce and respond to an enantiomeric blend of these compounds, or they may produce just one enantiomer of each (or a blend predominated by one enantiomer) and the other enantiomer is inactive. Even if *P. strobi* produced just one enantiomer each of grandisol and grandisal, it is unlikely that they would act allomonally and block the natural enantiomeric constitution of the *P. approximatus* pheromone. In other cases in which sympatric species display dual activity of enantiomeric semiochemicals, either separate enantiomers serve as a pheromone and an allomone for each species (e.g., ipsdienol for *Ips pini* and *I. paraconfusus*; Birch et al., 1980), or a synergistic blend of enantiomers is required by one species for pheromonal and allomonal activity, while the other species uses just

one enantiomer as a pheromone (e.g., sulcatol for *Gnathotrichus sulcatus* and *G. retusus*; Borden et al., 1980). All possible and probable types of enantiomeric specificity are reviewed by Silverstein (1979).

If our hypothesis above is correct, then male *P. strobi* must produce one or more other chemicals that block the response of *P. approximatus* to grandisol and grandisal. We have no evidence that *P. strobi* males produce an aggregation pheromone when feeding on white pine leaders (experiment 3; Phillips, 1981; Booth, 1978), and grandisol and grandisal, although produced by this species, are no more attractive to it than are white pine leaders (Booth et al., 1983; Phillips, unpublished data). It is possible that host location in *P. strobi*, which results in the finding of mates on suitable host leaders, is mediated primarily by host-related stimuli (e.g., Harris et al., 1983; VanderSar and Borden, 1977a,b). If the grandisol and grandisal produced by male *P. strobi* are qualitatively identical to the same compounds used as a pheromone by *P. approximatus*, then *P. strobi* would attract *P. approximatus* if it did not produce a blocking odor. We hypothesize that the evolution of a novel allelochemical signal in *P. strobi* was adaptive in the divergence and reproductive isolation of these two species. The blocking odor serves to prevent hybrid matings rather than to prevent competition for resources, a phenomenon known to occur in interspecific interactions of some bark beetles attacking the same host (Wood, 1982). *P. strobi* and *P. approximatus* use very different host resources. Any accidental interspecific matings would result in fertile hybrid progeny that would be unsuccessful in colonizing host material of either *P. strobi* or *P. approximatus*, thus reducing the fitness of both parents (Phillips and Lanier, 1983). *P. approximatus* aggregates on suitable host material in response to its aggregation pheronome; *P. strobi* aggregates on suitable host material via mechanisms that are not fully understood, and it produces a blocking odor to keep *P. approximatus* away. Because the blocking odor facilitates reproductive isolation and is adaptively favorable to individuals of both species, it could be referred to as an allomone-kairomone (Borden, 1977) or a synomone (Nordlund and Lewis, 1976).

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ELECTROANTENNOGRAM RESPONSES BY MOUNTAIN PINE BEETLES, *Dendroctonus ponderosae* HOPKINS, EXPOSED TO SELECTED SEMIOCHEMICALS

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Abstract—Electroantennograms (EAGs) were obtained for *D. ponderosae* to the bark beetle pheromones *trans*-verbenol, *cis*-verbenol, *exo*-brevicomin, *endo*-brevicomin, frontalin, verbenone; to the kairomones, α-pinene, β-pinene, camphene, Δ-3-carene, limonene, myrcene; to a blend (1 : 1 : 1) of *trans*-verbenol, *exo*-brevicomin, and myrcene; and to diacetone-alcohol. Male and female responses, in general, did not differ significantly over the whole EAG dose–response curves but differed at a few concentrations on many of the curves. There were more differences noted for pheromones than kairomones. The blend yielded among the largest EAGs in both sexes and appeared to show synergism. Responses of females were lower than those for males in most instances. Significant differences in responses by the two sexes were much fewer for the kairomones than the pheromones. EAG recovery rates tested at only one concentration showed significant differences between males and females for three pheromones, *trans*-verbenol, *cis*-verbenol, and verbenone, and two kairomones, camphene and Δ-3-carene. Thresholds were quite low for most of the odorants except *cis*-verbenol, camphene, verbenone, and diacetone-alcohol in females, and *cis*-verbenol, verbenone, α-pinene, and diacetone-alcohol in males. The results, using at least one EAG parameter, support behavioral and field studies involving *exo*-brevicomin, *trans*-verbenol, frontalin, and the blend.

Key Words—Mountain pine beetle, *Dendroctonus ponderosae*, Coleoptera, Scolytidae, electroantennograms, pheromones, kairomones, inhibitors, host selection, aggregation.

INTRODUCTION

The mountain pine beetle (MPB), *Dendroctonus ponderosae* Hopkins, colonizes host trees in response to a complex blend of semiochemicals of both insect

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and host origin (Vité and Gara, 1962; McCambridge, 1967; Pitman and Vité, 1969). Pitman et al. (1968) identified *trans*-verbenol as the main female-produced pheromone, and Pitman et al. (1969) found large amounts of brevicomin (not identified as *exo*- or *endo*-brevicomin) in the hindguts of feeding males.

Field studies of MPB semiochemicals are extensive. Host colonization by the beetles is dependent upon the interaction of host volatiles and beetle-produced pheromones (Pitman et al., 1968; McKnight, 1979). *trans*-Verbenol, although ineffective by itself (Pitman and Vité, 1969; Billings, 1974), is an effective aggregative pheromone when used in conjunction with the host-tree terpenes α-pinene and especially myrcene (Merrified, 1972; Billings, 1974; Billings et al., 1976; Pitman et al., 1978). Recent studies (Borden et al., 1983a, b; Conn et al., 1983) have shown that a blend of *trans*-verbenol, myrcene, and *exo*-brevicomin is highly effective in attracting beetles of both sexes and mediating mass attack on several hard-pine species in British Columbia.

There is a need to investigate chemicals found to be associated with the MPB and its host, lodgepole pine, on a neurophysiological basis. Until now no studies have been made on the perception of MPB semiochemicals at this level. This paper presents the results of an experiment in which electroantennograms (EAGs) were obtained from beetles of both sexes exposed to selected single semiochemicals (mostly racemic) and a blend similar to the one cited above. An attempt is made to correlate the results to behavioral and field studies by other workers.

METHODS AND MATERIALS

Adult beetles were obtained from laboratory-infested bolts of lodgepole pine, *Pinus contorta* var. *latifolia* Engelmann, cut in Logan Canyon, Utah. After emergence, the beetles were sexed according to a method described by Lyon (1958) and placed on moist strips of filter paper in Petri dishes at 5°C until use within 30 days after emergence.

Electroantennogram (EAG) techniques were modified from those described by Dickens et al. (1983). Ag–AgCl capillary electrodes were filled with insect saline (Yamasaki and Narahashi, 1959). A technique modified from Payne (1970) and Angst and Lanier (1979) was used to secure the beetle. Whole beetles were inserted in an appropriately sized hole drilled in a Plexiglas block. A thin brass plate drilled with a hole just larger than the maximal diameter of the largest beetle's head was attached to the top of the block so that the hole could be swiveled over the test-beetle's head. The plate was then pushed back against the ventral side of the head capsule forcing the head acutely backwards. This action secured the beetle in the block. A narrow strip of double-sticky tape was placed on the plate, and the antenna was pressed lightly onto its surface. The

TABLE 1. SOURCES AND PURITIES OF SEMIOCHEMICALS USED

Compound	Source of supply	Purity (%)[a]
trans-Verbenol	A[b]	90.5
cis-Verbenol	B	89
exo-Brevicomin	B	100
endo-Brevicomin	B	97.7
Frontalin	B	99.6
Verbenone	B	97.3
Diacetone-alcohol	C	98.1
(+)-α-Pinene	C	98.4
(−)-β-Pinene	C	98
Camphene	C	91.7
Δ-3-Carene	D	89.9
(+)-Limonene	C	97.5
Myrcene	D	89.4

[a] Purity of compounds determined by gas chromatography.
[b] A, PMG/Stratford Products, Vancouver, British Columbia B, Albany International, Willoughby, Ohio; C, Aldrich Chemical Co., Milwaukee, Wisconsin; D, SCM Terpene Products, Jacksonville, Florida.

recording electrode was inserted into the distal end of the antennal club following puncture with a sharpened tungsten needle. The indifferent electrode was inserted into the mouth. The semiochemicals (Table 1) were diluted in *n*-pentane in decade steps. Ten microliters of test solution were applied to a slightly folded 6 × 20-mm filter-paper strip and placed in a glass cartridge (80 mm long, 5 mm ID). After loading, the cartridge was immediately inserted into the dispenser apparatus for application to the insect. The dispenser was constructed to deliver a 1-sec stream of filtered air through the cartridge at 1.7 liter/min. The cartridge tip was placed about 1 cm from the preparation. At least 4 min elapsed between stimulations. Five replicates (different beetles) for each sex were made at each concentration. Most preparations would last 2–3 hr without degradation, thus allowing a full concentration series for one chemical to be tested. Stimulus dilutions were presented in order from the lowest to the highest concentration. *n*-Pentane (10 μl on filter paper) was used as control, and frontalin (10 μg on filter paper) was used as a standard.

Electrical responses were amplified by a Grass P-16 amplifier connected to an oscilloscope and an Apple II computer which digitized the responses via an 8-bit analog to digital converter board (Mountain Hardware), and plotted them [using a modified Waveman™ program (original available from Stolting Co., Chicago, Illinois) supporting an HP-7470A plotter] as regular EAGs on graph paper. EAGs were measured for amplitude and were shown as a percent of the mean of the two standards applied before and after two applications of

test chemicals. Any responses to air or *n*-pentane were subtracted from those of the odorants (Dickens et al., 1983). Threshold was considered as the lowest stimulus concentration at which the lower limit of its standard error did not overlap the upper limit of the standard error for the responses, if any, at lower concentrations (Dickens, 1978). Tests of the responses for significant differences between sexes for each chemical and between chemicals for each sex were made using Duncan's multiple range test at each concentration.

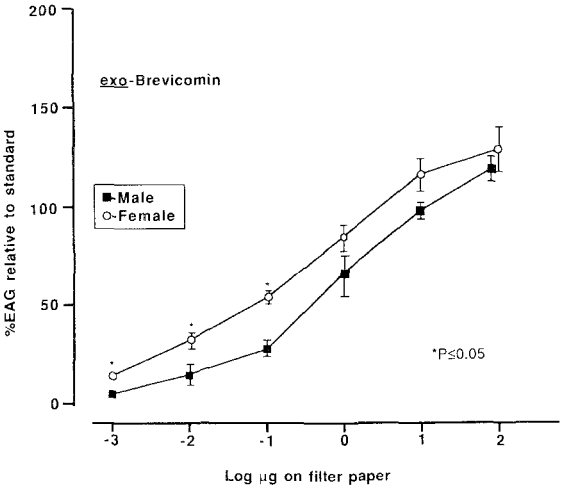
Only 10-μg applications of each chemical were quantified to produce recovery rates which were measured by having the computer calculate the total areas enclosed by the EAG traces and baselines as a percentage compared to those of the 10-μg frontalin standards.

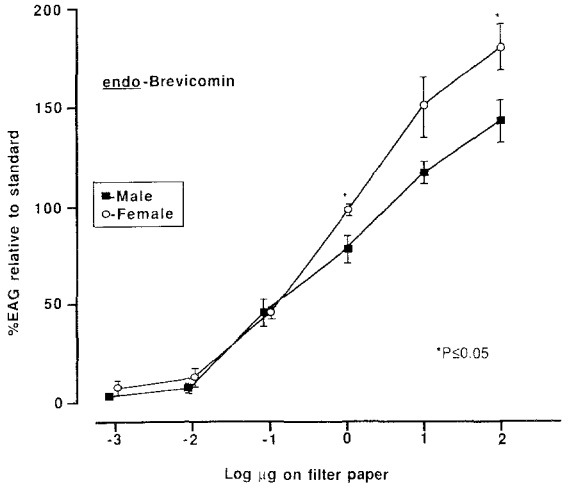
Sample purity (Table 1) was determined using an HP-5880A gas chromatograph fitted with a 20-m $\times$ 0.3-mm ID SE-54 capillary column. The temperature program initial value was set at 40°C for 2 min, increasing at the rate of 4°C/min until 150°C final temperature was reached.

RESULTS AND DISCUSSION

The EAGs recorded in response to the test chemicals were similar to those from other bark beetles (Angst and Lanier, 1979; Grant and Lanier, 1982; Dickens, 1981; Dickens et al., 1983). An increase in stimulus concentration yielded an increase in peak response amplitude (Figures 1–14) and a slower recovery rate. Saturation did not appear to be reached at the highest concentration of any of the chemicals. Shapes of the dosage–response curves were very similar for a given compound for each sex, which may indicate that the receptor mechanisms may be similar for each compound in both sexes (Dickens et al., 1983). The pentane controls produced responses of 10.7 $\pm$ 1.2% of the frontalin standards. Few responses to air were recorded.

In general, EAG amplitude of males versus females did not differ significantly over the entire concentration range of any one compound; however, in several instances a significant difference in response was noted for individual concentrations of most of the pheromones (Figures 1–6) and only one concentration of the kairomone α-pinene (Figure 8). Many of the sexual differences occurred at the higher concentrations, and with the exception of the brevicomins (Figures 1 and 2), female responses at the higher concentrations were lower than the male responses. Females had significantly higher responses to *exo*-brevicomin than males at lower concentrations (Figure 1). This supports findings of Rudinsky et al. (1974) from laboratory-walkway experiments and Conn et al. (1983) from field-trapping studies that females are preferentially attracted over males to lower concentrations of *exo*-brevicomin in combinations with





FIGS. 1 and 2. Dosage–response curves made from EAGs of MPB males (solid squares) and females (open circles) to serial dilutions of semiochemicals. $N = 5$; error bars indicate $\pm$SEM. *Significant difference between male and female response at 0.05 level.

other semiochemicals; at higher concentrations, females are inhibited. Significant differences between sexes were not noted for the other kairomones.

Dickens (personal communication) suggested that EAG recovery rates (represented as %EAG areas in Figures 15 and 16) may reflect the size of the

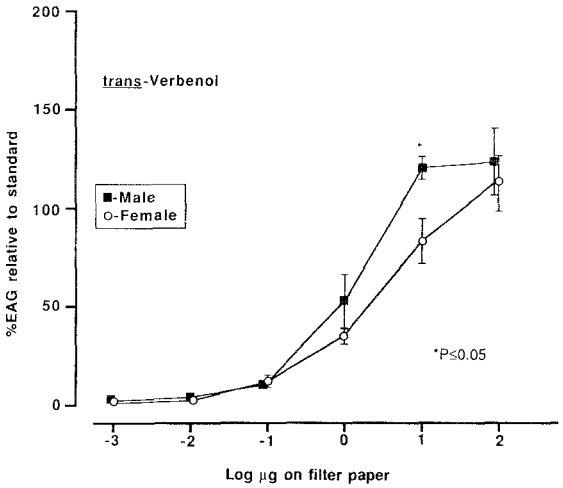


FIG. 3. Dosage–response curves made from EAGs of MPB males (solid squares) and females (open circles) to serial dilutions of *trans*-verbenol. N = 5; error bars indicate ±SEM. *Significant difference between male and female response at 0.05 level.

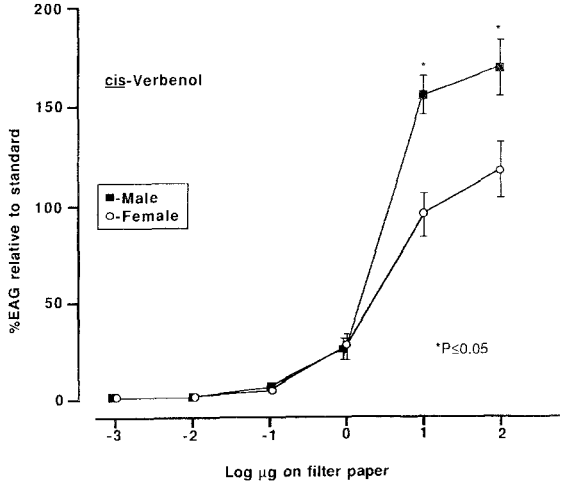


FIG. 4. Dosage–response curves made from EAGs of MPB males (solid squares) and females (open circles) to serial dilutions of *cis*-verbenol. N = 5; error bars indicate ±SEM. *Significant difference between male and female response at 0.05 level.

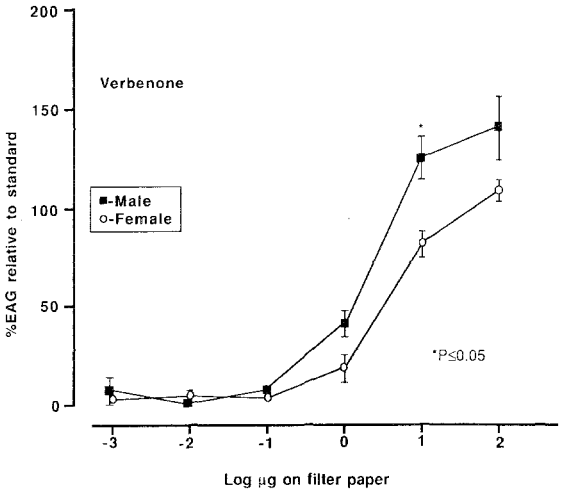


FIG. 5. Dosage–response curves made from EAGs of MPB males (solid squares) and females (open circles) to serial dilutions of verbenone. N = 5; error bars indicate ±SEM. *Significant difference between male and female response at 0.05 level.

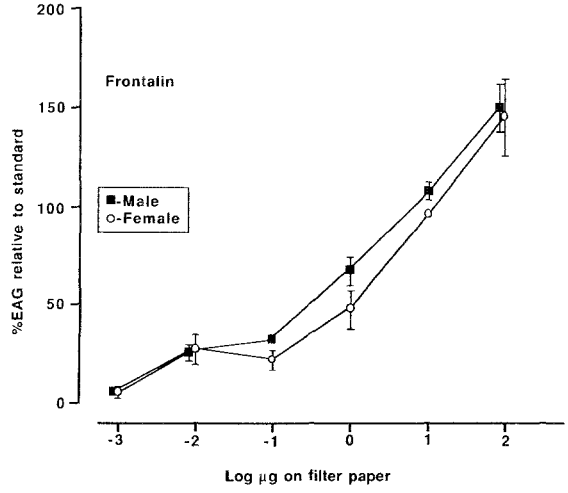


FIG. 6. Dosage–response curves made from EAGs of MPB males (solid squares) and females (open circles) to serial dilutions of frontalin. N = 5; error bars indicate ±SEM. *Significant difference between male and female response at 0.05 level.

FIG. 7. Dosage–response curves made from EAGs of MPB males (solid squares) and females (open circles) to serial dilutions of diacetone-Alcohol. N = 5; error bars indicate ±SEM. *Significant difference between male and female response at 0.05 level.

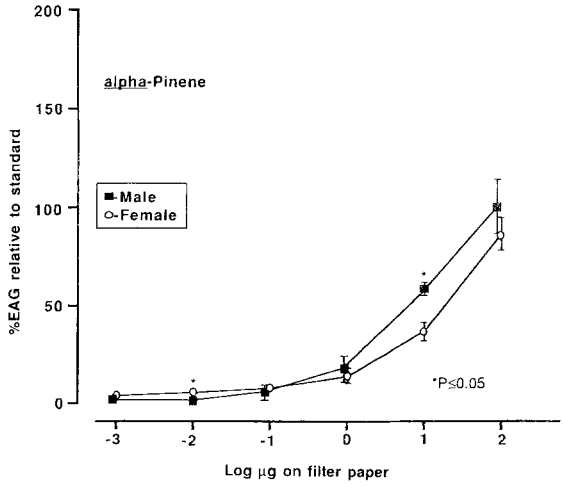


FIG. 8. Dosage–response curves made from EAGs of MPB males (solid squares) and females (open circles) to serial dilutions of *alpha*-pinene. N = 5; error bars indicate ±SEM. *Significant difference between male and female response at 0.05 level.

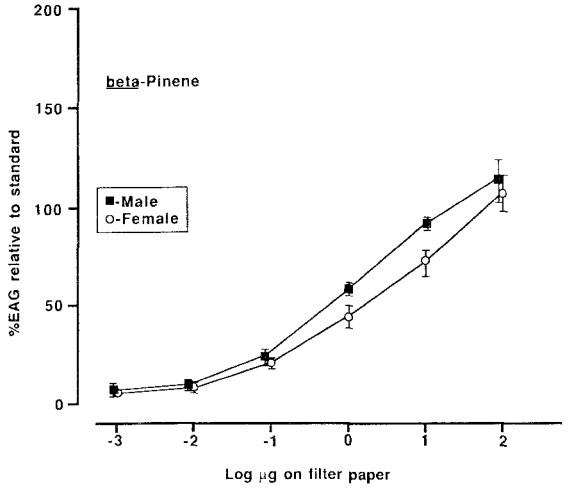


FIG. 9. Dosage–response curves made from EAGs of MPB males (solid squares) and females (open circles) to serial dilutions of *beta*-pinene. N = 5; error bars indicate ±SEM. *Significant difference between male and female response at 0.05 level.

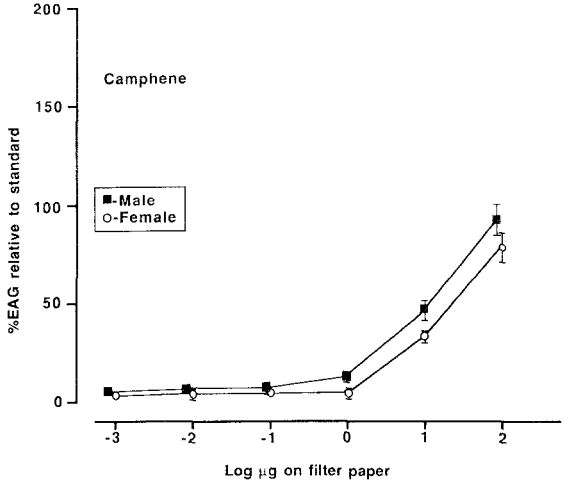


FIG. 10. Dosage–response curves made from EAGs of MPB males (solid squares) and females (open circles) to serial dilutions of camphene. N = 5; error bars indicate ±SEM. *Significant difference between male and female response at 0.05 level.

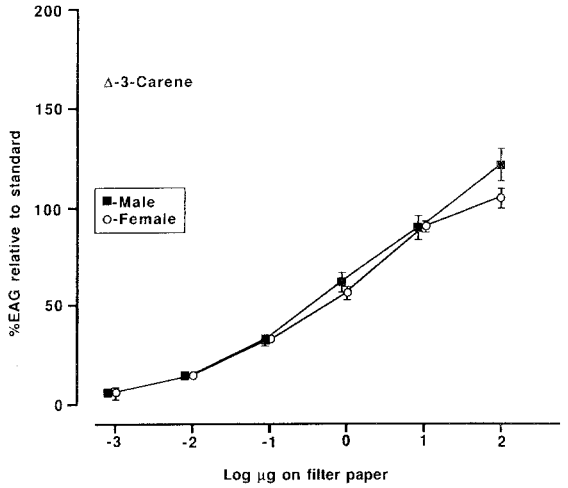


FIG. 11. Dosage–response curves made from EAGs of MPB males (solid squares) and females (open circles) to serial dilutions of -3-carene. N = 5; error bars indicate ±SEM. *Significant difference between male and female response at 0.05 level.

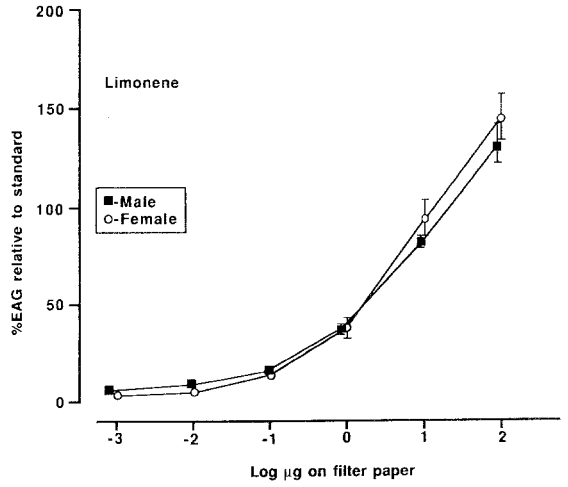


FIG. 12. Dosage–response curves made from EAGs of MPB males (solid squares) and females (open circles) to serial dilutions of limonene. N = 5; error bars indicate ±SEM. *Significant difference between male and female response at 0.05 level.

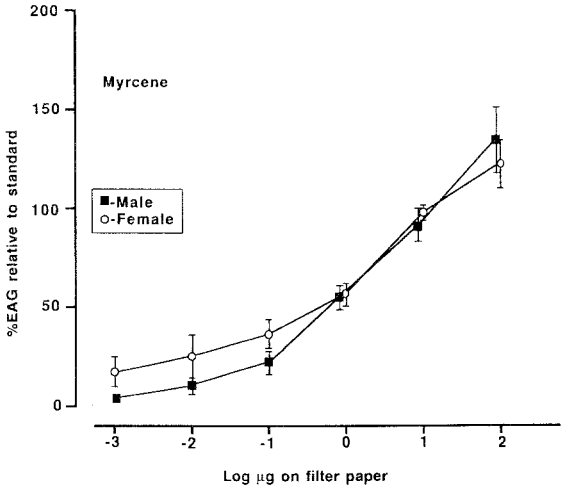


FIG. 13. Dosage–response curves made from EAGs of MPB males (solid squares) and females (open circles) to serial dilutions of myrcene. N = 5; error bars indicate ±SEM. *Significant difference between male and female response at 0.05 level.

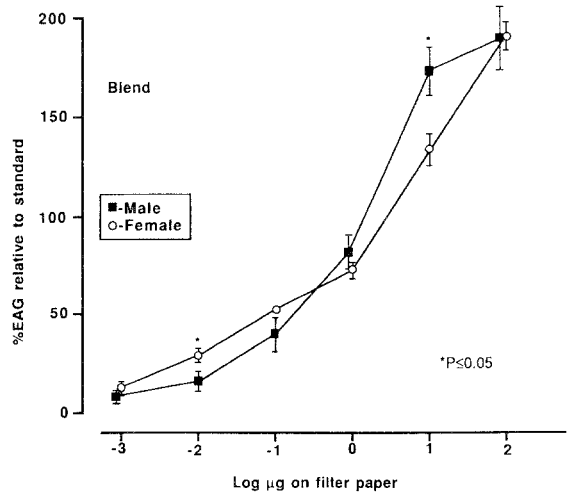


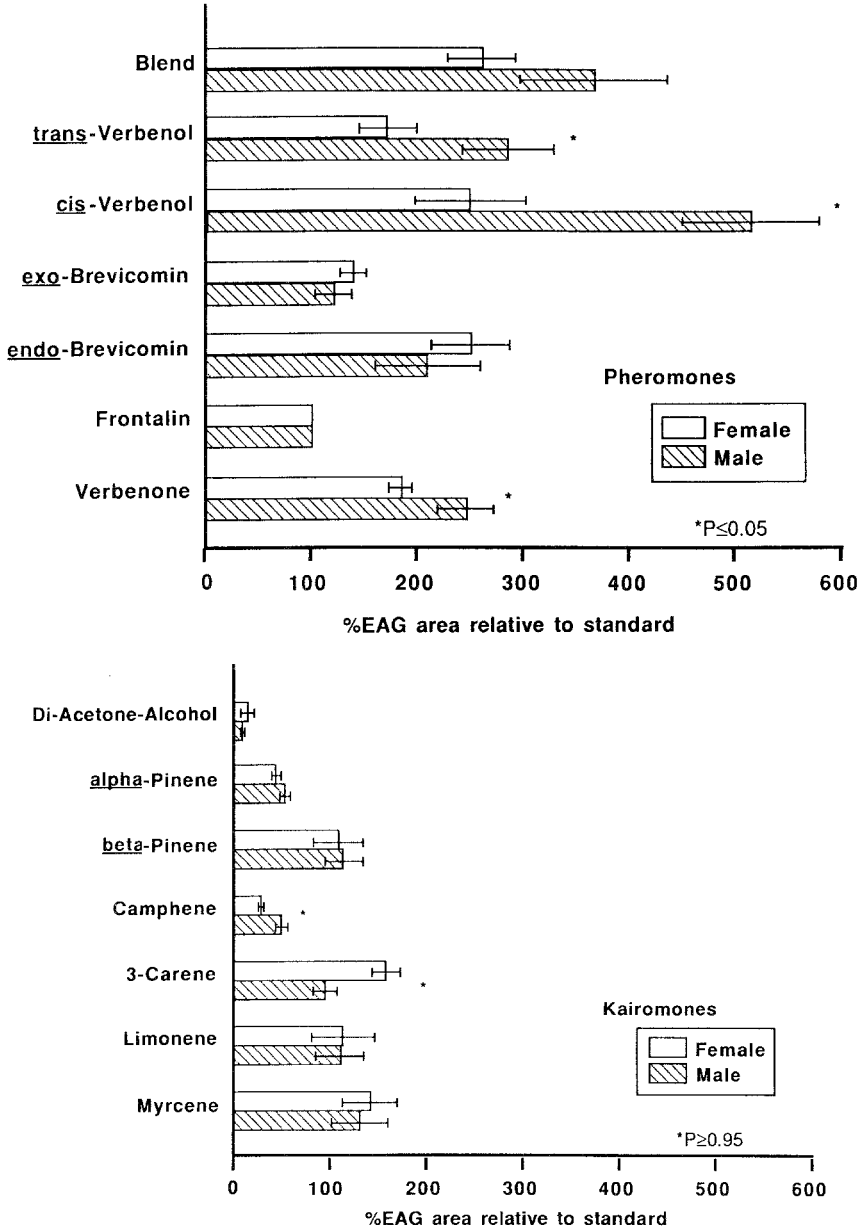
FIG. 14. Dosage–response curves made from EAGs of MPB males (solid squares) and females (open circles) to serial dilutions of blend. N = 5; error bars indicate ±SEM. *Significant difference between male and female response at 0.05 level.

receptor population. Recovery rates may also be related to the amount of time required for the impulse frequency of individual neurons in the population of responding sensilla to return to the nonstimulated state. Roelofs and Comeau (1971) hypothesized that recovery rates are affected by differences in the amount of time that a stimulant molecule spends bound to membrane receptors, and Kaissling (1974) indicated that they may also be affected by intrinsic differences in the time required for deactivation of the molecules. In the MPB, these rates differed significantly between the sexes for three pheromones: *trans*-verbenol, *cis*-verbenol, and verbenone (Figure 15); two kairomones, camphene and Δ-3-carene, also showed differences (Figure 16). Figure 15 also indicated that, of the pheromones, *cis*-verbenol applied to males effected the slowest recovery; and frontalin caused the most rapid recovery in both sexes. The most rapid recovery to all the tested chemicals occurred upon application of diacetone-alcohol (Figure 16). Most of the kairomones had recovery rates only just slightly slower than frontalin.

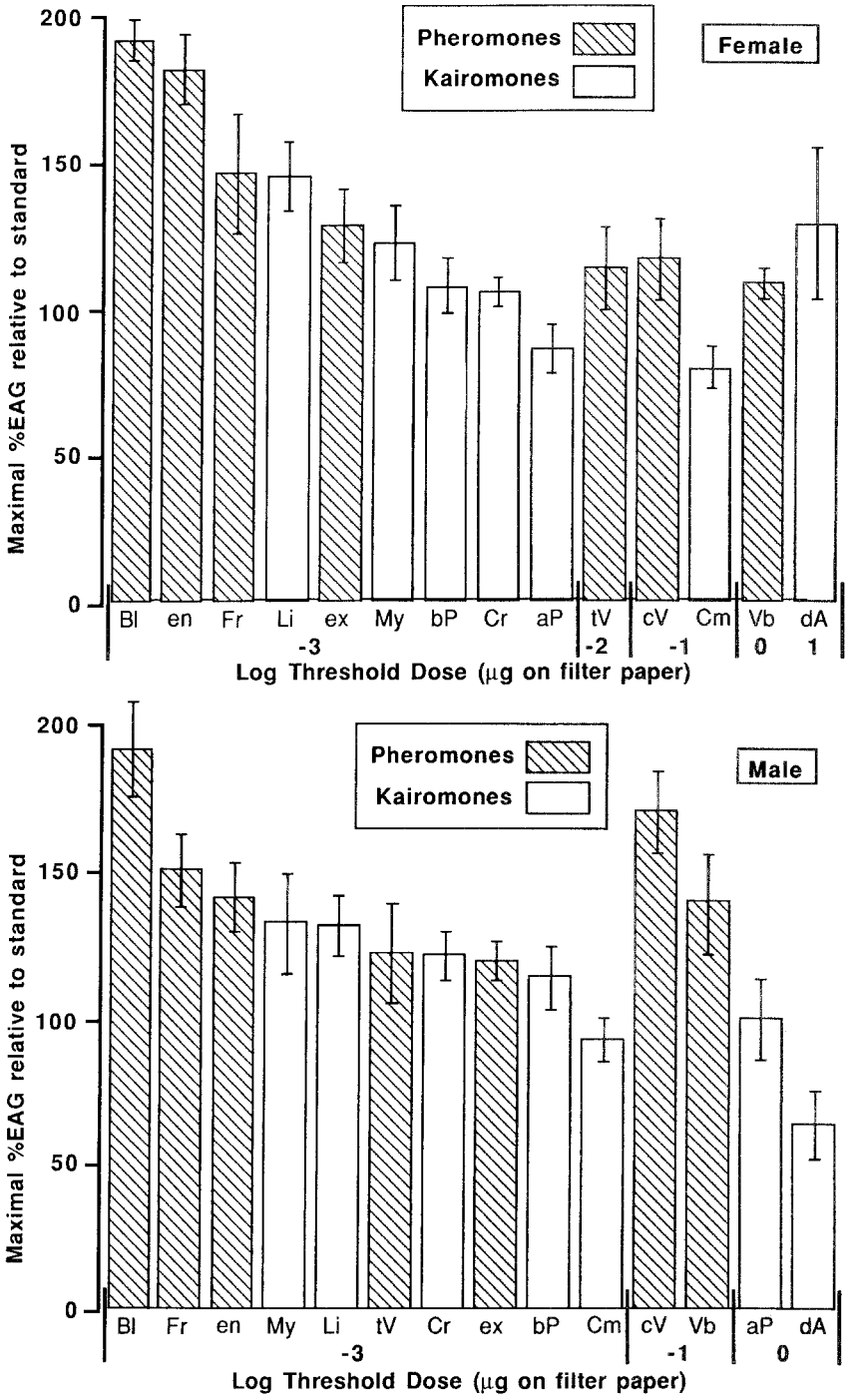
Figures 17 and 18 show the semiochemical threshold concentration plotted against the maximal EAG amplitude for each odorant. Most of the chemicals having the highest maximal EAGs (usually at the 10 μl dose) also had the lowest thresholds. The most obvious exceptions to this trend were *cis*-verbenol and verbenone in the males. Also, males showed lower thresholds to more of the chemicals than females, including *trans*-verbenol. Conn et al. (1983) captured significantly higher numbers of males than females in traps baited with *trans*-verbenol when combined with other host tree monoterpenes. These data appear to correlate with the need of the males to find the females in the large tracts of trees.

Male response to *cis*-verbenol did not have the lowest threshold but did have the longest recovery rate (Figure 15) and one of the highest maximal EAG amplitudes (Figure 18). It also had a significantly higher male response at higher concentrations than *trans*-verbenol but not at lower concentrations (Figures 3 and 4). Although Pitman et al. (1969) failed to find major quantities of *cis*-verbenol in either sex, it has been identified in female frass (Libbey, Ryker, and Rudinsky, unpublished data as quoted in Ryker and Rudinsky, 1982). In *D. pseudotsugae*, *cis*-verbenol stimulates more generalized synergist cells (Dickens et al., 1984). Thus, the large EAGs elicited by this compound could reflect its interaction with receptors on similar cells in the MPB.

Responses of both sexes to frontalin were almost exactly the same, showing no significant differences (Figures 6, 17 and 18). Frontalin is a major pheromone in several species of *Dendroctonus* (Ryker and Libbey, 1982). Rudinsky et al. (1974) isolated frontalin from pairs of unfed MPB but did not identify the sex producing it. Ryker and Libbey (1982) feel that until males join females, they produce no frontalin. In their laboratory studies, they found that arrestment occurred in males exposed to frontalin in the presence of *trans*-verbenol and other terpenes; furthermore, in their field studies frontalin acted as an antiag-



FIGS. 15 and 16. Recovery rates of semiochemicals expressed as percent of EAG areas related to frontalin standard for MPB males (hatched area) and females (clear area); stimulus chemicals 10 μg on filter paper. The larger the %EAG area, the slower the recovery rate. $N = 5$; error bars indicate $\pm$SEM. *Significant difference between male and female response at 0.05 level.



FIGS. 17 and 18. Calculated thresholds for significant EAG responses as related to maximal EAGs (usually at the 100-μg doses) to pheromones (hatched area) in kairomones (clear area) recorded from MPB. $N = 5$; vertical bars represent $\pm$SE.

gregative pheromone for both sexes; however, they were unable to determine the exact role of frontalin in the MPB. On the other hand, Chatelain and Schenk (1984) induced attack of the MPB on frontalin-baited trees. These data lead to the probable conclusion that frontalin is a multifunctional pheromone in the MPB.

Both sexes are sensitive to low levels of *exo*- and *endo*-brevicomin (Figures 17 and 18). These pheromones are reported by Rudinsky et al. (1974) to be male produced and possessing antiaggregative properties; furthermore, supported by results from their bioassays, they found that very low levels of *exo*-brevicomin, when combined with Pondelure™ (*trans*-verbenol and α-pinene blend), would attract large numbers of beetles. They designated *exo*-brevicomin (in various blends of other semiochemicals) as a multifunctional pheromone in both the MPB and *D. frontalis* since it is aggregative at low levels and antiaggregative at high levels. The multifunctionality of *exo*-brevicomin has been supported by more recent research where it was reported to increase attack on lodgepole pine baited with *trans*-verbenol and monoterpenes, but it was antiaggregative on similarly baited white pines (Pitman et al., 1978; McKnight, 1979). Furthermore, Ryker and Rudinsky (1982) suggested that high levels of both racemic *exo*- and *endo*-brevicomin may act as interruptive pheromones preventing aggregation in populations of the MPB infesting lodgepole pine.

Responses of males to the blend composed of *trans*-verbenol, *exo*-brevicomin, and myrcene (1 : 1 : 1 ratio of $\frac{1}{3}$ quantities of each making total concentration equal to those of the single chemicals tested) were significantly greater than those of females at 10-μl doses and lower than females at 10^{-2}-μl doses (Figure 14). Both sexes had low thresholds (Figures 17 and 18) and relatively long recovery rates (Figures 15 and 16). Female responses to the blend were significantly greater than to *trans*-verbenol at all concentrations, whereas male responses were significantly higher at all but the two lowest concentrations. Females showed significantly larger reponses to the blend than to the other pheromones at most doses except *exo*-brevicomin (blend higher at highest dose) and *endo*-brevicomin (blend higher at 10^{-2}-μl and lower at 1-μl doses). Male response to the blend compared to the other pheromones was less marked than that in the female with only verbenone being significantly less for more doses except at the lowest one. Only at the two highest doses did the blend show a significantly greater response in both *exo*-brevicomin and *endo*-brevicomin. However, as a general rule, the blend in both sexes is one of the best EAG stimulants compared to the other odorants tested.

This appears to correlate with field studies which show a blend of the same chemicals to be the best for attracting both sexes and mediating mass attack on trees (Borden et al., 1983a) and in field-trapping studies (Conn et al., 1983). Male responses for the individual components of the blend at 10-μl doses were mathematically extrapolated down to a theoretical mean of 118.6% of the standard at 3.3 μl. The mean for the blend response at 10-μl doses was 173.3% of

the standard or 46% higher than the theoretical mean of 3.3 μl. Computation of a theoretical mean for females at 3.3 μl was 94.5% of the standard compared to 132.6% of the standard for the blend at 10 μl or 40% higher. These large differences between the blend and the theoretical individual responses imply a synergistic relationship between the components of the blend possibly caused by each one stimulating separate sensory cells in the antenna or by occupying multiple receptor sites on single sensory-cell membranes. Recordings from single cells on MPB antennae might be useful in corroborating the occurrence of synergism. Furthermore, since the actual concentrations of odorants reaching the antennae of the beetles tested in both the field experiments and in the present EAG study are unknown, further work needs to be done in adjusting the ratios of the odorants in blends for maximal behavioral and/or electrophysiological responses while monitoring stimulus concentrations.

All of the monoterpenes tested are present in the resin of the lodgepole pine (Smith, 1964). Although not readily apparent from their dose-response curves (Figures 10–13), Figures 17 and 18 show that the lowest thresholds were seen in both sexes to limonene, myrcene, carene, and β-pinene. α-Pinene had a threshold higher in the male than in the female (Figure 17 and 18). In combination with *trans*-verbenol, α-pinene is highly attractive to MPB infesting white pine (Pitman, 1971) but not to MPB infesting lodgepole pine (Borden et al., 1983a; Conn et al., 1983) which may correlate with the fact that lodgepole pine resin has lower concentrations of α-pinene than resins of other species of pines attacked by the MPB (Cole et al., 1981). Camphene had a lower threshold in the male than in the female (Figures 17 and 18).

Diacetone-alcohol has the highest threshold and most rapid recovery time of all the chemicals tested (Figures 16–18). In the female, it tested at the highest concentration with many of the other odorants when compared to that of the male (Figure 7). Since diacetone-alcohol has a much higher evaporation rate than either the pheromones or kairomones, it is likely that much of the odorant at lower concentrations is lost to the atmosphere before a stimulation can be effected. This material has not been reported to be produced by either the beetle or lodgepole pine. However, Ryker (personal communication) indicated that it was effective in attracting beetles to traps in Oregon but not in British Columbia.

Since a gas chromatograph was not available to control the actual concentrations of the stimulants at the antenna (Wadhams, 1982), the assumption was made that all of the odorants (except diacetone-alcohol) have similar vapor pressures and surface-binding characteristics. Since this assumption may not be true, response values and threshold estimates may depend not only upon neuron sensitivity but upon the volatility of the chemicals as well.

The EAG is probably a composite potential resulting from the activity of different populations of sensory neurons. The amplitude and recovery rate of the EAG is likely dependent upon differential responses from these populations

depending on the type of stimulant and its concentration. Furthermore, lack of understanding of the response parameters of each type of neuron in these populations makes interpretation of the EAG difficult. In the long run, behavior of the insect is a result of CNS interpretation of sensory input.

Although it is a valuable tool for investigating insect responses to possible semiochemicals, care must be taken in the interpretation of EAG responses as they relate to behavioral and field studies. Compounds related to or derived from the primary pheromone components may also produce excellent antennal responses as is shown in the *cis*-verbenol response of the MPB in this report; furthermore, it is impossible from the EAG approach to determine if a compound is excitatory (aggregative) or inhibitory (antiaggregative) in terms of insect behavior (Roelofs, 1977). Although good EAG responses can correlate well with effective field odorants (Dickens et al., 1983), this may not always prove to be true. While "the ultimate bioassay is to challenge the natural population with a faithful reproduction of the test material..." (Silverstein, 1984), much can be gained from more subordinate studies such as those involving EAGs if one keeps these problems in mind.

All of the chiral compounds tested were racemic except three kairomones (Table 1). McKnight (1979) found significant differences in the behavior of the MPB to optical isomers of several semiochemicals, especially *trans*-verbenol. Further studies are planned to investigate EAGs of the MPB to the enantiomers of the pheromones tested in this paper.

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PHEROMONE-MEDIATED COPULATORY RESPONSES OF THE SCREWWORM FLY, *Cochliomyia hominivorax*

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Abstract—A bioassay based on male copulatory responses occurring on contact with dead decoy insects was used to confirm the existence of a sex pheromone in the screwworm fly, *Cochliomyia hominivorax*. Males responded to female but not male decoys. Mated and virgin females were equally stimulatory. Activity was abolished when females were washed with hexane but partially restored by treatment with crude hexane extract of females. Responses decreased when extracts were diluted and when the number of females extracted per milliliter of hexane was decreased from 20 to 1 in the preparation of extracts concentrated to 0.4 female/μl. Sexually mature female decoys of the 009 strain, the most laboratory-adapted of three strains examined in intrastrain tests, produced few copulatory attempts compared with those of Aricruz or DE-9 strains. However, newly emerged 009 as well as Aricruz females elicited responses from about 80% of sexually mature males. Those of the DE-9 strain stimulated fewer than 1%. The observation that 009 females were maximally stimulatory before becoming receptive to mating suggests that these strain differences resulted from laboratory colonization.

Key Words—Screwworm fly, *Cochliomyia hominivorax*, Diptera, Calliphoridae, sex pheromone, mating-stimulant pheromone.

INTRODUCTION

Aggregations containing sexually active males of the screwworm fly, *Cochliomyia hominivorax* (Coquerel), have been observed on vegetation (Krafsur, 1978), often in association with flowers of specific types (Guillot et al., 1978; Mackley and Long, 1983). The sexually active males were identified by their position within the vegetation, posture, territoriality, and striking behavior toward screwworms, flies of other species, butterflies, and even small pebbles.

Considering the variety of targets struck, vision appears to be an important stimulus eliciting strikes. However, a contact sex pheromone may play a role in the release of male copulatory behavior (Mackley and Broce, 1981).

In many Diptera, including the housefly, *Musca domestica* L. (Rogoff et al., 1964), and the tsetse fly, *Glossina morsitans morsitans* (Westwood) (Langley et al., 1975), female-produced sex pheromones are located on the cuticle and stimulate male mating behavior either on contact or at close range [see Howard and Blomquist (1982) for additional examples]. Mackley and Broce (1981) suggested that female screwworm flies produce a sex pheromone because dead females elicited fewer male copulatory responses after washing in nonpolar solvent and dead males previously held with females produced more responses than those from all-male groups. However, tests with solvent extracts of females gave inconclusive evidence for a pheromone.

The present study was undertaken to confirm the existence in the screwworm of a female-produced sex pheromone and to examine effects of sex, mating, age, and strain on pheromone-mediated copulatory responses.

METHODS AND MATERIALS

Insects. Screwworms of the DE-9, Aricruz, and 009 strains were reared on a beef-based larval diet and maintained as adults on corn syrup at 25 ± 1°C under a 12:12 light–dark photoperiod (Hammack, 1984). Virgin adults collected within 24 hr of emergence and separated by sex under CO_2 anesthesia were assigned an age of 0 days and tested when 5–8 days old, unless stated otherwise. No anesthetic was administered to males tested before 3 days of age or to either sex after day 0. The three strains had been developed by the former ARS Screwworm Research Laboratory, Mission, Texas, for use in the screwworm sterile-male release program and had been established from multiple collections of egg masses made in the field in 1979 (DE-9, Tamaulipas, Mexico), 1978 (Aricruz, Arizona and Veracruz, Mexico), or 1974–1975 (009, Texas). The DE-9, Aricruz, and 009 strains had been maintained at Fargo, North Dakota, since December 1979, May 1979, and December 1977, respectively. The present tests were conducted with the DE-9 strain between July 1980 and June 1982 and with the Aricruz and 009 strains between February 1980 and February 1981.

Bioassay and Pheromone Extraction. Screwworm mating behavior under laboratory conditions has been described previously (Broce, 1980; Mackley and Broce, 1981). The bioassay used here focused on behavior occurring once the sexes make physical contact and measured the percentage of individually tested males that attempted copulation with decoy insects when males and decoys were paired for a period of 60 sec. The decoys were screwworm flies that had been killed by freezing at −20°C for 30 min on the morning of testing. Dead decoys

were used to eliminate auditory and visual (motion) cues that might contribute to the release of copulatory behavior. Each was pinned through the thorax, dorsal side up, to the base of an inverted paper cup with an insect pin (000). The head of the pin was exposed on the decoy's dorsum. Unwashed decoys were thawed and mounted for testing.

For the other treatments, female decoys warmed to room temperature after freezing were first washed with hexane to prepare the pheromone extracts (as described below) and then washed again in two 0.5-ml streams of hexane dispensed from a pipete. They were air dried for at least 30 min after washing and mounted. Female extract was applied to the dorsal surface of the thorax and abdomen of the mounted washed decoys with a microsyringe at volumes of 5, 10, or 50 μl (air dried for about 60 sec between each of 5 successive 10-μl applications). Washed decoys treated only with hexane served as controls. The decoys were air dried for at least 15 min after the final solvent application. However, they were all generally prepared at the same time and held mounted at 25°C for as much as 5 hr before assay.

Each test male was transferred from its holding cage to a 20-cc glass vial immediately prior to assay. The vial was inverted over the decoy and contact between the male and decoy accomplished by tapping the cup and vial against the substrate. Positive responses were scored when males mounted the decoy dorsally (usually with wing buzzing), flexed their abdomens, and probed the decoy's abdominal tip or occasionally the head with their genitalia. Males were tested once at 25 $\pm$ 1°C within 1–6 hr after the start of a 12-hr photophase and discarded. This interval was used because Crystal (1971) reported high mating rates for screwworm flies throughout the first half of a 12-hr photophase. Light intensity on the test surface was about 700 lux supplied by daylight fluorescent lamps.

In one test, male and female unwashed virgin decoys were assayed to determine their relative effectiveness in eliciting male copulatory attempts. In another, virgin and mated unwashed female decoys were compared. Mated females had been held with males from emergence until the day of testing at eight days postemergence. Insemination was confirmed after assay by detection of sperm in spermathecae of the decoys. These sex and mating status comparisons were done with the Aricruz strain.

Hexane-washed decoys treated with 2 FE of standard female extract in 5 μl of hexane and washed controls treated only with 5 μl of hexane were tested along with unwashed female decoys to confirm that chemical stimuli are involved in eliciting male copulatory responses. The standard extracts were prepared by individually dipping 40 females for 15 sec each in the same 2-ml aliquot of glass-distilled hexane and then reducing the solvent volume in a stream of CO_2 to 0.1 ml [0.4 female equivalent (FE)/μl]. Insects of the DE-9 strain were used here and in all of the following extraction tests.

The pheromone activity in standard extracts was low, and two modified extraction procedures were tried. In one series of tests, the activity recovered when standard hexane extracts were prepared was compared with that recovered when each of 40 females was dipped for 15 sec in a separate 1-ml aliquot of hexane and the 40 aliquots pooled and concentrated to 0.4 FE/μl. Five replicates were tested per treatment in one test done at a dose of 2 FE in 5 μl. Extraction and assay procedures were repeated in a second test, except that decoy pairs were treated with 20 FE in 50 μl and each of two decoys per treatment was tested with 25 males.

Pheromone activity declined with the decrease in the number of females extracted per milliliter of hexane, and dose–response relationships were investigated with extracts prepared by successively dipping 100 females for 15 sec each in the same 4-ml aliquot of hexane and then reducing the solvent volume to 0.1 ml (1 FE/μl). The extract was applied in 10 μl of hexane to washed decoys at five doses ranging from 4.1 to 10 FE. Five decoys (one per dose) were tested simultaneously with matched males, and the process repeated with seven more sets of decoys. The relationship between dose and mean response based on the eight replicates was analyzed by linear regression. The extracts used in the dose–response test were stored at $-20°$C for as much as 20 hr before assay, whereas extracts used in all other tests were assayed immediately after preparation. Storage was minimized because preliminary tests indicated that the pheromone activity of whole females was nearly lost when they were stored at $-20°$C for three to four weeks.

Unwashed female decoys of the DE-9, Aricruz, and 009 strains that ranged in age from 1 to 15 days were assayed to determine their ability to stimulate copulatory attempts in males of the same age and strain. Intrastrain tests were also conducted in which the response of 6- to 8-day-old males to unwashed female decoys of various ages was determined. Zero-day-old females used in the latter tests were frozen within 4 hr after emergence.

Unless stated otherwise, decoys were each assayed with 10 males exposed individually, and at least 10 decoys (replicates) were tested per treatment. Except for the analysis of dose–response relationships, Wilcoxon's signed rank test (T statistic) or the Kruskal and Wallis test (H statistic distributed as χ^2) (Steel and Torrie, 1960) was used to determine whether the null hypothesis of no treatment effects was valid.

RESULTS AND DISCUSSION

Unwashed female decoys elicited copulatory attempts from significantly more test males (73%) than did unwashed male decoys (0%) ($T = 0$, $n = 10$, $P < 0.01$). However, there was no significant difference between the percentages responding to mated and virgin female decoys (48 and 50% respectively,

$T = 25$, $n = 10$, $P > 0.05$). Thus, the sex of decoys significantly affected activity, but the mating status of female decoys did not.

Hexane-washed females treated with 2 FE of standard extract induced significantly more copulatory attempts (32% of test males responding) than controls treated only with hexane (0% responding) ($T = 0$, $n = 10$, $P < 0.01$). Although the extract treatment did not fully restore the activity of unwashed females (64% responding), these results clearly implicated a female-produced chemical stimulus or set of stimuli in the release of male copulatory behavior. This conclusion was strengthened by the demonstration of a significant dose–response relationship (Figure 1). The percentage of males that attempted copulation with extract-treated decoys (Y) varied linearly with the logarithm of the concentration of female extract (X): $Y = 50.3 \log_{10} 100X - 107.8$, $r^2 = 0.80$, $P < 0.05$.

When concentrated to 0.4 FE/μl and tested at 2 FE, an extract prepared by dipping 40 females in 40 1-ml aliquots produced no responses, although a standard extract prepared by dipping 40 females in 2 ml stimulated 38% of test males. An extract prepared by the former method also failed to produce any responses when assayed at 20 FE, although a standard stimulated 50% of males at this dose. The absence of pheromone activity when increased amounts of solvent were blown off suggests that the screwworm pheromone is volatile and thus might have attractant functions. However, the bioassay technique used here was based upon responses elicited on contact because no evidence exists as yet to suggest that female screwworm flies are attractive to males over any distance (Mackley and Broce, 1981; Hammack, unpublished).

Other possible explanations for the observed lack of activity include pheromone lability or, because the tests were done with crude extract, the produc-

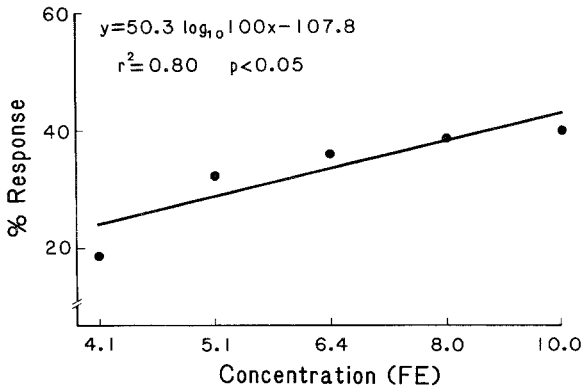

FIG. 1. Percentage of males attempting copulation with dead, hexane-washed, females treated with different concentrations of crude extract of females. Concentrations are plotted on a logarithmic scale and expressed in female equivalents (FE).

tion or more thorough extraction of contaminants interfering with the response to pheromone. When chemical identifications have been achieved in other Diptera, the female-produced sex pheromones that act on contact or at short range have generally proved to be hydrocarbons showing little if any volatility, usually alkenes or methyl branched alkanes with a chain length in the range of 23–37 carbons (Antony and Jallon, 1982; Carlson et al., 1984; see Howard and Blomquist 1982). The possibility of a more thorough extraction of interfering substances with an increase in the solvent volume used to wash screwworms was raised by reports that inactive alkanes that may be present in crude female extracts mask the activity of hydrocarbon sex pheromones in the face fly, *Musca autumnalis* De Geer (Uebel et al., 1975) and in tsetse flies (*Glossina* spp.) (Coates and Langley, 1982; Carlson et al., 1984). More data are clearly needed to explain the loss observed in the screwworm with extraction in the larger solvent volume. It was not prevented by the use of N_2 instead of CO_2 to blow off hexane (Hammack, unpublished data using a screwworm strain designated FC96 from Chiapas, Mexico).

When pairs of males and decoy females of the same age and strain were tested at various intervals after emergence, the maximum response among 009 groups was only 27% of males attempting copulation, a value less than any DE-9 or Aricruz response obtained after 1 day of age (Figure 2). No copulatory attempts occurred in any strain when both sexes were 1 day old, but this could be attributed to immaturity of the male in the Aricruz and 009 strains because newly emerged female decoys of these strains stimulated about 80% of 1-week-old males in intrastrain tests (Figure 3). In contrast, newly emerged females of the DE-9 strain stimulated less than 1%. Interstrain tests are needed to determine whether these differences arose because newly emerged females of differ-

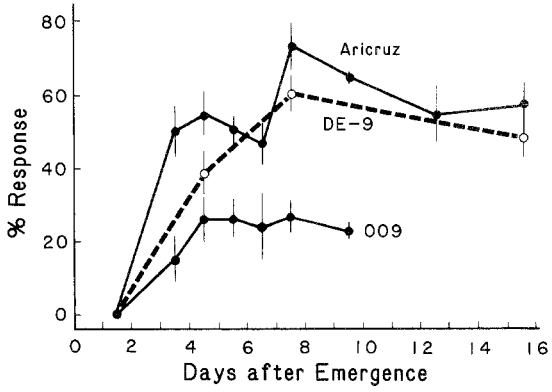

FIG. 2. Percentage ± SE of males attempting copulation with unwashed female decoys in intrastrain tests conducted with dead screwworm flies of the DE-9, Aricruz, and 009 strains. Test males and decoy females were the same age at the time of assay.

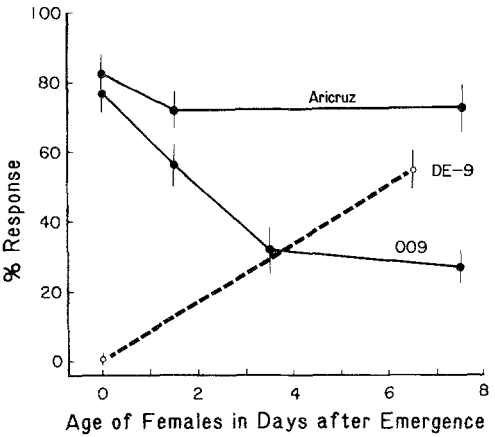

FIG. 3. Percentage ± SE of 6- to 8-day-old males attempting copulation with unwashed female decoys of various ages in intrastrain tests conducted with dead screwworm flies of the DE-9, Aricruz, and 009 strains.

ent strains vary in their ability to stimulate male copulatory attempts and/or whether male responsiveness to a pheromone stimulus varies with strain. Preliminary unpublished data (Hammack) suggest the latter explanation, but the point to be made here, in addition to the low response among 009 pairs of the same age, is that the mating-stimulant activity of females changed differentially with age depending on strain.

Activity increased significantly with age after emergence in the DE-9 strain ($T = 0$, $n = 15$, $P < 0.01$), remained unchanged in the Aricruz strain (corrected $H = 2.4$, $df = 2$, $P > 0.05$), and declined significantly in the 009 strain (corrected $H = 21.6$, $df = 3$, $P < 0.001$). The decline in the 009 strain was evident before 2–3 days of age when female screwworm flies first become receptive to mating (Adams, 1979; Hammack, unpublished data for the 009 strain). For females to be maximally stimulatory when not yet receptive to mating appears wasteful of resources, unless the pattern in the long-colonized 009 strain (and perhaps the Aricruz strain) resulted from adaptation to laboratory conditions. It is of interest in this regard that Pomonis and Mackley (1985) reported changes with colonization in cuticular lipid profiles in female but not male screwworm flies. Thus, colonization seems a likely explanation for the strain differences observed here, although an explanation based on the existence of reproductively isolated field populations, as hypothesized by Richardson et al. (1982), cannot yet be excluded.

A loss or masking of the screwworm pheromone with age might well be adaptive in colonized populations considering the common practice of holding the sexes together in breeding stocks until the time of oviposition. Most insects

in such stocks are the same age within 1.5 days. Mating begins days before females are ready to oviposit, but males, unlike females, are polygamous and continue to court inseminated females (Bushland, 1960). Data from the present study indicating no effect of insemination on female pheromone activity are consistent with Bushland's (1960) observations. Therefore, considering the artificially high density of caged populations, male harassment of females between the times of mating and oviposition could lead to selection for reduced pheromone activity. Indeed, female mortality in mixed-sex laboratory populations has been used as an indicator of the sexual aggressiveness of screwworm males (Crystal and Ramirez, 1975; Crystal and Whitten, 1976).

Additional research is needed to clarify the effects of colonization on the production of and responsiveness to sex pheromone, optimize pheromone extraction and/or its presentation during bioassays, and identify the active chemical(s). Knowledge derived from such research may prove useful for monitoring the quality of screwworms mass-reared for sterile releases and evaluating their mating competitiveness with field populations from different ecological and geographical sources.

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Editors' Note

This special issue is devoted to the International Conference on Chemical Communication and Regulation of Sexual Reproduction, Growth, and Maturation of Schistosomes held at Johns Hopkins University, Baltimore, Maryland 21205, on May 1–3, 1985. The papers are introduced with a Foreword by Dr. Everett L. Schiller who served also as the conference director and as one of two reviewers for each manuscript. The editors are grateful to several conference participants who also served as second reviewers: Drs. David A. Erasmus, L. K. Eveland, Bernard Fried, Eugene G. Hayunga, Walter M. Kemp, and Irene Popiel.

We acknowledge the cooperation of Plenum Publishing Corporation in producing extra copies of this issue to accommodate registrants of the symposium and others interested.

R.M. Silverstein
J.B. Simeone,
Editors
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FOREWORD

The Johns Hopkins University International Conference on Chemical Communication and Regulation of Sexual Reproduction, Growth and Maturation of Schistosomes was a three-day conference organized for the purpose of bringing investigators in the field of schistosome biology together as a specialist group to thoroughly review the subject, provide an update on research findings, critically evaluate ongoing research, and set guidelines for future research. This conference, supported by a grant from the Edna McConnell Clark Foundation, provided an opportunity to learn about a diversity of systems for investigating the complex sequence of events involved in schistosome sexual reproduction and to identify potential targets for interfering with these processes. This publication of the proceedings is expected to focus the attention of other scientists on these important problems.

In the introductory session, reviews are presented of interspecific chemical communication between parasite and host, intraspecific chemical communication between parasites, and internal chemical communication within individual parasites. Chemical communication in parasitic nematodes is reviewed by L.W. Bone. Using *Nippostrongylus* and *Trichostrongylus* as models, he demonstrates an investigative approach which is germane to the study of schistosome chemical communication. Of particular relevance is the emphasis placed on establishing the proper physical system for the in vitro bioassay of responses to pheromonal gradients. Chromatographic partial purification of two pheromones is reported.

G.C. Kearn describes the role of chemical substances from fish hosts in hatching and host finding in monogeneans. The behavioral responses to both host and environmental cues are described. The precise chemical nature of the active host-derived components awaits elucidation.

B. Fried shows that some adult hermaphroditic Digenea tend to pair in vitro and in vivo. Pairing is both intra- and interspecific, suggesting that nonspecific factors are involved. From the considerable evidence presented, lipophilic substances function in aggregation and pairing in these parasites.

The subject of invertebrate internal coordinating mechanisms dependent on "hormonal" regulatory substances is reviewed by P.F. Basch. Attention is drawn to the fact that evidence for these processes occurring in flatworms is observational and few physiological studies have been made. The presence of

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ecdysteroids in schistosome tissues is demonstrated and their possible role in morphogenetic processes is discussed.

In the session devoted to the subject of chemical regulation of schistosome behavior, L. K. Eveland, M. Haseeb, D. Shirazian, J.E. Childs, and E.L. Schiller present evidence that adult schistosomes attract each other in vitro and that the attraction is chemically mediated. Lipid release from the tegument of adult worms is described by M. Haseeb and its role in chemoattraction is discussed.

J. Gloer reports on a serial stepwise extraction of schistosome tissues using pentane, diethyl ether, ether acetate, methanol, and water and the incorporation of the extracts into a bioassay system for chemoattraction. He shows that the least polar extracts are attractive heterosexually but not homosexually. The major components of this fraction are steroids.

Evidence that a chemoattractant compound(s) in female extracts may serve as a mating pheromone is presented by J.E. Childs. E.L. Schiller describes a time-lapse video tape system for documenting sexual behavior of *S. mansoni* in vitro. Tapes made during various experiments concerned with chemical orientation of these parasites reveal that the opposite sexes are attracted to each other by some means of premating communication that culminates in aggregation and copulation of sexual pairs within 24 hr. He reports that attractivity is significantly greater in heterosexual than in homosexual pairs. Male worms move towards females more rapidly and more vigorously than females move towards males. Clustering and directional preferences of male worms towards test compounds (prepared by J. Gloer) indicate the presence of chemoattractant substances in female extracts that serve as a mating pheromone.

The presentations in this session generated much discussion with the following recommendations: purification and further chemical characterization of the attractant substances should be continued; unisexual adults and sexually immature worms should be cultured in vitro and their excretory-secretory (ES) products and tissue extracts bioassayed for chemoattractant activity; and an in vivo bioassay method should be developed for investigating chemoattraction.

The session on developmental physiology and reproduction is devoted to a detailed consideration of the morphogenetic and biochemical changes which take place in female schistosomes immediately after pairing with males.

Explanations for the nature of male-stimulated female maturation are reviewed by I. Popiel. Attempts to demonstrate direct chemical transfer between members of worm pairs are considered, and the exchange of cholesterol within worm pairs is demonstrated. She reports on differences in the extent of maturation and in responsiveness to male stimulation between unisexual female infections from two different laboratories. Of particular interest to the study of female morphogenesis is the observation that when unisexual females are paired with transected segments of male worms, stimulation of vitellogenesis is local and cannot be propagated internally.

Details of the process of vitellogenesis are provided by D. Erasmus, who also reports on anomalies such as vitelline gland development in unisexual females and in mature males. Some biochemical changes which take place in females after pairing with males are described. Of significance is the demonstration that an increase in thymidine incorporation in unisexual females occurs 24 hr after pairing with mature males derived from bisexual infections. When such females pair with mature males from unisexual infections, the increase in thymidine incorporation is delayed for 24 hr.

M. Shaw reports on changes in the elemental composition of male and female schistosomes in relation to age postinfection and sexual status. An important finding in his investigation is the significant increase in calcium and phosphorus levels in females from bisexual infections which begins about day 28 postinfection, i.e., at the onset of female maturation.

Next, E. Cornford deals with the influence of mating upon surface exchange in schistosomes. He reports that paired males and females take up more glucose than separated individuals, and that glucose is transferred from males to females *in copula.*

G. and C. Wu report that factors present in portal blood, but not in peripheral blood, stimulate egg production in vitro. Sera from nonpermissive hosts were used in these experiments. They show the active fraction of rabbit serum to be between molecular weights 2000 and 50,000 daltons and that of rat serum to be between 2000 and 30,000 daltons. Their results represent the first demonstration of a reproductive response to portal versus peripheral blood. The biochemical nature of the stimulatory factor is not known. They suggest that this factor may be involved in site location during schistosome migration and that it may be a host hormone associated with digestive physiology.

D. Cioli considers the variation in extent to which growth and maturation of schistosomes occur in a spectrum of nonpermissive hosts. In nonpermissive rats, portal worm elimination takes place in the fourth week of infection. The few worms that survive do not develop to full sexual maturity. P. Knopf discusses the effect of host factors in this inhibition of maturation. Thyroidectomy makes rats more permissive; portal worm elimination is delayed for four weeks, maturation of worms proceeds, and miracidia can be hatched from liver eggs. Administration of T3 or T4 to thyroidectomized rats completely restores their nonpermissive status.

In a comparative analysis of schistosome antigenic composition, J. Rotmans demonstrates the detection of a large number of female-specific, but few male-specific, antigens. Unisexual females are antigenically quite different from females derived from bisexual infections. The preparation of monoclonal antibodies to these antigens is described.

W. Kemp describes receptors on the schistosome surface with immunological function. These are identified as hostlike C3 and Fc receptors, and their possible functions are discussed. He raises an important fundamental question

that arises from the fact that schistosomes are totally covered with host components, namely: does the worm surface present effector cells of the immune system with "self" or with idiotype?

The final session is concerned with characterization and mode of action of schistosome metabolic products. E. Hayunga reviews the analytical methods involved in the isolation and characterization of pheromones and other chemicals which may influence schistosome maturation. Special attention is given to the technical problems that arise when investigating parasite molecular biology. He presents data to define the role of an isolated antigen in hepatic egg granuloma attenuation. Evidence of diminished fecundity of female worms in experimental infections supports the theory of a "crowding effect" in murine schistosomiasis and suggests the possibility that toxic or chemotactic secretions by worms may inhibit reproduction.

P. Nirde describes the detection of ecdysteroids by radioimmunoassay of HPLC-separated lipid extracts in schistosomes. He finds higher levels of ecdysone and 20-hydroxyecdysone in juveniles of both sexes than in adults and implicates these compounds as factors in schistosome morphogenesis. Their precise role is yet to be determined.

D. Thompson and J. Bennett report on the effects of steroids and steroid synthesis inhibitors on *S. mansoni* fecundity in vitro. Cholesterol and dianisidine, an inhibitor of cholesterol metabolism to hormonal products, are both ineffective in blocking egg production. The data show that, although various progesterones depress egg production, estrogenic and androgenic hormones have no effect.

P. Morrison and E. VandeWaa show that *S. mansoni* schistosomulae rely on cyanide-sensitive respiratory metabolism for at least 3 hr after transformation from cercariae. After 24 hr, the parasites are metabolically similar to adults and depend on lactate fermentation for most of their energy requirements. The biochemical switches for metabolic transformation are not yet known.

The conferees drew a number of useful conclusions and recommendations from these sessions:

It is not clear whether the male stimulus for female maturation is chemical, physical, or a combination of both. Future investigations should address both possibilities.

Vitellogenesis is the first visible sign of female maturation, and it begins approximately two days after worm pairing. A better understanding of the overall process of male-stimulated female development requires a knowledge of the developmental processes that occur immediately after pairing and before vitellogenesis.

It is important to establish whether or not the variation in extent of maturation in unisexual females observed in different laboratory populations is genetically determined.

These investigations have indicated that sexually mature schistosome pairs are, to some extent, biochemically interdependent. The precise nature and significance of this interdependence is yet to be determined.

Comparative biochemical investigations on males and females should include a consideration of unisexual worms and those from bisexual infections which are not yet sexually mature.

Further information is required on the properties of the schistosome surface membrane with respect to changes which take place during development and especially during the critical period of sexual maturation, i.e., 28–40 days postinfection.

There is a noticeable paucity of characterized chemicals which may function in the regulation of schistosome developmental and reproductive processes. For example, only one class of biochemically characterized ecdysteroids was mentioned in this conference. It was reported that ecdysone and 20-hydroxyecdysone have been extracted from immature and mature worms. These compounds have demonstrated roles in insect morphogenesis. They are now being implicated as factors in the morphogenesis of schistosomes and other helminths, but their specific role has yet to be determined. An overriding recommendation drawn from a consideration of schistosome biochemistry, as related to development and reproduction, was the need to identify associated macromolecules and to establish their precise functions. This presents a formidable task because only small amounts of tissue or worm ES products are available for analysis and biochemical purification often results in only minute quantities of biologically active substances.

New approaches to the control of schistosomiasis are needed. A knowledge of the pheromones that regulate sexual attraction and reproduction in schistosomes could lead to the development of pharmacologic and/or immunologic means of interfering with them. Preventing worms from mating would preclude egg production, thereby eliminating the main cause of pathology in this disease. Interfering with the processes of sexual reproduction also would eliminate the excretion of eggs into the external environment, thereby significantly reducing the transmission potential of the parasite.

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NEMATODE REPRODUCTIVE AND INGESTIVE RESPONSES TO HELMINTH AND HOST CHEMICAL STIMULI

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Abstract—Current knowledge of the involvement of chemical stimuli in the reproductive and ingestive physiology of zooparasitic nematodes is reviewed. The habitat of zooparasitic nematodes, coupled with their sensory reduction, indicates that chemical stimuli may modulate most aspects of their behavioral physiology. Nematodes respond to the feeding status of the host so that the helminth's pharyngeal pumping, site selection, and sexual activity may be altered. Biogenic amines from the host, such as histamine, dopamine, or serotonin, may synchronize host and helminth ingestion. Octopamine, which is released by nematodes, depresses ingestion by male, but not female, worms. Biogenic amines may also regulate ovipositioning by female helminths. Nematodes release pheromones that enable precopulatory location of a mate. The male helminth is activated by both components of the female's pheromone while movement by the female is probably arrested by male-produced chemicals. Continuation of spectral analyses may allow future identification of female pheromone. The male's pheromone receptor binds lectin that is specific for mannose residues. Host immunity also alters the behavior of nematodes. Ingestion by nematodes is depressed by serum, particularly IgG_1, from infected animals. Similar results occur after immunization of noninfected hosts. Immune serum has no effect on pheromone communication between the sexes of helminth, but depresses ovipositional behavior of female worms. Exploitation of these chemical signals and the helminths' responses may allow novel techniques for disease control.

Key Words—Nematode, pheromone, mating attraction, oviposition, reproduction, ingestive activity, host immunity, chemical reception.

INTRODUCTION

The habitat of zooparasitic nematodes, coupled with their sensory reduction, indicates that chemical stimuli from the host or helminth may modulate most

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aspects of their behavioral physiology. Croll and Sukhdeo (1981) proposed that nematode behavior consisted of only 22 activities; 14 of these patterns were responses predominantly to chemical stimuli. Chemoreception by nematodes may be highly specific since Dusenbery (1975) reported that *Caenorhabditis elegans* showed sterospecificity for D- and L-tryptophan. Adult parasites are characterized by a tremendous fecundity that ensures transmission of the infective stage in the environment and reinfection of another host. The demands of nematode reproduction require significant nutrient acquisition; hence ingestion by helminths may be of primary importance in the maintenance of reproductive status. Accordingly, the host and helminth stimuli that regulate the interrelated functions of feeding and reproduction are reviewed.

SEXUAL COMMUNICATION STIMULI

Pheromones are intraspecific chemical stimuli that regulate diverse physiological or behavioral events in an organism's life history. Pheromones are implicated, commonly for reproductive communication, in over 25 species of nematode from free-living, plant-parasitic, and animal-parasitic habitats (see Bone and Shorey, 1978; or Bone, 1982 for recent reviews).

Chemotaxis by male nematodes to pheromone from their females is affected by various biological and physical factors. Communication between the sexes is disrupted with helminth age, which suggests that host immunity in older infections may impair pheromone production and responsiveness. The age-dependent loss of communication is greater in vitro than in vivo, which indicates that the artificial bioassay systems may have some minor effect on communication (Glassburg et al., 1981). Pheromone reception by males is coupled to the final larval molt. Time-of-day and mating history have little effect on chemical communication. Light, culture sterility, and host food deprivation for 24 hr also have no apparent influence on pheromone production. Anaerobism greatly reduces pheromone production, while optimum chemical production (or less degradation) occurs at pH 7.1. Decreased temperature and osmolarity reduce production of pheromone by females (Ward and Bone, 1983a).

Several in vitro studies have examined the specificity of nematode pheromones. Roberts and Thorson (1977) reported interspecific pairing between *Nippostrongylus brasiliensis*, *Nematospiroides dubius*, and *Trichinella spiralis*. More recently, Belosevic and Dick (1980) have examined three *Trichinella* isolates and reported differential patterns of chemical attraction. However, from the above studies, only *T. spiralis* was tested in a dosage–response format to detect subtle differences.

One pheromone component from *N. brasiliensis* and *Trichostrongylus colubriformis* is similar in HPLC retention. This component is absent in *Toxocara canis*. Hybridization between nematodes occurs in several genera, but the via-

bility of hybrid offspring is low. Thus, closely related species may employ pheromones with similar functional groups so that other mechanisms may control species isolation. Pheromone-based control of helminths would be very appealing if several commonly occurring species of worm were affected by a single synthetic pheromone.

Chemical gradient formation is known to influence male responses to pheromone. In vitro assays have used a 2-hr period for gradient formation with steeper or shallower gradients reducing male responsiveness. In vivo assays revealed that chemical communication via pheromone transmission in the intestine occurs with the direction of peristaltic flow. Adequate male responsiveness was found over a 5- to 11-cm distance in the intestine. Thus, distance influences chemical communication in the aqueous environment of the host (Glassburg et al., 1981).

An unresolved area of research involves the chemical responsiveness of female nematodes to their males. Most investigations have reported a more predictable response of males to females than vice versa. Some available evidence suggests females of *N. brasiliensis* are rapidly arrested by the presence of males (Glassburg et al., 1983). Thus, the stationary females become a pheromone point-source for the approaching male worm. Such a system seems more rational than the collision of two moving organisms and would account for the less consistent reports in the literature regarding attraction of the female. The behavior of males and females differed in the intestine, so that males disperse and females localize. Food deprivation altered female behavior in the intestine, but the female's movement was arrested by males. As discussed in a subsequent section of this review, female nematodes may partition their responses between mating and feeding while males are more driven by their sexual status than by nutrition.

Chemical studies of the pheromone from *N. brasiliensis* have revealed two compounds that account for the biological activity in incubate from females (Ward and Bone, 1983b). One component may be a peptide, based on pronase sensitivity, with an estimated weight of about 600 by gel filtration while the other component is apparently a hydrocarbon (mol wt 375). Isolation of this compound by HPLC has revealed that the chemical is moderately polar and soluble in water and alcohols. Behavioral and chromatographic analyses of pheromone release versus content indicate a rapid biosynthetic mechanism. Elemental analysis suggests the presence of one nitrogen, while spectral analyses seemingly indicate that an amino acid accounts for about 50% of the molecular weight. Mycotoxin inhibitors of amino acid transport in the host intestine yield a 72% increase in pheromone production by female *N. brasiliensis* while depletion of the amino acid in the host's diet causes a 34% reduction in pheromone production. Studies in progress with radiolabeled amino acid may contribute to pheromone identification and ultimately allow insight into pheromone biosyn-

thesis. Pharmaceutical blockage of the biosynthetic pathway may offer new avenues for drug control of helminthiasis.

OVIPOSITIONAL STIMULI

Little information is evident regarding chemical stimuli that govern egg release or oviposition by helminths. Phillipson (1974) proposed that physiological changes in the host's digestive system regulated oviposition by nematodes, but no stimuli were identified. Oviposition by *Haemonchus contortus* was maximal at pH 4–4.5, which suggests that the concentration by hydrogen ion may influence oviposition (Honde and Bueno, 1982). Oviposition by nematodes was stimulated by serotonin, 5-hydroxytryptophan, and epinephrine, while histamine had a moderate effect (Croll, 1975). Serotonin and octopamine had antagonistic effects on oviposition by *Caenorhabditis elegans* and, thus, stimulated and inhibited, respectively, egg release (Horvitz et al., 1982). If these biogenic amines are released by helminths, or used as chemical cues from the host, a regulatory role in oviposition could result. Huang et al. (1982) observed an inverse relation between esophageal and vulvar contractions in *C. elegans*. Thus, oviposition and ingestion may be antagonistic.

Host hormones influence helminth fecundity through an unknown mechanism. Release of larvae by *Trichinella spiralis* was decreased by diethylstilbesterol and increased by testosterone (Reddington et al., 1981). Swanson et al. (1984) found a testosterone-dependent production of eggs by *N. brasiliensis* which was eliminated by ligation of the bile duct. Estrogen decreased egg production by *N. brasiliensis*, while host pregnancy caused greater oviposition which suggests a stimulatory role for progesterone. However, the broad physiological effects of these hormones on the host obscures their direct action as stimuli for reproduction.

Host immunity may also directly affect parasite fecundity by blocking the release of eggs by females. Bosshardt and Damian (personal communication) found that IgG from the baboon host inhibited oviposition in vitro by the blood fluke *Schistosoma mansoni*. Bone and Klesius (unpublished) found that serum from infected animals reduced in vitro oviposition by female *T. colubriformis*. Greater inhibition occurred as the duration of the infection was lengthened. Serum from animals that were immunized with adult worms or their IgG component also reduced oviposition in vitro by 80–90%. Indirect immunofluorescence studies showed that IgG was bound to the cephalic region of the worm, but binding and the inhibition of egg release were eliminated by absorption of serum with whole worms. Vaccination of lambs with irradiated *T. colubriformis* larvae and subsequent infection also decreased fecundity (Wagland et al., 1984).

Charley-Poulain et al. (1984) found a temporal relation between elevated host IgA and depressed oviposition by *Haemonchus contortus*. Host immunity

also reduces fecundity in *Trichinella spiralis* (Kennedy and Bruce, 1981) and *Angiostrongylus cantonensis* (Techasoponmani and Sirisinha, 1980). The lower egg counts typically found in older infections may be the result of these immune influences.

FEEDING STIMULI

Feeding by intestinal nematodes is predominantly by oral ingestion of host tissue or chyme, although a transcuticular uptake of solutes occurs in the filarial nematode *Brugia pahangi* in vitro (Chen and Howells, 1979). *B. pahangi* may also feed orally in vivo (Howells and Chen, 1981). Transcuticular absorption of cholesterol and glucose are reported also for *Ascaris suum* (Fleming and Fetterer, 1984).

Little information is available regarding the stimuli that may regulate pharyngeal pumping, and thus oral ingestion, by nematodes. Several authors (Fernando and Wong, 1964; Roberts and Fairbairn, 1965) have reported that serum stimulated feeding by the hookworm *Ancylostoma caninum*.

Recent studies on *Nippostrongylus brasiliensis* and *Trichostrongylus colubriformis* have used fluorometric analysis of the fluorescent dye rhodamine B to determine in vitro and in vivo ingestion by these worms (Bottjer and Bone, 1984, 1985a,b). Both species increased their feeding as the time of removal from the host was prolonged, which suggests a response to food deprivation. Starvation of the host up to 36 hr reduced in vivo feeding by *N. brasiliensis* which then increased its feeding during subsequent fasting of the host. Feeding by the worms resumed within 15 min after feeding the fasted hosts. Females ingest more and compensate for food deprivation better than do males.

These results indicate that nematodes monitor their nutritional environment and may rapidly adjust their ingestion accordingly. However, the regulatory chemical cues are somewhat elusive. In vitro ingestion by *T. colubriformis* was not stimulated by various sugars, amino acids, enzymes, host chyme or bile, serum from eight mammalian or avian species, seven bacterial species, and nine salt or supplemented culture media (Bone and Bottjer, 1985a; Bottjer and Bone, 1985c).

In contrast, selected biogenic amines altered feeding by *T. colubriformis* in vivo and in vitro, which suggests a synchrony of host and helminth ingestion. Histamine and dopamine elevated nematode feeding, dosage dependently, while serotonin had a less significant effect (Bone and Bottjer, 1985a). Serotonin may coordinate tapeworm movement and host feeding (Mettrick and Cho, 1981). Pharyngeal pumping in the free-living nematode *Caenorhabditis elegans* was stimulated and depressed, respectively, by serotonin and octopamine (Horvitz et al., 1982). Croll (1975) found that histamine elevated pharyngeal activity in several nematodes. The cephalic receptor for histamine-induced feeding may

contain a mannose residue, based on lectin binding and subsequent loss of increased feeding (Bone and Bottjer, 1985b).

In vitro crowding of *T. colubriformis* or *N. brasiliensis* reduced feeding by both helminth sexes (Bottjer and Bone, 1984, 1985a). Ingestion by males was depressed more by the presence of females than by equal numbers of concomitant males. In vitro doses of octopamine decreased ingestion by male, but not female *T. colubriformis*. Horvitz et al. (1982) reported that *C. elegans* contained octopamine. If this biological amine is released by nematodes, females may feed preferentially to males under competitive conditions so that fecundity is maintained.

Host hormones may also affect the feeding status of intestinal nematodes since *N. brasiliensis* ingested more dye in vivo in normal male mice than in female animals (Bone and Bottjer, unpublished). Implantation of gonadectomized mice with testosterone-filled capsules elicited a dose-dependent increase in in vivo ingestion by the nematode. Ligation of the bile duct eliminated the testosterone-mediated feeding and reduced in vivo ingestion to a low level. Thus, the hormone's effect on the worms may be via biliary transport. Implants with estrogen or progesterone increased or decreased, respectively, in vivo ingestion by *N. brasiliensis*. High levels of implanted cholesterol caused some elevation of helminth ingestion.

In vivo feeding by *N. brasiliensis* and *T. colubriformis* declined significantly with helminth age as the duration of infection was increased. Removal of the older helminths from the host showed that in vitro feeding was similar to younger nematodes. Thus, stimuli that are associated with host immunity may become more pronounced with enduring infections and disrupt the ingestive activity of worms (Bottjer and Bone, 1985a). This repression of in vivo feeding was reversible, based on the worm's recovery of expected feeding rates under in vitro conditions.

Additional data also reflect that helminth feeding was deleteriously altered by host immunity (Bottjer et al., 1986). Serum from uninfected goats had no effect on in vitro feeding by *T. colubriformis*. However, serum from infected goats inhibited ingestion by worms. The suppression of feeding increased with the duration of the infection and was dosage dependent. Preexposure of worms to immune serum also decreased subsequent feeding in basal medium, but rigorous washing of the helminths restored normal levels of ingestion.

Fractionation of immune serum and indirect immunofluorescent studies indicated that immunoglobulin G from the host bound to the cephalic region of *T. colubriformis* and was responsible for the inhibition of ingestion. Immunization of goats with adult worms elicited a host response so that serum from these animals reduced in vitro ingestion. In vivo feeding by worms also declined by about 50% in immunized animals. Antiparasite activity by IgG from immune host serum has been shown in vitro in trichostrongylid infections. Pritchard et al. (1983) suggested that *Nematospiroides dubius*-specific IgG from mice me-

diated cell adherence to adult stages. Jones and Ogilvie (1972) found anti-*Nippostrongylus brasiliensis* acetylcholinesterase (AChE) antibody in infected rat serum IgG. Rothwell and Merritt (1974) reported that antibodies in IgG fractions from serum of sheep infected with *T. colubriformis* possessed activity against worm AChE. However, the role of AChE in helminth feeding is unknown.

Oxygenation of a basal medium caused a concentration-dependent elevation of feeding by *N. brasiliensis*, which suggests that a significant quantity of oxygen may be obtained by oral uptake in addition to transcuticular diffusion. Oxygenation of serum also elevated ingestion by the nematode, but oxygen-stimulated feeding was decreased in serum from animals with 14- to 16-day-old infections (Bone et al., 1985). Thus, deleterious effects of host immunity on nematode ingestion may also depress their respiration.

The above alterations of helminth feeding, mediated by host immunity, were consistent with previous reports. Ogilvie and Hockley (1968) and Lee (1969) reported that the intestinal cells of *N. brasiliensis* were damaged severely by host immunity. Older infections of *T. colubriformis* also reveal degenerative changes in the worm's intestinal cells (Rothwell et al., 1980). Ballantyne et al. (1978) proposed that changes in feeding reduced the adenylate energy charge in immune-damaged *N. brasiliensis* and *Nematodirus battus*. Thus, suppression of helminth feeding is apparently one effector mechanism of host immunity.

Phagostimulants may have a potential for control of parasitic helminths. Alphey (1983) found that nematicide was more effective on starved versus fed nematodes. Bottjer and Bone (unpublished) found that *T. colubriformis* ingested four- to five-fold greater quantities of radiolabeled antihelminthic in vitro when stimulated by histamine. In contrast, drug uptake was decreased in immune serum. Discovery of phagostimulants for nematodes with little to no pharmacological effect on the host may permit novel means of improving drug efficacy through enhanced uptake by the parasite.

RECEPTION OF CHEMICAL STIMULI

Most studies of chemical reception by nematodes have been indirect. Wright (1983) noted the resemblance of nematode chemosensilla to vertebrate olfactory sense cells. Samoiloff et al. (1973) used laser microbeam irradiation to determine that the spicules of male *Panagrellus silusiae* contained pheromone receptors. Orientation to females required comparison of pheromone concentrations on both spicules. In contrast, Ward (1973) suggested that *C. elegans* compared chemical concentrations with its cephalic receptors. Croll (1977) proposed that a cephalic nerve in only male *C. elegans* was a likely source of pheromone reception.

Pheromone receptors of *N. brasiliensis* are subject to chemical "swamping" or molecular saturation with subsequent loss of the males' responsiveness,

in vitro and in vivo (Bone and Shorey, 1977; Glassburg, et al., 1981). However, excessive rinsing of *T. colubriformis* males restored their responsiveness to female pheromone at an enhanced level, which suggested that sensory adaptation was the basis of the diminished response. Interestingly, exposure of *T. colubriformis* to immune serum also diminished male responsiveness to female pheromone, which suggests immune-mediated disruption of orientation to chemical stimuli (Bone and Bottjer, 1984).

Bone and Bottjer (1985b) reported that mannose residue was involved in pheromone and histamine-induced feeding reception on *T. colubriformis*, based on lectin binding and behavioral assays. Jansson et al. (1984) reported that enzymatic treatment of *C. elegans* with mannosidase or sialidase inhibited attraction of the worm to a bacterial food source but that subsequent receptor turnover renewed responsiveness. Zuckerman and Jansson (1984) summarized a series of steps in nematode chemoreception that are initiated by the binding of chemoattractants to cephalic surface carbohydrates which were exudates of the amphids or other sensilla. Similarly, binding of immunoglobulins to the surface carbohydrates may account for immune-mediated alterations of nematode behavior and physiology. Continued studies of chemoreception by nematodes may reveal important effector mechanisms of host immunity and means of helminth control by sensory disruption.

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ROLE OF CHEMICAL SUBSTANCES FROM FISH HOSTS IN HATCHING AND HOST-FINDING IN MONOGENEANS

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Abstract—Hatching responses to chemical stimuli appear to have evolved independently in different kinds of monogenean skin and gill parasites of fishes, particularly in those parasites associated with bottom-dwelling hosts. Some monogeneans, such as *Entobdella soleae*, have two hatching strategies, responding readily to host skin mucus but hatching spontaneously in small numbers in the absence of the host. Other monogeneans, such as *Acanthocotyle lobianchi*, have abandoned spontaneous hatching and rely entirely on a "sit-and-wait" strategy, but improvements in the speed of hatching provide opportunities to take advantage of brief periods of contact between the eggs and the host. This has led to the loss of ciliated epidermal cells and to the inability to swim. Comparison of the eggs and hatching responses of two unrelated monogeneans, *Leptocotyle minor* and *Hexabothrium appendiculatum*, which share the same dogfish host, reveals evidence of convergence. Small, stable molecules such as urea, excreted by the host, have been implicated as hatching stimulants in monogeneans. There is evidence that host recognition in *E. soleae* is by chemoperception but, in contrast with the lack of specificity of the chemical hatching stimuli, this appears to be of a specific nature.

Key Words—Monogeneans, fish parasites, chemical hatching factors, host-finding.

Most monogeneans are parasites of the skin and gills of fishes and display strict host specificity. In most monogeneans the eggs leave the host and develop on or in the bottom sediment (Kearn, 1986). The eggs have shells of tanned protein and a ciliated larva or oncomiracidium develops within them over a period of time that usually falls within the range of 10–40 days. It is this ciliated larva that has the task of finding the fish host, there being no intermediate host.

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The fish hosts of monogeneans have diversified greatly; they occupy many different kinds of habitat and display a variety of behavior patterns. It is perhaps not surprising that the eggs and larvae of monogeneans have responded to this diversity by adaptations that enhance their chances of infecting their specific hosts. Fishes advertize their presence in a variety of ways. As they swim, their movements disturb the surrounding water, and they may cast shadows on the bottom sediment. It has already been established that the eggs of certain monogeneans hatch when mechanically disturbed, e.g., *Microcotyle salpae* (see Ktari, 1969), and larvae emerge when the eggs of *Entobdella diadema* are plunged into darkness (Kearn, 1982). Fishes are also the source of chemical substances in their skin or gill secretions and in their urine or feces, and there is evidence that the eggs of several monogeneans are stimulated to hatch by such chemical cues (Table 1).

The first evidence that monogenean larvae might respond to chemical hatching factors of host origin was presented by Euzet and Raibaut (1960). They collected and incubated eggs of a polyopisthocotylean monogenean, *Squalonchocotyle torpedinis*, from the gills of an electric ray, *Torpedo marmorata*. They observed that eggs kept in seawater, uncontaminated by the host, hatched only in small numbers but, in the presence of either an electric ray or isolated gills from such a ray, a large number of larvae emerged. Similar evidence for a chemical hatching stimulus was reported by Ktari (1969) in the gill-parasitic polyopisthocotylean *Microcotyle salpae*. A significant feature of the freshly hatched larvae of both *S. torpedinis* and *M. salpae* is that these larvae lack ciliated epidermal cells (see below).

More detailed knowledge of the role of hatching factors from the host has emerged from a study of the skin-parasitic capsalid monogenean *Entobdella soleae*. Its bottom-dwelling, flat-fish host, the common sole (*Solea solea*), survives well in aquaria and the life cycle of the parasite continues in these aquaria, providing a supply of adult parasites and eggs for experimental work.

The larva of *E. soleae* is ciliated, and it has been established that hatching occurs in the absence of the host (Kearn, 1973). When eggs are exposed to the natural cycle of illumination or to an artificial light regime, e.g., 12 : 12 light–dark, hatching is rhythmical, most larvae emerging on each successive day during the first few hours after dawn or after the artificial illumination is switched on. However, when sole body mucus is added to fully developed eggs at any time during the illumination cycle, hatching is greatly enhanced, indicating that sole mucus contains a potent hatching stimulant (Kearn, 1974).

It has been shown by Kearn (1975) that hatching is preceded by ciliary activity on the part of the fully developed oncomiracidium; this activity begins spontaneously in the absence of the host or as a result of stimulation by host mucus. The beating cilia produce rotation of the larva about its longitudinal axis, the rotating head of the larva occupying the corner of the tetrahedral egg

Table 1. MONOGENEANS REPORTED TO RESPOND TO CHEMICAL HATCHING FACTORS FROM THE HOST

Species	Relationship	Microhabitat	Host	Oncomiracidium	Author
Squalonchocotyle torpedinis	Hexabothriid polyopisthocotylean	Gills	*Torpedo marmorata*	Unciliated	Euzet and Raibaut, 1960
Hexabothrium appendiculatum	Hexabothriid polyopisthocotylean	Gills	*Scyliorhinus canicula*	Ciliated	Whittington (in progress)
Microcotyle salpae	Mazocraeidean polyopisthocotylean	Gills	*Box salpa*	Unciliated	Ktari, 1969
Leptocotyle minor	Microbothriid	Skin	*Scyliorhinus canicula*	Ciliated	Whittington (in progress)
Acanthocotyle lobianchi	Acanthocotylid dactylogyridean	Skin	*Raja* spp.	Unciliated	Macdonald, 1974
Acanthocotyle greeni	Acanthocotylid dactylogyridean	Skin	*Raja clavata*	Unciliated	Macdonald and Llewellyn, 1980
Entobdella soleae	Capsalid dactylogyridean	Skin	*Solea solea*	Ciliated	Kearn, 1974
Pseudodactylogyrus bini	Uncertain	Gills	*Anguilla japonica*	Ciliated	Chan and Wu, 1984

where the lid or operculum is situated. A considerable body of evidence suggests that erosion of the cement attaching the operculum to the rest of the shell is brought about by secretory material from gland cells in the head region of the larva. The interval between the commencement of rotation and the escape of the larva from the egg may be as short as 4–5 min.

Thus, *E. soleae* appears to have alternative hatching strategies depending on whether or not there are soles in the vicinity of the fully developed eggs of the parasite. If there is no host in the vicinity, hatching takes place soon after dawn, but relatively small numbers of larvae emerge. Natural selection might be expected to favor spontaneous hatching of the parasite during this period of the day because the sole host rests on the bottom during the hours of daylight and slow-moving oncomiracidia seem more likely to successfully locate and attach themselves to an inactive host than to one that is actively swimming. Prolonged close contact is likely to occur between the host and the eggs of the parasite; the host spends long periods of time resting on the sea bottom and the eggs of the parasite are attached to sand grains by adhesive material on a long appendage (Kearn, 1963a,b). In these circumstances, the ability of the larvae to respond at any time of day to chemical hatching factors from the host will enhance the survival of the parasite.

If the response of the larva of *E. soleae* to chemical hatching factors from the host is to be effective in ensuring host invasion, it is important that the larvae are infective immediately after hatching. This has been tested by placing fully developed eggs on the upper surface of a sole. Kearn (1981) observed that some freshly hatched larvae attached themselves by the sticky areas on the head region before the posterior, hook-bearing attachment organ or haptor had been withdrawn from the shell. After emergence, the haptor was then attached to the skin and the sticky areas were released and this was followed, within about 30 sec, by the loss of all the ciliated epidermal cells of the larva. Thus the oncomiracidia of *E. soleae* appear to be infective immediately after hatching, and there is no requirement for an initial period of free-swimming before infection takes place.

There appears to have been a further development in the monogenean *Acanthocotyle lobianchi*, a skin parasite of rays (*Raja* spp.). The eggs of this parasite, like those of *E. soleae*, have adhesive material on their appendages and stick to sand grains. Macdonald (1974) discovered that these eggs fail to hatch spontaneously when kept in seawater free of contamination by host mucus. Nevertheless, the larvae are able to survive within the egg for as long as 80 days at 13°C. When the eggs are treated with ray mucus, hatching occurs but, unlike *E. soleae*, emergence of the larva takes place with great rapidity (within a few seconds). The larva responds by greatly extending its body and in so doing pushes off the operculum; the opercular cement is already weak in

anticipation of the thrust of the larva. Thus, *A. lobianchi* has speeded up the hatching process in response to a chemical hatching stimulus and appears to rely entirely on a "sit-and-wait" strategy, having abandoned the ability to fall back on spontaneous hatching as a "last-ditch" means of locating the host. Because of the adoption of this strategy, there is no longer a requirement for free-swimming and ciliated epidermal cells are absent. The host must virtually make contact with the eggs if infection is to take place, but the speed of hatching ensures that the larva can take advantage of exceedingly brief periods of contact. A similar evolutionary pathway may have led to the absence of ciliated cells in the larvae of *S. torpedinis* and *M. salpae* (see above).

Parasitologists working at the laboratory of the Marine Biological Association of the United Kingdom at Plymouth, England, have encountered the eggs of some other monogeneans that, apparently, fail to hatch spontaneously. Whittington (work in progress) has found that hatching in the skin parasite *Leptocotyle minor* and the gill parasite *Hexabothrium appendiculatum*, both from the dogfish *Scyliorhinus canicula*, resembles hatching in *A. lobianchi*. Eggs of these parasites fail to hatch spontaneously in the absence of the host but survive for at least 80 days; the opercular cement is weakened in readiness for hatching, which is rapid and stimulated by seawater from a tank containing dogfish. Whittington has observed that the eggs of *L. minor* and *H. appendiculatum* are small, with exceptionally long and slender appendages lacking adhesive droplets. These small eggs are readily kept in suspension even by weak convection currents, and the slender appendage makes a contribution to their slow rate of sedimentation because eggs sink faster if the appendage is experimentally removed. Thus the appendages may serve to keep the eggs in suspension, like the silk threads produced by small aerial spiders.

This has led Whittington to suggest that currents produced by dogfish foraging near the bottom may lift resting eggs off the bottom; these eggs may then drift sufficiently close to swimming dogfish for hatching to be stimulated by chemical substances from the host's body. The retention of the ciliated cells and the ability of the larvae of *L. minor* and *H. appendiculatum* to swim, may reflect the fact that drifting eggs are unlikely to make intimate contact with the bodies of moving hosts. Whittington has suggested that the striking similarity between the shapes and sizes of the eggs and the hatching behavior of these unrelated parasites (*L. minor* is a microbothriid and *H. appendiculatum* is a polyopisthocotylean) may be an example of convergent evolution that has arisen because of the exertion of similar selection pressures on the two parasites by the same host.

In contrast with this apparent convergence between dogfish parasites, the skin parasite *A. lobianchi* and the gill parasite *Rajonchocotyle emarginata*, both of which inhabit *Raja* spp., appear to have evolved different strategies for host

invasion. Previous workers had failed to hatch the eggs of *R. emarginata*, but Whittington and Kearn (1986) have found that the ciliated larvae of this parasite do hatch spontaneously but have a relatively long incubation period (44–47 days at 13–14°C). Moreover, these eggs fail to respond when treated with mucus from ray skin or gills.

There are indications that the chemical hatching factors in the skin mucus of soles and rays are small and stable molecules (Kearn, 1974; Kearn and Macdonald, 1976). Exploratory tests with potential hatching stimulants implicated urea, ammonium chloride, and arginine in the hatching of *E. soleae*, but the only substance tested that stimulated hatching in *A. lobianchi* was urea (Kearn and Macdonald, 1976). Incubation with urease was found to be a most effective way of destroying the stimulatory properties of ray mucus for *A. lobianchi* eggs, but similar tests with sole skin washings and eggs of *E. soleae* gave inconsistent results. Recent work by Whittington (in progress) suggests that extrapure urea may be less effective as a hatching stimulant for *E. soleae* eggs than less pure grades, and he has found that lower grade urea rarely proves as effective as sole mucus. This suggests that a trace substance present in commercial urea may stimulate hatching in the eggs of *E. soleae* or, perhaps more likely, that the effectiveness of urea may be enhanced by the presence of one or more other substances. This contrasts with the situation in *A. lobianchi*, the unhatched larvae of which respond equally readily when treated with extrapure urea or lower grade urea. Furthermore, urea appears to be just as effective as ray mucus. Preliminary observations by Whittington (work in progress) indicate that the eggs of *L. minor* and *H. appendiculatum* respond to urea in a similar way.

Kearn and Macdonald (1976) estimated the levels of urea in ray and sole skin mucus. The levels of urea in ray skin mucus are relatively high, providing a strong hatching signal for the eggs of *A. lobianchi*. The levels of urea in sole skin mucus are much lower, and this may have led to the development of sensitivity on the part of unhatched oncomiracidia of *E. soleae* to a combination of substances present in mucus, one component of which may be urea.

Kearn (1974) showed that the chemical hatching stimulus for the eggs of *E. soleae* is nonspecific, the eggs readily hatching if treated with skin washings from a variety of fishes including whiting (*Merlangius merlangus*) and rays. This lack of specificity seems at first sight to be distinctly disadvantageous since hatching might be induced by nonhost fishes or by other bottom-dwelling organisms. However, this nonspecific stimulus may not be disadvantageous if soles are locally abundant, because, in these circumstances, the hatching stimulus is more likely to be provided by a sole than by any other organism. Moreover, even if hatching were stimulated by a nonhost organism, it is most unlikely that the larvae would attach themselves to it. When oncomiracidia are presented experimentally with a choice of skin samples, either in the form of

detached scales or small pieces of skin from more than one fish species, the larvae of *E. soleae* show a strong preference for sole skin, even in total darkness (Kearn, 1967). Larvae also show a preference for agar blocks which have been in contact with sole skin rather than similar blocks soaked in seawater. This indicates that chemoperception may be important in host identification and suggests that the chemical cues provided by the host are specific, in contrast with the nonspecific nature of the hatching stimulus. However, there is not yet any evidence that oncomiracidia are attracted to the sole by chemotaxis, and recognition of the host may rely on contact chemoperception.

The monogeneans that are known to respond to chemical hatching factors from the host are listed in Table 1. Three points are worth stressing. First, the phenomenon appears to have arisen independently several times. Secondly, the hosts that provide the chemical hatching cues may be teleosts or elasmobranchs but are usually flat-fishes or round-bodied hosts that feed on the bottom. This is perhaps not surprising because most monogenean eggs come to rest on the bottom, and the effectiveness of a hatching response to chemical substances of host origin depends on contact or close proximity between the hosts and the fully developed eggs. Thirdly, there is a tendency for the oncomiracidia to dispense with ciliated cells.

Hatching responses to chemical substances from the host of the kind described here have rarely been reported in other flatworm parasites. Shinn (1983) has presented evidence to suggest that in the parasitic turbellarian, *Syndisyrinx franciscanus*, the intestinal fluid of its sea-urchin host stimulates the production of a hatching fluid by the parasite. Host digestive secretions play an important role in hatching of some cestode eggs, perhaps partly by stimulating larval activity, but their main role is concerned with erosion of the egg envelopes (see, for example, Caley, 1975; Holmes and Fairweather, 1982) or the opercular cement (Kennedy, 1965). The molluscan hosts of digeneans are slow-moving, bottom-dwelling organisms, and contact between these hosts and digenean eggs seems likely to be a frequent occurrence. In similar circumstances, several monogeneans have independently acquired a hatching response to chemical substances from the host, and it is rather surprising that there appear to be no reports of a similar phenomenon in those digenean eggs that hatch in water. However, some digeneans have exploited the opportunities offered by accidental ingestion of their eggs by their molluscan hosts, hatching apparently being stimulated by host digestive enzymes and/or physicochemical conditions in the gut (Smyth and Halton, 1983). Smyth and Halton have pointed out that this area has received little attention and further study seems likely to be rewarding.

Host location by digenean miracidia that hatch in water has been studied more intensively than in monogeneans (Smyth and Halton, 1983). Striking changes in the behavior of the free-swimming miracidia have been reported on

the addition of snail-conditioned water, for example in *Schistosoma mansoni* but comparable changes in the behavior of oncomiracidia have not been reported.

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CHEMICAL COMMUNICATION IN HERMAPHRODITIC DIGENETIC TREMATODES

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Abstract—Adult hermaphroditic digenetic trematodes show a tendency to pair or aggregate in vivo, on the chick chorioallantois, and in vitro. Intraspecific pairing studies on *Echinostoma revolutum*, *Leucochloridiomorpha constantiae*, and *Amblosoma suwaense* are reviewed. Lipophilic excretory–secretory products are involved in chemical communication in hermaphroditic digeneans. Free sterols are involved in chemical attraction in *L. constantiae* and *E. revolutum* and sterol esters play a similar role in *A. suwaense*. In vitro pairing between *E. revolutum* and various other digenean species suggest that interspecific pairing occurs in Digenea and that nonspecific factors are involved.

Key Words—Platyhelminths, Trematoda, hermaphroditic digeneans, intraspecific pairing, interspecific pairing, behavior, chemical communication, pheromones, lipids, sterols.

INTRODUCTION

Except for the schistosomes and some didymozoids, digeneans are hermaphroditic. This paper examines studies on chemical communication in adult hermaphroditic digeneans. Some information on this topic has been presented in Nollen's (1983) review on sexual reproduction in parasitic flatworms, and Bone's (1982) review on reproductive chemical communication in platyhelminths.

Digenetic trematodes have complex life cycles and various larval stages. The first intermediate host in the digenean cycle is usually a specific snail, in which intramolluscan stages develop. In monoecious digeneans, cercariae released from sporocysts or rediae encyst on a substratum or in a second inter-

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mediate invertebrate or vertebrate host. The encysted metacercariae (cysts) are infective to the definitive vertebrate host and are usually acquired by the feeding habits of the host. In the laboratory, cysts are administered per os to experimental definitive hosts and metacercariae usually excyst in the intestine. Excysted metacercariae establish in more or less specific sites in the intestine or its ramifications. How excysted flukes locate specific sites is not well understood, and this subject has been reviewed by Ulmer (1971) and Kemp and Devine (1982).

Laboratory studies on chemical communication have used monoecious digeneans that have little or no economic significance, i.e., *Echinostoma revolutum*, *Zygocotyle lunata*, *Haematoloechus medioplexus*, *Leucochloridiomorpha constantiae*, and *Amblosoma suwaense*. In the future, representative fasciolids may provide interesting models for chemical communication studies. Interference with chemical communication in fasciolids could provide a means for biological control of these important veterinary and medical trematodes.

PHEROMONES

Chemical communication in animals is mediated by pheromones. The parasitology literature indicates that helminthologists use the term essentially as defined by Karlson and Luscher (1959), i.e., pheromones are substances emitted by an organism which can elicit a specific behavioral or developmental reaction in another of the same or closely related species. Both primer and releaser pheromones have been described for behavioral activities associated with digeneans (Bone, 1982). Primers cause physiological changes without immediate accompanying behavioral responses and influence behavior through hormonal changes. Releasers cause an immediate change in the behavior of the recipient and act directly on the recipient's nervous system (Wilson and Bossert, 1962). Other pheromones described for insects and arachnids, i.e., alarm, trail, marking, have not been examined in trematodes.

The term excretory-secretory products or simply ES products has been used in trematode chemoattraction studies (Fried and Shapiro, 1975; Fried et al., 1980a, 1983; Bennett and Fried, 1983). This term refers to excretions and secretions released by trematodes either in vivo or into an in vitro cultivation or maintenance medium (Fried, 1978). Some of these ES products have pheromonal information. Because of the small size of most digeneans, it is difficult to associate the release of ES products with a particular system (von Brand, 1973; see Source of Pheromones, below). ES products have also been described in trematode immunology studies where they serve as a source of antigens (Rotmans et al., 1981; Sandeman and Howell, 1981; Irving and Howell, 1982).

RECEPTORS

Studies on the reception of stimuli in hermaphroditic digeneans are sparse (Kemp and Devine, 1982). Scanning electron microscopy (SEM) and light level argentation studies have shown that larval and adult digeneans have various types of sensory papillae, some of which are probably chemoreceptors (Hoole and Mitchell, 1981; Fried and Fujino, 1984). However, chemoreceptors have not been unequivocally identified in monoecious digeneans, and there are no electrophysiological studies that elucidate their role in chemoreception. In insect physiology, the use of the electroantennogram has allowed for quantitative studies in sensory physiology. Until similar methodology is developed in trematode physiology, sensory studies will remain speculative.

METHODOLOGY

Pairing, i.e., the tendency of two monoecious digeneans to come together, and clustering, i.e., the aggregation of three or more worms have been studied in vivo, on the chick chorioallantois and in vitro (Fried and Roberts, 1972; Fried et al., 1980a, b; Fried and Alenick, 1981; Fried and Robinson, 1981).

For in vivo studies on *Echinostoma revolutum*, experimental hosts were fed metacercarial cysts (Fried and Weaver, 1969); in brachylaimid studies, free (unencysted) metacercariae were inoculated per cloaca into domestic chicks (Fried and Harris, 1971; Fried and Schnier, 1981). At necropsy, observations were made on pairing or clustering of worms in their normal sites (Fried and Roberts, 1972; Fried and Alenick, 1981; Fried and Robinson, 1981).

To eliminate host factors associated with worm migration and establishment in vivo, pairing and clustering have been studied on the chick chorioallantois of domestic chick embryos maintained in ovo or in vitro (Fried and Roberts, 1972; Fried et al., 1980b).

Pairing studies that exclude organisms other than the parasites have been done in vitro in 3.5-, 5.0-, and 8.5-cm-diameter plastic Petri dishes usually maintained in an incubator or on a slide warmer at 35–41°C (Fried and Roberts, 1972; Fried et al., 1980a; Fried and Robinson, 1981; Fried and Pallone, 1984). In this design, a 1% agar substratum is prepared in the dish, since worms move better on the agar than the plastic. A sterile Locke's (Paul, 1975) solution overlay is usually used, and worms migrate at the agar–Locke's interface. In this nonbarrier design, worms show a maximal tendency to pair. However, this design does not exclude worm thigmotaxis, i.e., worm-to-worm contact pairing. In the nonbarrier design worms are usually started 2 cm from each other and the tendency of one worm to attract the other is examined at various intervals up to 24 hr.

Worms in contact with or within 5 mm of each other are considered paired (Fried and Roberts, 1972; Fried et al., 1980a; Fried and Pallone, 1984). This criterion for pairing has also been used by Perkins and Fried (1982) in studies on free-living planarians in the genus *Dugesia*. Both *Dugesia* and the digeneans often show noncontact pairing, i.e., worms come within 1–2 mm of each other, do not make contact, and then retreat to about 5 mm from each other. In nonbarrier studies initial starting distance is a factor in subsequent pairing. *E. revolutum* started more than 8 cm apart did not pair, whereas those started less than 8 cm did (Fried and Pallone, 1984). Other factors involved in *E. revolutum* pairing are temperature and the overlay medium. More pairing occurred at 39°C than 35°C, and an overlay of sterile Locke's was better than the defined medium NCTC 135 (Fried and Pallone, 1984). In our nonbarrier studies with monoecious digeneans and planarians, a single worm did not pair with a dead worm or an inert object.

Attraction of a single worm toward either a filter paper disk or silica gel square containing worm homogenate or ES products has been studied in Petri dish cultures (Fried and Gioscia, 1976; Fried, et al., 1980a; Fried and Robinson, 1981). These studies begin by placing the worm equidistant between the experimentally impregnated disk or square and its control. Migration toward or contact with the experimental disk or square is considered attracted.

In barrier designs, one worm is isolated from the other, thus eliminating the possibility of worm thigmotaxis. Such designs have been used to study heterosexual and homosexual chemical attraction in schistosomes maintained in polycarbonate linear chambers (Imperia et al., 1980; Eveland et al., 1982, 1983). The barrier usually consists of a chimney made of dialysis tubing (Imperia et al., 1980) or a dialysis sac (Fried et al., 1980a). Single *Echinostoma revolutum* adults migrated significantly toward a dialysis sac containing other *E. revolutum* (Fried et al., 1980a). In these designs worms isolated in the sac are designated "releasers" and those outside, "migrators" (Belosevic and Dick, 1980; Fried and Wilson, 1981a). In a recent study, *E. revolutum* pheromonal dosage was examined in a barrier design where either two, four, or six releasers were isolated in a cylindrical stainless steel screen (40-mesh) chimney and a single migrator was allowed to move toward or away from the barrier (Figure 1). The results showed that four and six releasers were better at attracting a single migrator than two releasers (Figure 2) (Fried and Wilson, 1981a).

A recent study has allowed us to track *E. revolutum* adults in an 0.8 agar–Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Worms survived up to 48 hr in the medium and left distinct acetabular tracks in the agar. Some worms showed directed movement toward each other, suggesting that chemotaxis was involved (Fried and Vates, 1984).

In most studies statistical significance of pairing and chemoattraction data at each time period were determined using either the chi-square test or the two-

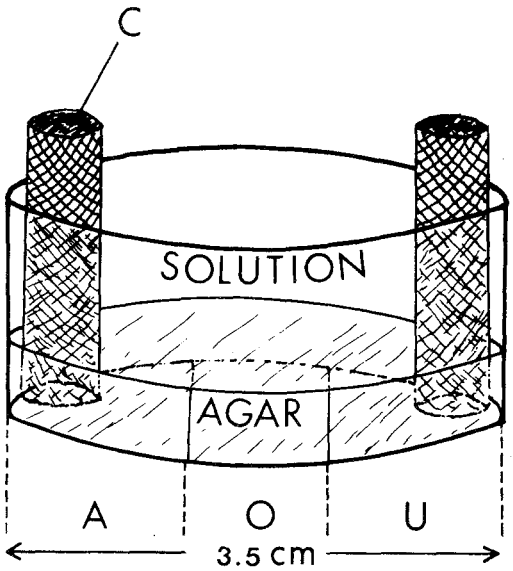

FIG. 1. Petri dish chamber used in pheromonal studies. C = chimney; A = attraction zone; O = neutral zone; U = unattraction zone. Reprinted from Fried and Wilson (1981a) with permission of the Helminthological Society of Washington.

sample Z-test for proportions (Book, 1977). Based on computer simulations, the probability that two randomly selected points in a 5-cm Petri dish are within 5 mm of each other is less than 0.15 (Wagner, 1975).

SOURCE OF PHEROMONE

An adult hermaphroditic digenetic trematode is a complex metazoan with numerous systems; i.e., tegumentary, nervous, male and female reproductive, digestive, excretory, and others (Smyth and Halton, 1983). Each worm has several pores through which ES products may be released, i.e., mouth, gonopore, excretory. The worm has a complex tegument which may release ES products that play a role in chemical communication. Most adults are less than 1 cm and are not easy to manipulate experimentally. Attempts to collect ES material from the mouth will also yield products from the gonopore (Figure 3) (Bailey and Fried, 1977). Intubation of worms is difficult, although Lutz and Siddiqi (1971) cannulated and expressed fluids from the excretory system of *Fasciola gigantica*. Recently, Gallo and Fried (1984) designed a micropipet apparatus that allowed for the collection of ES products from the excretory system of *E. revolutum*. Ligation experiments have also been used to block certain systems (Fried and Appel, 1977), but the results of ligation experiments

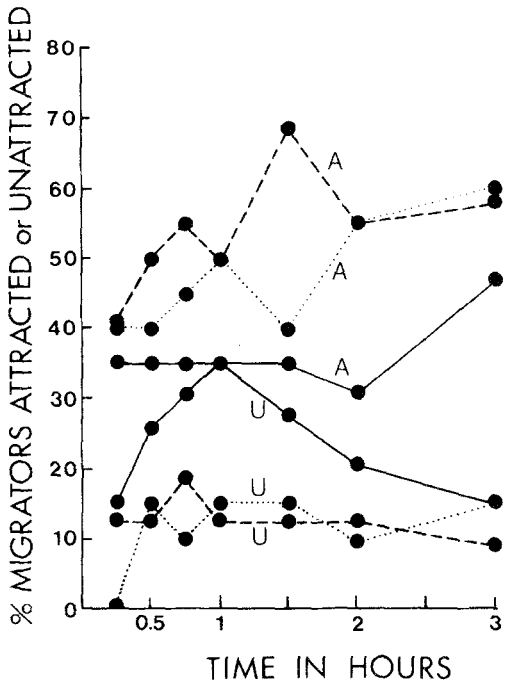

FIG. 2. Summary of migrators attracted (A) or unattracted (U) to releasers in chimney. Solid line = two releasers in chimney; broken line = four releasers in chimney; and dotted line = six releasers in chimney. Reprinted from Fried and Wilson (1981a) with permission of the Helminthological Society of Washington.

should be accepted with caution since Halton and Arme (1971) and Nollen and Nadakavukaren (1974) have shown that ligation of adult trematodes may cause tegumentary damage.

PAIRING PHENOMENA—ADVANTAGES OF PAIRING

Observations on adult hermaphroditic digeneans both in the wild and the laboratory indicate that isolated worms are rare. Digeneans are usually found paired or clustered in their natural host site. In 15 of 16 cases, the zoogonid, *Deretrema* sp., was found in pairs in the gallbladder of naturally infected flashlight fish, *Anamalosis katopteron*, and in only one case was a solitary fluke found (Burn, 1980).

In single-worm infections of various digeneans, normal growth and development does not occur (Nollen, 1983). Fried (1962) found that the eyefluke, *Philophthalmus hegeneri*, when maintained singly in experimentally infected domestic chicks, stopped growing after 20 days, and failed to produce fertile

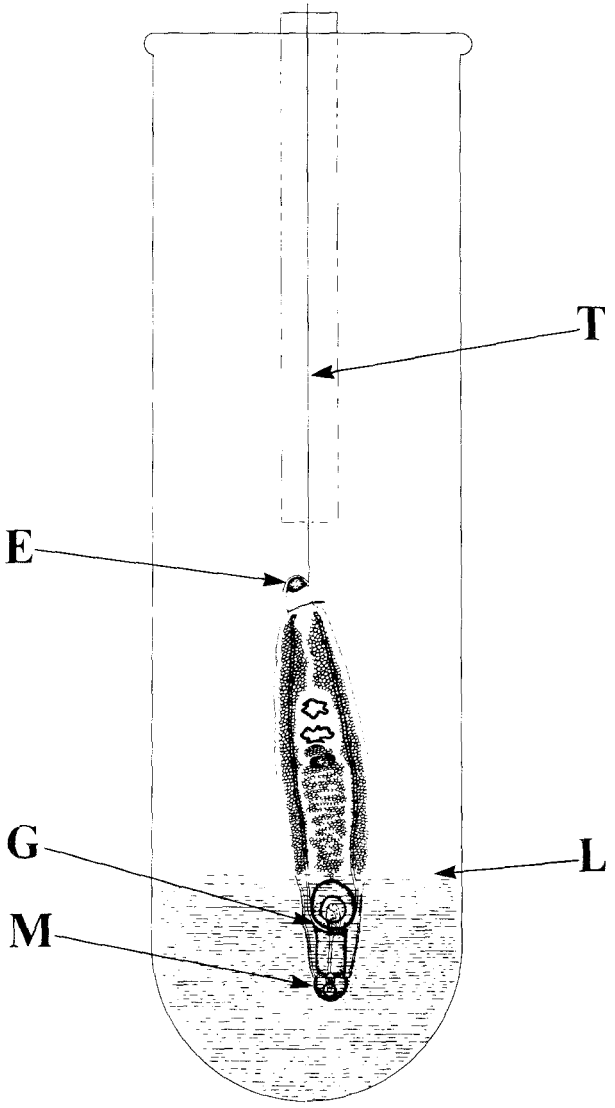

FIG. 3. *Echinostoma revolutum* adult suspended by a thread (T) from its posterior end into Locke's solution to collect excretory–secretory products from the mouth (M) and gonopore (G). The worm can also be suspended from its anterior end to collect ES products from the excretory pore (E).

eggs. An eyefluke of birds, *P. burilli*, described in Australia by Howell and Bearup (1967), showed a pattern similar to *P. hegeneri*. Moseley and Nollen (1973) confirmed Fried's (1962) findings on *P. hegeneri* and Nollen (1983) suggested that contact with another eyefluke of the same species is required for normal growth and sexual maturity of *P. hegeneri*. This may be due to mutual tactile stimulation by worms, or stimulation of growth by the presence of sperm in the seminal receptacle (Nollen, 1983).

Sogandares-Bernal (1966) observed that monometacercarial infections in the domestic cat with *Paragonimus kellicotti* never developed beyond the wandering preadult stage in the abdominal cavity. When these hosts were later infected with other metacercariae, preadults paired and developed to mature worms in the lungs. Sogandares-Bernal postulated that chemotactic factors are involved in both worm attraction and sexual maturation in *P. kellicoti*. Worms do not find each other by chance alone and factors other than "host mediated" are involved in worm chemoattraction.

Foreyt et al. (1977) studied the pairing tendency of *Fascioloides magna* in naturally infected white-tailed deer, *Odocoileus virginianus*. Pairing of worms was important for normal capsule formation in the liver and for worm maturation.

Leucochloridiomorpha constantiae AND *Amblosoma suwaense*

In the early 1970s my students and I became interested in following Sogandares-Bernal's (1966) study, but with a simpler and less expensive model than his *Paragonimus*-cat system. Through the courtesy of Dr. William J. Bacha, Jr., of Rutgers University, Camden, New Jersey, we obtained viviparid snails, *Campeloma decisium*, that were naturally infected with free (unencysted) metacercariae of *Leucochloridiomorpha constantiae* (Brachylaimidae). The metacercaria of *L. constantiae* is precocious and when inoculated per cloaca in the domestic chick achieves sexual maturation within three or four days in the bursa of Fabricius (Harris et al., 1972). In our chick studies we found that this organism invariably paired or clustered in the bursa of Fabricius (Fried and Harris, 1971; Harris et al., 1972). Paired worms produced a significantly greater number of eggs and were larger than singles (Fried and Harris, 1971; Fried et al., 1980b). Single worms were capable of autocopulation, but in situations where worms were paired, we observed cross- but not autocopulation (Fried and Harris, 1971). We concluded that there were genetic and reproductive advantages associated with pairing in *L. constantiae*.

To eliminate definitive host-mediated factors, Fried and Roberts (1972) examined pairing and clustering in the chick chorioallantois of in ovo prepared embryos (Figure 4). In about 50% of the trials, worms were either paired or clustered, suggesting that this reaction could occur in the absence of bursa of

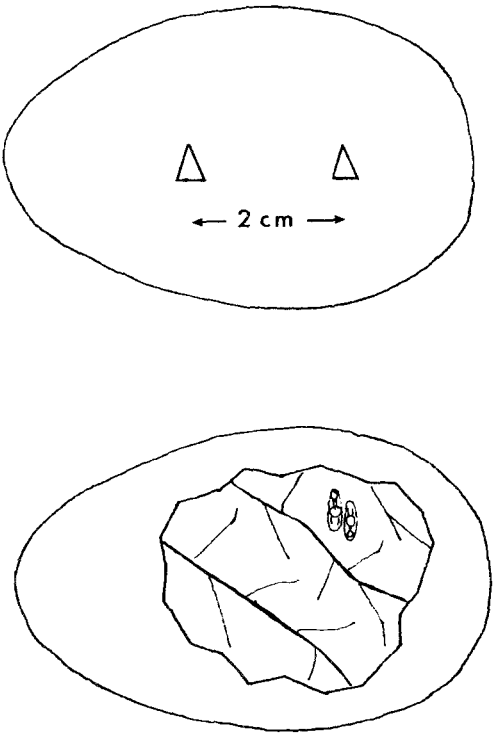

FIG. 4. In ovo chorioallantois technique in which single *L. constantiae* worms were placed in each window, 2 cm from one another (top). Worms often paired on the chorioallantois (bottom). See Fried and Roberts (1972) for details.

Fabricius factors. This study was later confirmed using chick embryos cultivated in vitro (Figure 5) (Fried et al., 1980b).

Fried and Roberts (1972) also examined worm-to-worm attraction in vitro at 37.5°C in 5-cm Petri dishes containing an agar substratum and a Locke's overlay. Metacercariae were started 2 cm from each other and worms in contact or within 5 mm of each other were considered paired (Figure 6). These experiments were maintained up to 24 hr, and we observed overall about 60% pairing. In vitro pairing was very similar to what we had already observed both in the domestic chick and on the chorioallantois.

In unpublished histochemical studies, Tom Roberts found most obvious change in lipids and little change in proteins, carbohydrates, and mucosubstances of *L. constantiae* metacercariae incubated in the Petri dish bioassay. Harris and Cheng (1973) using oil red O histochemical staining procedures reported neutral lipid accumulation in the intestine and release from the mouth in *L. constantiae* metacercariae maintained in Locke's solution. These histochem-

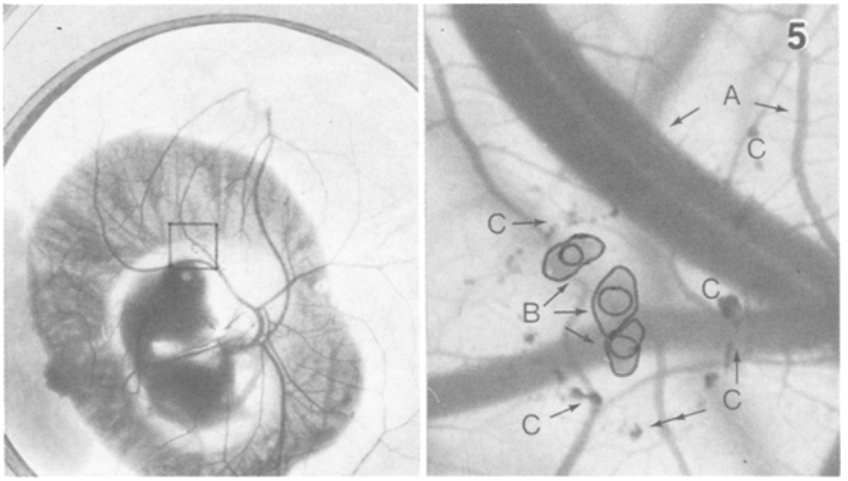

FIG. 5. *L. constantiae* maintained in chick embryos cultivated in vitro. Photo on the left shows a chick embryo cultivated in vitro in a Petri dish. Details of the inset are shown in the photograph on the right. Three *L. constantiae* worms (B) are clustered on the chorioallantois. Worms have been outlined in India ink for clarity. Abbreviations: A = blood vessels; C = hematin-like material associated with worms. See Fried et al. (1980b) for details.

ical findings were confirmed by Fried and Shapiro (1975), who also used thin-layer chromatography (TLC) to show accumulation and release of lipids by this metacercaria. TLC showed that free sterol was the major neutral lipid accumulated and egested by this organism and argentation-TLC of the free sterol fraction showed only cholesterol.

Fried and Gioscia (1976) obtained ES products from *L. constantiae* metacercariae and extracted them in chloroform–methanol (2:1) (Folch et al., 1957). TLC analysis of the material showed only free sterol in 12 of 15 trials. When this material was placed on filter paper disks in the Petri dish bioassay, a significant number of worms were attracted to the disks. Larger amounts of material generally produced greater worm attraction than smaller amounts. We stated that free sterol, presumably cholesterol, was a presumptive chemoattractant for *L. constantiae*. Subsequently, Berger and Fried (1982) used quantitative densitometric TLC to confirm that free sterol was the major ES lipophilic product of *L. constantiae* metacercariae and reported that worms released an average of 3.2 ng of free sterol/metacercaria/hr.

Through the courtesy of Dr. William F. Font, University of Wisconsin, Eau Claire, Wisconsin, we obtained another brachylaimid, *Amblosoma suwaense*, from *C. decisum* snails in Wisconsin. This free metacercaria is larger

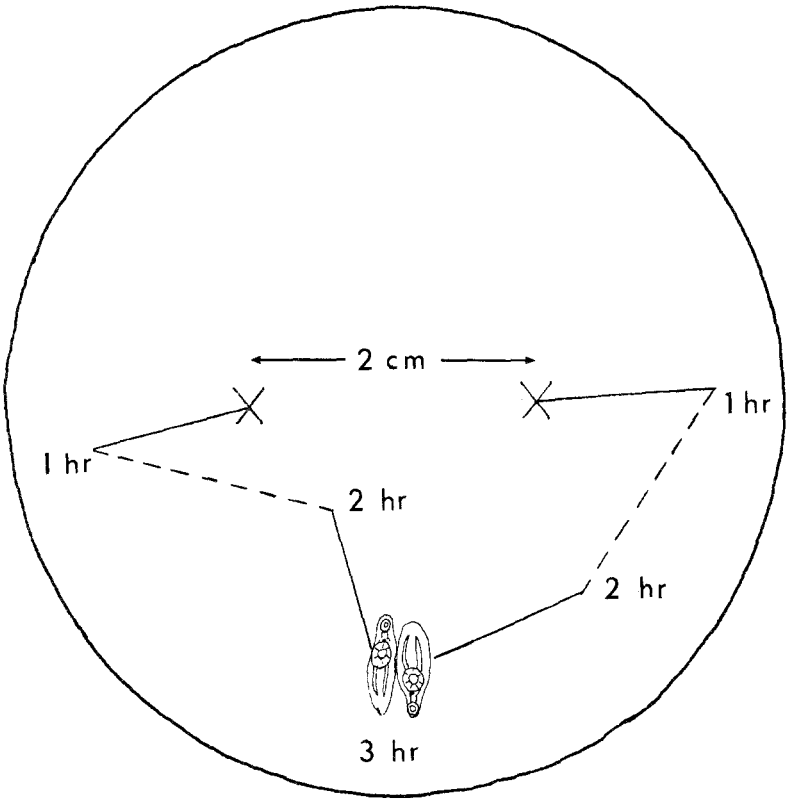

FIG. 6. A diagram showing worm positions of *Leucochloridiomorpha constantiae* metacercariae in the Petri dish culture at the start (X), and at 1, 2, and 3 hr. In this experiment worms paired at 3 hr. See Fried and Roberts (1972) for details.

and more robust than *L. constantiae* and lives between the shell and digestive gland of the snail. This organism also shows a tendency to pair and aggregate in Petri dish bioassays (Fried and Robinson, 1981). Interestingly, its major lipophilic ES product is sterol ester and free sterol is a minor fraction (Figure 7) (Fried and Robinson, 1981; Berger and Fried, 1982). When *A. suwaense* lipids were preparatively isolated and tested in the Petri dish bioassay, *A. suwaense* metacercariae were attracted to sterol esters, but not free sterols (Fried and Robinson, 1981). Berger and Fried (1982) used densitometric TLC to quantify these lipids and found that *A. suwaense* released a mean of 128 ng of sterol ester/metacercaria/hr compared to a mean release of free sterol of 49 ng/metacercaria/hr.

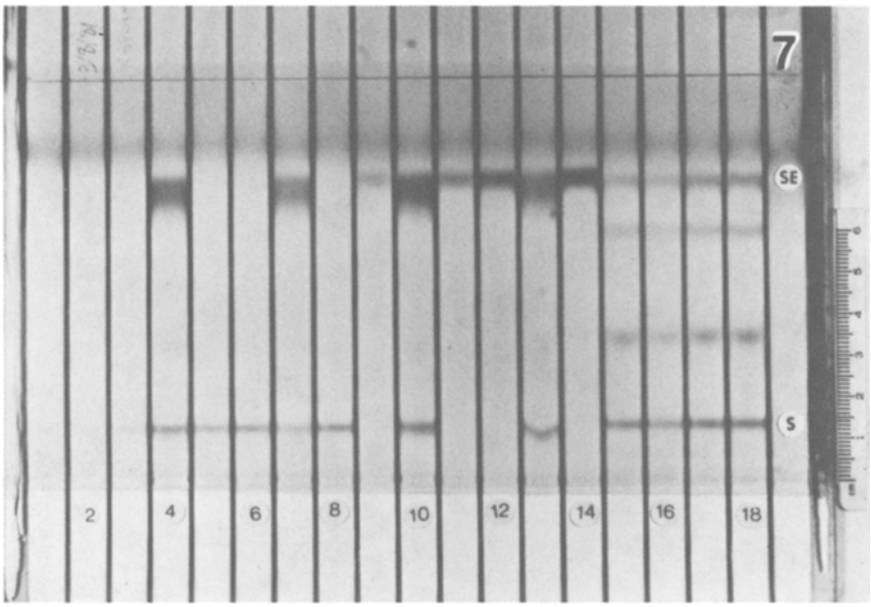

FIG. 7. A thin-layer chromatogram showing sterol ester (SE) and free sterol (S) excretory–secretory products of *Amblosoma suwaense* (lanes 4, 7, and 10). Other lanes contain various neutral lipid standards. See Berger and Fried (1982) for details.

Echinostoma revolutum

Echinostoma revolutum has been maintained in my laboratory since the late 1960s (Fried and Weaver, 1969). This robust fluke reaches 1–2 cm in length and 5–10 mg in experimentally infected domestic chicks. It becomes sexually mature in about nine days and survives for up to six weeks in the domestic chick (Fried, 1984).

Monometacercarial studies of *E. revolutum* in the domestic chick have produced adults with viable eggs, indicating that this species can self-fertilize under conditions that preclude cross-fertilization (Fried and Alenick, 1981). Worms from multiple infections were mainly paired or clustered, and both the number and percentage of hatched eggs were greater in multiple- than single-worm infections. Thus, at least in the domestic chick, reproductive advantages were associated with the tendency to pair or cluster.

Fried et al. (1980a) studied pairing of *E. revolutum* adults in vitro in agar–Locke's Petri dish cultures. Worm-to-worm pairing of adults was highly significant. Chemically excysted metacercariae examined under similar conditions did not pair.

In a modified Petri dish bioassay (cover slips placed in the dish to provide a linear channel), Fried et al. (1980a) observed significant migration of a single

E. revolutum adult to echinostomes in dialysis sacs. TLC analysis of fluids released into the medium from worms in the sac in the absence of another worm outside the sac showed lipids. Oil red O histochemical studies of *E. revolutum* adults have demonstrated neutral lipids in the excretory tubes and bladder and the intestinal ceca were lipid negative (Fried and Morrone, 1970; Fried and Appel, 1977; Butler and Fried, 1977). The *E. revolutum* lipid excretory pattern is different from that of brachylaimids, but is similar to that of *Fasciola hepatica* where lipids are excreted across the epithelial cells of the excretory system (Burren et al., 1967).

To study the activity of ES products released from *E. revolutum*, ES products were collected in Locke's, extracted in chloroform–methanol (2 : 1) (Folch et al., 1957), and separated into hydrophilic and lipophilic fractions. Silica gel squares impregnated with these fractions were tested in modified Petri dish bioassays. The results showed that single adults of *E. revolutum* were very significantly attracted to lipophilic but less significantly attracted to hydrophilic fractions (Fried et al., 1980a).

To further analyze lipophilic fractions, *E. revolutum* adults were extracted in chloroform–methanol (2 : 1) and individual lipophilic fractions were isolated preparatively (Fried and Sherma, 1982) on silica gel sheets developed in the Skipski et al. (1965) dual-solvent system which provides good separation of free sterols from glycerides. When the sterol, triglyceride-free fatty acid, sterol ester, and phospholipid fractions were tested in the bioassay, attraction only occurred with the sterol band. The band that remained at the origin contained phospholipid and other polar compounds and acted as a chemorepellent (Fried et al., 1980a).

Analyses of free sterols in *E. revolutum* by GLC and TLC have shown that the major free sterol is cholesterol (Barrett et al., 1970; Fried and Boddorff, 1978; Fried and Shapiro, 1979). An attempt to use authentic cholesterol as an attractant for *E. revolutum* in the Petri dish bioassay was equivocal (Fried et al., 1980a), a finding similar to that of Roberts and Thorson (1977a) in their heterosexual chemoattraction studies on *Nippostrongylus brasiliensis*.

With the help of Dr. David J. Chitwood and his colleagues in the Insect Physiology Laboratory, United States Department of Agriculture, Beltsville, Maryland, we have begun to examine *E. revolutum* sterols with the goal of determining if individual sterols are pheromones. Using the powerful technique of capillary GLC as well as capillary GLC–mass spectrometry, Chitwood et al. (1985) have identified the relative percentage of sterols in *E. revolutum* adults as follows: cholesterol, 96.73; 24-methylcholesterol, 1.06; 24-ethylcholesterol, 0.87; desmosterol, 0.72; 24-methylenecholesterol, 0.28; 24*Z*-ethylidenecholesterol, 0.13; lathosterol, 0.09; 24-methylcholesta-5,22-dienol, 0.04; 24-ethylcholesta-5,22-dienol, 0.04; cholesta-7,24-dienol, 0.04. Chitwood et al. (1985) concluded that one should not speculate that any of the sterols identified are the lipophilic substance or substances involved in chemoattraction in *E.*

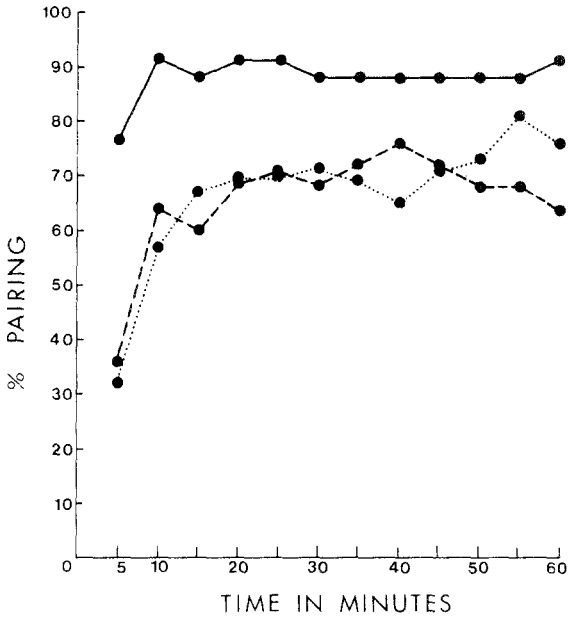

FIG. 8. Summary of interspecific and intraspecific pairing studies with *Echinostoma revolutum* and *Zygocotyle lunata*. Solid line = *E. revolutum* intraspecific studies; broken line = *Z. lunata* intraspecific studies; dotted line = interspecific studies. Reprinted from Fried and Wilson (1981b) with permission of the Helminthogical Society of Washington.

revolutum. Because of the possible occurrence of additional components in column chromatographic or TLC-prepared free sterol fractions other than free sterols, positive identification of the lipophilic chemoattractive substance requires further purification and bioassay.

INTERSPECIFIC PAIRING

Interspecific pairing in vitro has been reported by Fried and Jacobs (1980) for *Echinostoma revolutum* matched against either *Zygocotyle lunata* or *Leucochloridiomorpha constantiae*. Fried and Wilson (1981b) observed that interspecific pairing between *E. revolutum* and *Z. lunata* more closely matched the intraspecific pattern of *Z. lunata* than that of *E. revolutum* (Figure 8). Fried and Leiby (1982) observed significant interspecific pairing of *Haemotoloechus medioplexus* and *E. revolutum* at both 21 and 30°C. Whereas transience of interspecific pairing was noted in nematodes (Roberts and Thorson, 1977b), this was not the case in some trematode interspecific combinations (Fried and Wilson, 1981b; Fried and Leiby, 1982). Both *Z. lunata* and *H. medioplexus* also emit significant amounts of free sterol into a nonutrient medium, indicating that

free sterols may be involved in interspecific as well as intraspecific pairing. Interspecific pairing of trematodes indicates that nonspecific substances are involved in chemoattraction of hermaphroditic digeneans.

CONCLUDING REMARKS

The studies reported herein suggest that monoecious digeneans pair or aggregate both in vivo and in vitro. SEM observations on *E. revolutum* show no damage in the tegument of worms maintained in the agar–Locke's bioassay for 2 hr (Fried and Pallone, 1984). Presumably in vitro observations are indicative of what is happening in the host.

Pairing and aggregation of digeneans may have nutritive, social, developmental, and reproductive advantages for digeneans. In vitro observations on pairing in *L. constantiae* or *E. revolutum* have failed to show copulatory behavior. Perhaps this reflects inadequacies in the in vitro environment, or possibly copulatory phenomena are independent of aggregative behavior in trematodes as reported in nematodes (Bonner and Etges, 1967; Marchant, 1970).

The studies reported herein suggest the importance of worm lipophilic factors in pairing and aggregative behavior in monoecious digeneans. Of particular importance are free sterols which are involved in chemoattraction in *L. constantiae* and *E. revolutum* and sterol esters which play a similar role in *A. suwaense*. Whether lipids are pheromonal in hermaphroditic digeneans or serve as carriers, as described by Sonenshine et al. (1981) for the *Dermacentor* sex pheromone, 2,6-dichlorophenol, is not known.

Lipids also play a role in chemical communication of dioecious trematodes. Extracts of *Schistosoma mansoni* males in organic solvents (presumably lipids) enhance growth and vitellogenesis of unisexual females maintained in vitro (Shaw et al., 1977). Lipids released from the tegument of male *S. mansoni* may be involved in heterosexual chemical attraction (Haseeb et al., 1984, 1985).

Saladin (1979) proposed "behavioral parasitology" as a discipline that would combine ethology, sensory physiology, and parasitology. He also invoked the Timbergen (1963) caveat, which suggests that familiarity with an organism is essential before problems can be attacked by experimentation. This suggestion is important for a better understanding of chemical communication in trematodes. With the aid of videotechnology it should be possible to examine more closely digenean behavior in vitro. Observations on single versus paired worms should elucidate further on migration, locomotion, oviposition, and mating behavior.

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INTERNAL CHEMICAL COMMUNICATION WITHIN FLATWORMS

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Abstract—An understanding of reproductive function is important for control of parasitic helminths. In cestodes and trematodes virtually nothing is known about regulatory and coordinating mechanisms that control maturation, gamete formation, egg production, and related processes. Neurosecretory neurons have been reported in various species but specific modes of action of neurohormones have yet to be demonstrated. The role of ecdysone is being investigated.

Key Words—Schistosomes, nerve–hormone interactions, platyhelminths, differentiation reproductive maturation, *Schistosoma mansoni*, ecdysteroids.

In all multicellular animal groups from sponges to vertebrates, hormonal and neural processes play an important role in control and coordination of reproduction and other somatic functions. Reproduction is almost the key to the existence of trematode parasites; maturation and egg production account for much of their metabolic activity. The egg is the basis of transmission and distribution, and in schistosomes is also the agent of pathogenesis, yet little is known about the control or reproduction in these parasites. There is need for a broad understanding of the coordination of sexual development and reproduction in trematodes and cestodes, with comparisons to invertebrates in general. Such knowledge may aid in the development of methods to frustrate egg production and thus to minimize and control trematode diseases. A specific area of interest is the study of relationships between neural and hormonal conditions needed for the attainment and maintenance of reproductive maturity.

Both of the major groups of "hormonal" regulatory substances—steroids and peptides—are almost universally distributed in living organisms. Study of

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these materials in parasitic helminths has barely begun and has so far been limited to a scattering of reports of their discovery in one or another species. Functional investigations are almost nonexistent. Knowledge of neurotransmitters, while still insufficient, is far more advanced (Hillman, 1983).

In a larger context, the classical distinctions between the nervous and endocrine systems are, as reported by Bloom (1981) "now in disarray." It has been suggested, for example by Le Roith et al. (1982), that there is an early phylogenetic precursor common to both systems so that conventionally conceived neuronal and endocrine functions are manifestations of a single primitive coordinating mechanism. As discussed by Fujita (1983), Krieger (1983), and others, different roles have been assigned to (1) neurons that function through classical neurotransmitters for very local signals; (2) paracrine cells, for short-range transfer of chemical messages; and (3) endocrine cells, which produce chemical substances generally active at a remote site and transmitted through body fluids. Many types of intermediate cells with blended functions are now coming to light. Moreover, it is now clear that insulin and various other polypeptide hormones, generally considered as endocrine substances, may also be products of neural tissues. Both classical brain and classical gut peptides are now known to be widely produced and distributed within vertebrates, so that the distinctions of site origin are largely blurred (Roth et al., 1982; Krieger, 1983). As relatively primitive acoelomate organisms lacking a circulatory system, platyhelminths would have no endocrine functions (as defined above) but may be good candidates for study of paracrine and nerve–hormone interactions.

In their excellent review, Maddrell and Nordmann (1979) provide the following partial list of neurosecretory functions in invertebrates: growth, differentiation, regeneration, body water balance, pigment production, blood sugar levels, heart rate, molting, sexual development, ovulation, and oviposition. The regulatory substances are generally peptides varying from a few amino acid residues to about 10 kd in molecular weight. In starfish, Kanatani (1983) has identified a molecule as small as 1-methyl adenine as a potent regulatory hormone inducing oocyte maturation and spawning in all species so far tested. Recent years have seen an explosion of interest in neurobiology and thousands of papers have dealt with neurohormones and neurotransmitters. Although the parasites in general have not yet shared in this informational wealth, it seems clear that future analyses will deal with basic biological phenomena such as metamorphic phase changes (e.g., from miracidium to sporocyst or cercaria to metacercaria to adult) as well as reproductive maturation. Metamorphosis and maturation are phenomena certainly not peculiar to parasites, but an understanding of their biological and hormonal bases could have practical application through pharmacologic development of specific inhibitors.

An extraordinary phylogenetic conservation of functional peptide configurations is becoming apparent so that many of the peptide hormones once

thought characteristic of mammals are being found in lower organisms. For example, thyrotropin-releasing hormone in gastropods (Grimm-Jorgensen and Connolly, 1983); cholecystokinin/gastrin activity in coelenterates, ectoprocts, crustaceans, insects, and annelids (Larson and Vigna, 1983); and neurophysins, ACTH, and angiotensin in planaria (Remy, 1982). Certain "invertebrate" peptides have also been found in mammalian brain. The phylogeny of neurosecretory functions has been reviewed by Scharrer (1976) and more comprehensively by Remy (1982), who described numerous relationships between vertebrate and invertebrate neuropeptides. Insulin, the subject of concentrated study, has been found in mollusks where it serves in regulation of carbohydrate metabolism (Fritsch et al., 1976; Plisetskaya et al., 1978), and also occurs in insects and annelids (Le Roith et al., 1983).

A great number of papers, which cannot be reviewed here, have described neurohormonal regulation of growth, development, molting, and reproduction in arthropods, particularly insects and crustaceans. Ecdysteroids, best known as molting hormones of arthropods, have recently been reported in schistosomes by Torpier et al. (1982) and Nirde et al. (1983, 1984), and in the cestode *Moniezia expansa* by Mendis et al. (1984). Interrelations between peptide and steroid hormones are seen in such processes as neurosecretory control of production and release of ecdysone by the prothoracic gland, and it is not unlikely that similar functional cascades, feedback loops, and other control mechanisms are to be found in platyhelminths.

In flatworms, work has concentrated on morphological description of neurosecretory cells and granules in planarians and other turbellarians (Grasso and Quaglia, 1970a,b, 1971; MacRae, 1967; Reuter, 1981; Reuter et al., 1980). Experimentally, Sakurai (1981) has demonstrated that feeding of sexually reproducing worms to a normally agamic strain that reproduced only by fission caused the latter to develop gonads. It was proposed by Grasso and Benazzi (1973) that sexualizing factors in planaria are neurosecretory in origin, as nerve plexi containing many neurosecretory cells innervate the gonads. Grasso et al. (1975) reported that sexuality could be induced in agamic planaria by feeding isolated neurosecretory granules concentrated by density gradient centrifugation from homogenized sexual planarians.

In cestodes, Davey and Breckenridge (1967) demonstrated neurosecretory cells in the scolex of *Hymenolepis diminuta* and described a cycle of secretion related to development of the adult and to strobila formation. In *Hymenolepis microstoma*, Webb (1977) showed many neurosecretory cells in the neck region by electron microscopy and followed the release of the electron-dense neurosecretory granules into the intercellular spaces from sites resembling synapses. Gustafsson and Wikgren (1981a,b) and Gustafsson et al. (1983) demonstrated numerous neurosecretory cells in *Diphyllobothrium dendriticum*, showing that both peptidergic and aminergic nerves occur in this species. A rapid activation

of the peptidergic NS system takes place when the plerocercoid larvae are transferred from poikilothermic fish to homeothermic environments in vivo and in vitro. After 1 hr of cultivation, large numbers of granules were evident.

Among trematodes, Ude (1962) first demonstrated neurosecretory granules in the cerebral ganglia of *Dicrocoelium*. Gresson and Threadgold (1964) reported that the beta neurons of adult *Fasciola hepatica* had a NS function, and Dixon and Mercer (1965) showed neurosecretory granules in the nervous system of its cercariae. Grasso and Quaglia (1972, 1974) and Shyamasundari and Rao (1975) suggested, but did not prove, a connection between neurosecretory cells and reproductive maturation in *Fasciola*. A scattering of morphological studies on neurosecretory cells and neurosecretory granules has appeared for other species of trematodes: *Leucochloridiomorpha constantiae* (Harris and Cheng, 1972); *Gastrothylax* sp. (Mehrotra and Bhutia, 1979); *Proalarioides tropidonotus* (Kalyankar and Kankal, 1981); and *Opisthodiscus diplodiscoides* by Matskasi (1970), who described a clear circadian cycle in the increase and reduction of neurosecretory granules within the neurosecretory cells. Sharma and Sharma (1981), studying *Ceylonocotyle scoliocoelium*, stated that "our current investigations indicate that the activities of the neurosecretory cells in a trematode are responsible for initiating growth in juveniles and maturation in adults." An unpublished thesis (Steele, 1971) discussed neurosecretion and development in *Acanthoparyphium spinulosum*. Increased numbers of neurosecretory cells appeared just before large numbers of eggs and sperm were found in the reproductive system. In the author's opinion, neurosecretory cells were responsible for gametogenesis and possible differentiation of the entire reproductive system in this trematode.

In the same year that Ude first described neurosecretory cells in a trematode, Gönnert (1962) reported on the histological structure of the egg-forming region (oogentop) in *Fasciola hepatica*. He discussed the reproductive coordination system as follows (freely translated).

> The oogentop is . . . complicated in structure. Therefore, there must be a coordinating system, whose function it is to regulate the flow and the proper sequence of individual functions. To this question there are as yet no data available. . . . In my investigations I have seen nerve cells in the region of the Mehlis' gland. Precise examination of serial sections revealed the existence of two groups of nerve cells, which are called plexus I and plexus II. Plexus I consists of usually four and at most five nerve cells which lie in the region of the ovovitelline duct and presumably coordinate the functions of the oviduct, vitelline duct, ovovitelline duct, and glands of the upper ootype and Mehlis' gland. . . . Plexus II consists of only two nerve cells. These lie close to the upper uterus. One of these is found very near the ootype valve and appears to be connected to it by a process. The second cell lies further towards the uterus. I presume that plexus II inner-

vates the lower ootype and the ootype valve as well as the upper uterus section, thus the region of eggshell formation.

Specifically regarding schistosomes, much work has been done on neuropharmacology in connection with drug development and assessment (recent reviews by Hillman, 1983; Mellin et al., 1983), but this has little direct application to understanding the neurobiology of reproduction. The influence of host hormones on schistosomes has been reviewed by Knopf (1982), but the current state of knowledge contributes little to the study of schistosome reproductive biology.

Numerous studies have been carried out on the neurobiology of freshwater mollusks, particularly *Lymnaea*, by a large group of Dutch investigators; however, the possible involvement of regulatory peptides in mollusk–trematode interactions does not appear to have been investigated and will most likely prove to be a fertile subject for research. The complexity of studies on trematode neurohormonal regulation should not be minimized: these organisms must function effectively within molluscan and vertebrate milieux at appropriate times, interacting with and perhaps obtaining regulatory chemical cues from each of their hosts, while at the same time retaining their independent internal coordinating systems.

Despite the report and review by Dei-Cas et al. (1980) with diagrams of the neural ganglia and major nerve trunks, surprisingly little information is available on schistosome neuroanatomy. The few detailed studies made with the electron microscope (Silk and Spence, 1969; Reissig, 1970; Dei-Cas et al., 1980) agree in describing a variety of electron-dense granules, some of which were considered to be neurosecretory granules by all authors. However, no function was ascribed to these intraneuronal granules and no study has been made of their association with developmental states or with components of the reproductive system.

We are now studying the presence and distribution of neurosecretory cells in various life history stages of schistosomes. The association between the polypeptide neurohormones and steroid regulatory substances, well established in arthropods, is likely to be found elsewhere. As mentioned above, ecdysteroids have been found by chemical analysis in *S. mansoni*. Accordingly, we have investigated the distribution of immunoreactive ecdysteroids in the life history stages of *S. mansoni*. A rabbit antiserum to beta-ecdysone 2-hemisuccinate (Soumoff et al., 1981) kindly provided by Dr. J.D. O'Connor of UCLA, was used for primary incubation, with fluorescein-conjugated goat anti-rabbit IgG as secondary antiserum. Whole cercariae and paraffin sections of sporocysts, unisexual, paired, and cultured adults were utilized.

Immunoreactivity has been identified in all stages except miracidia. In intramolluscan sporocysts, activity is limited to portions with the maturing cercariae which, when liberated, show strong immunoreactivity in the median re-

gion and postacetabular glands. In adult males and unisexual females activity is concentrated in an assemblage of cell bodies of unknown function within the parenchyma around the intestinal ceca. Interconnections are suggestive of a neuronal network, but this has not been demonstrated. They may also represent ducts of the excretory system. In mature paired females immunoreactive nuclei are found peripherally, just beneath the tegument. An area of strong reactivity in both unisexual and adult females is located in the epithelial lining in the ootype area.

Despite a few tantalizing glimpses, the intricacies of maturational regulation in platyhelminthes remain almost completely unknown. However, the gradually emerging comprehension of generalized neuronal and hormonal control mechanisms in invertebrates will sooner or later be applied to this group. The resulting understanding of trematode and cestode reproductive biology will have profound consequences in new methods of control of these important parasites of animals and man.

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SCHISTOSOME BEHAVIOR IN VITRO

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Abstract—Schistosome adults attract each other in vitro and the attraction is chemically mediated. In *Schistosoma mansoni* adults, excretory-secretory (ES) products of worms of one sex attract worms of the opposite sex, and at least the lipophilic fraction is attractive. Intra- and interspecific attraction occurs in *Schistosoma mansoni*, *S. japonicum*, and *S. haematobium* adults. Current knowledge of schistosome behavior in vitro is reviewed.

Key Words—Schistosoma mansoni, Schistosoma japonicum, Schistosoma haematobium, trematode, digenetic, chemoattraction, sexual attraction, intraspecific attraction, interspecific attraction; heterosexual behavior, homosexual behavior, premating attraction, pheromonal response, pheromone production, behavior, pheromone, excretory-secretory products, lipophilic, hydrophilic.

Although schistosome chemoattraction has been the subject of many studies during the last two decades, most studies have focused on larval stages (MacInnis, 1965, 1976; Chernin, 1970, 1974; MacInnis et al., 1974; Stibbs et al., 1976; Roberts et al., 1979; Saladin, 1979). Many investigations have been done in vivo on ways that *Schistosoma mansoni* males and females affect each others growth and development. For example, Moore et al. (1954) suggested that substances responsible for female maturation may be transferred from male to female in sperm or through the tegument. However, Armstrong (1965) demonstrated that females paired with males castrated by X-irradiation reached sexual maturity and concluded that materials responsible for female maturation do not originate in the testes and must therefore be transferred through the tegument.

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Armstrong (1965) first examined the distribution of adult *S. mansoni* in unisexual infections by quick freezing mice in liquid nitrogen and dissecting them after partial thawing to prevent postmortem migration of worms. He found that 97% of the worms of both sexes were located in the liver or in the nearby portal vein, with their anterior ends directed toward the flow of blood. He observed homosexual pairing of males. The homosexual pairs showed a larger male harboring one to several sexually immature males in its gynecophoral canal. He also studied the pairing behavior of X-irradiated schistosome males and females, and observed that badly deformed and sterile males paired less frequently with females than did normal males. He believed that worms find each other because they are attracted to the liver and that both sexes are active in searching for each other, but that tactile attraction is responsible for bringing conspecific members together. In mixed infections of different species, choice initially appeared random, followed by separation into species after two to three months. He suggested that communication between male and female adults is evidenced by the migration of worm pairs from the liver and portal vein to the mesenteric venules and from venule to venule as the blood vessels fill with eggs. By contrast, homosexual pairs did not migrate to the mesenteric veins, and all were found in the liver or portal vein. Awwad and Bell (1978) further reported that human fecal material contains substances which attract paired *S. mansoni*.

Experiments have more recently been designed for direct in vitro observations of adult schistosome behavior. Michaels (1969) showed that when worms were transected into equal halves, and the halves were allowed to pair in vitro, like halves almost always paired normally and unlike halves almost always paired abnormally. Also, whenever an anterior section of a worm of either sex was placed near an intact worm, it resulted in abnormal pairing position, in contrast to the normal position achieved by posterior halves pairing with intact worms. These observations suggested that a posterior linear receptor system exists in schistosomes and that it is probably neurosecretory in nature. She also observed that when worms began to pair it was always posteriorly, and when worms were transected into thirds the middle third showed a pairing efficiency which was intermediate to the anterior and posterior thirds. These observations suggested that pairing involves activity on the part of both sexes. Males rarely paired with dead females, and females never paired with dead males, although both paired with living worms of the opposite sex 90% of the time. Michaels suggested, therefore, that a neurosecretory mechanism combined with tactile behavior is responsible for schistosome pairing.

Shaw (1977) cultivated male and female *S. mansoni* from mixed and unisexual infections and showed that female worms from unisexual infections usually paired with males from mixed or unisexual infections within 24 h.

From the observations of Michaels (1969), and Shaw (1977), it seemed probable that pairing is induced by a chemical mediator transmitted between

males and females. Shaw et al. (1977) further hypothesized the existence of a pheromone in *S. mansoni* associated with the lipid fraction.

Shirazian and Schiller (1978, 1982) reported that when adult *S. mansoni* males and females were separated, then put back together in an oscillating chamber with either their original partners or partners from different pairs, the worms "paired" most frequently with their original partners.

A bioassay system, with Earle's balanced salt solution (EBSS) containing 0.1% glucose and 0.5% lactalbumin hydrolysate (Clegg, 1965), was developed by Imperia et al. (1980) to study the effects of *S. mansoni* excretory-secretory (ES) products in vitro. Although Carlisle et al. (1983), and Weisberg et al. (1983) reported that morphologic changes are induced by maintenance of *S. mansoni* in vitro, Haseeb et al. (1983, 1984, 1985a) demonstrated that EBSS is an appropriate medium for short-term experiments in the bioassay, since it has no deleterious effects on worms and departure from normal morphology is not observed by either scanning electron microscopy (SEM) or transmission electron microscopy (TEM).

Bioassay designs were modifications based on previous models used for nematode behavior studies (Bonner and Etges, 1967; Salm and Fried, 1973). In the nonbarrier design, polycarbonate chambers with 14 linear channels, each channel 3 cm long, 1 cm wide, and 1.5 cm high, were used. The channels were marked into 0.5-cm zones. Each channel was filled to a height of 0.85 cm with 1% agar. The agar was overlaid with EBSS. The chamber was maintained in a controlled environment room at 37°C under subdued light. Worms were pipetted into the chamber 15 mm apart in separate channels. The orientation of worms was initially random, and although it changed within a few seconds, there was no apparent pattern of orientation. This distance allowed each worm to move either toward the other, which was initially 15 mm away, or 7.5 mm in the other direction, toward the end of the channel. Worm movement did not apparently follow any particular orientation, but could only be measured as distance traveled over time. Distances were measured between worm centers at various time intervals. At each time period, the distance between a worm couple was measured, and the percent attraction determined by the formula:

$$\frac{15 - D}{15} \times 100$$

where D is the actual distance between a worm couple at a particular time point. Observations were based on the mean $\pm$ SE values from the summation of individual trials. Thus, by this formula 0% attraction would indicate that worms remained at their original distance, and 100% attraction would indicate that all the worms were in contact.

The obvious advantage of the nonbarrier design is that by allowing both worms to migrate, any chemoattractive effect can be cumulative and therefore

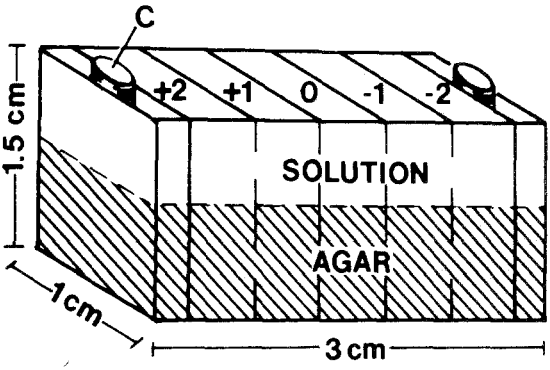

FIG. 1. Linear channel, barrier design of polycarbonate chamber used in pheromonal studies. Reprinted from Imperia et al. (1980) with permission of *The Journal of Parasitology*.

maximized. However, some limitations of this design are: (1) It does not permit determination of whether one or both worms are the emittors or responders; and (2) it cannot rule out the possibility that mating is accomplished by means of thigmotaxis and by trial and error rather than by chemotactic response, as was suggested by Armstrong (1965).

The barrier design, although somewhat less sensitive than the nonbarrier design, permits such distinctions. It also allows testing of excretory–secretory (ES) products, as described below. In this design, shown in Figure 1, channels contained 0.6-cm-diameter dialysis tube chimneys at both ends to restrain worms but allow the passage of ES products. Most experiments employed dialysis tubing with approximately 12,000 M_r exclusion. The chimney tops protruded above the saline. In this design, a worm was placed at the zero (starting) point, equidistant between the chimneys. Worms which moved toward occupied chimneys were considered attracted, whereas those moving toward unoccupied chimneys were scored unattracted. Control experiments have been carried out with single worms placed equidistant between empty chimneys and, at all time points, most of the "migrating" worms remained at the zero (starting) point, and the remaining worms were distributed equally on both sides of the zero point. Thus, a 50% attraction would be expected if migration were random and therefore only those responses greater than 50% are regarded as attraction. Observations were made at regular intervals up to 4 hr. Chi-square and Student's *t*-tests were used to analyze the data.

Initially, Imperia et al. (1980) tested the in vitro attraction of single female *S. mansoni* toward one or three males using the barrier design. Either one, three, or no males were placed in the chimney adjacent to the +2 zone, and one female was placed in the center of the starting zone. Observations on the

migration of the female toward or away from the experimental chimney were made at 0.5, 1, 2, 3, and 4 hr. Females in the +2 and +1 zones were considered attracted, whereas those in −2 and −1 zones were scored unattracted. Chi-square tests indicated that individual females were significantly attracted ($P < 0.025$) to a one-male source at all time periods. Females were not attracted to a three-male source at any time point in this barrier design.

Eveland et al. (1982) then showed heterosexual and homosexual attraction, both in the absence and presence of barriers. Attraction was seen in all cases. No significant differences occurred between male–female, male–male, or female–female combinations in the nonbarrier design. At least during the first hour, attraction was clearly a function of time, with most attraction occurring within 0.5 hr. Also, in no case of heterosexual or homosexual attraction did worms migrate past each other.

Experiments were also done to compare heterosexual attraction in worms from same verses different pairs in the absence of barriers. When males and females were separated, then put into the bioassay with either their original partners or partners from different pairs, there was significantly more heterosexual attraction between same than different pairs.

When heterosexual and homosexual attraction were tested in the presence of barriers, the results were different. Most attraction occurred within 0.5 hr, and the greatest attraction was heterosexual, with males moving toward females. Heterosexual attraction with females moving toward males was approximately equal to homosexual female attraction, and male homosexual attraction was not seen.

It is noteworthy that most of the chemoattraction was demonstrated in this bioassay system, both with and without barriers, within 30 min, in contrast to the "pairing" shown by Shirazian and Schiller (1978, 1982), which took place only after 6 hr. This is not surprising, however, but strongly suggests that chemoattraction and pairing are distinct phenomena.

Eveland et al. (1983) showed heterosexual attraction when one but not two male or female worms were placed behind the barrier. One or two male or female worms, or a male–female in copula were placed in one of the chimneys and either a male or female worm was placed at the zero (starting) point. The results of one vs. two worms in the chimney are shown in Figure 2. Males were significantly attracted to one, but not two females. Beginning at 1 hr, males were significantly repelled when more than one female was in the chimney. Likewise, one male attracted a female, but two males repelled. These results were in agreement with the observations by Imperia et al. (1980) that females are less significantly attracted to a three-male source than to a one-male source at all time periods.

The results comparing a single worm with an in copula pair in the chimneys are shown in Figure 3. Although males and females were significantly attracted

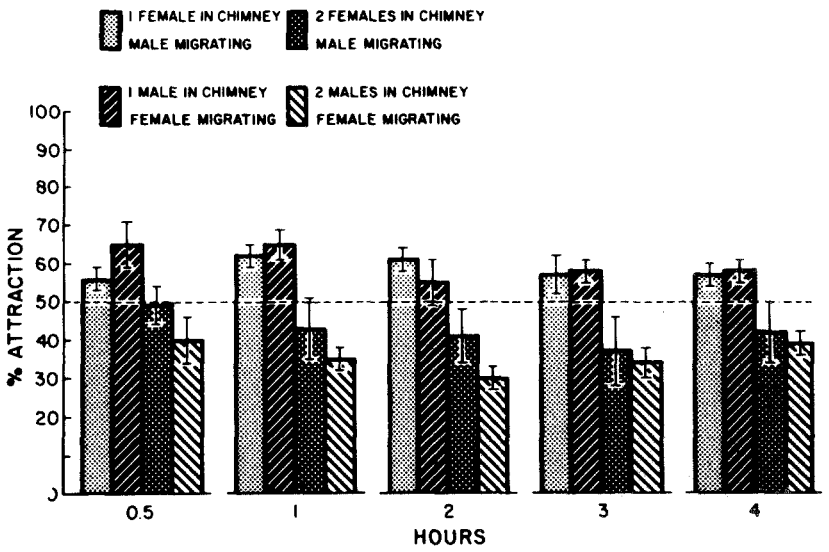

FIG. 2. *Schistosoma mansoni* worms were attracted to one, but not two of the opposite sex. For each time period, 100–110 trials were done with each combination shown. Standard error bars are shown. Reprinted from Eveland et al. (1983) with permission of *Experimental Parasitology*.

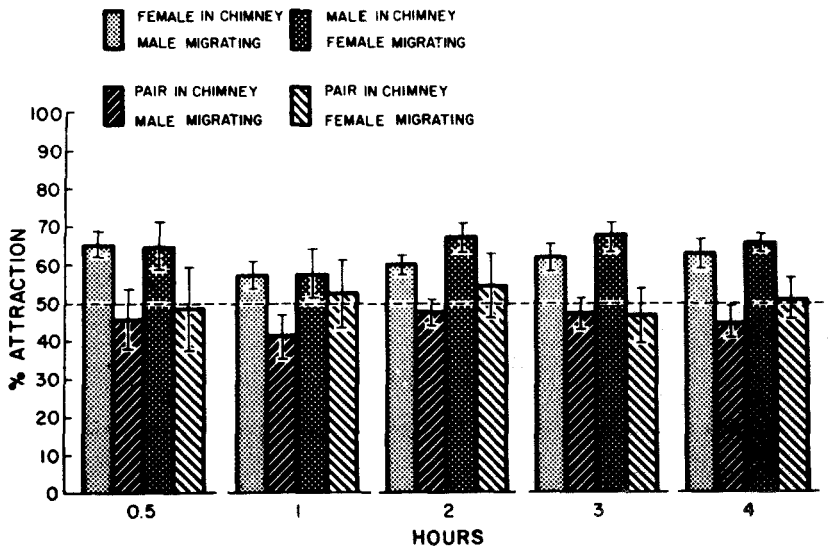

FIG. 3. *Schistosoma mansoni* worms were attracted to single worms but not to worm pairs. For each time period, 100–110 trials were done with each combination shown. Standard error bars are shown. Reprinted from Eveland et al. (1983) with permission of *Experimental Parasitology*.

to single worms in chimneys, they were not attracted to pairs. While no definite explanation can be given for the fact that two or more worms in the chimney were less effective than one in attracting males and females, it is interesting in this regard that Fried et al. (1983) showed that when male worms were incubated singly for 0.5 hr, they released cholesterol, but they did not appear to release cholesterol when incubated in groups of ten. Likewise, Haseeb et al. (1985b) demonstrated that *S. haematobium* males incubated singly released more free sterols than a similar number incubated in groups.

Initially, our studies were done with perforated chimneys to allow free passage of substances of all sizes (Imperia et al., 1980). In the perforated design, barriers were prepared by puncturing dialysis tubing of approximately 12,000 M_r exclusion size with common pins. Unperforated chimneys with approximately 50,000, 12,000, and 1000 M_r exclusions were compared with perforated chimneys. Either a single male or female was placed behind the barrier. No reduction in attraction was seen when unperforated dialysis tubing with a cutoff of approximately 12,000 M_r was used, although a cutoff of 1000 M_r prevented attraction (Eveland et al., 1983).

Similar results were obtained in EBSS containing 10% fetal calf serum, and when 50% fetal calf serum was used, attraction took longer to occur.

Attraction between *S. japonicum* or *S. haematobium* males and females from same or different male–female worm pairs was tested. Also, *S. mansoni* males or females of opposite sex but from different pairs were compared with *S. japonicum* or *S. haematobium* worms of opposite sex for their attraction to *S. mansoni* (Eveland and Fried, 1986).

Experiments were carried out using nonbarrier designs. For each time point, distances between worms were measured in millimeters and the percent attraction was determined by the formula $15 - D/15 \times 100$, as described previously. At 0.5 hr, *S. japonicum* from same pairs were 64% attracted, compared to 45% attraction between worms from different pairs ($P = 0.06$) and at 1.0 hr, *S. japonicum* from same pairs were 71% attracted to each, compared to 53% attraction between worms from different pairs. However, the differences were not significant ($P > 0.05$). Likewise, differences between same and different *S. haematobium* pairs were not significant at any time point. Interspecific attraction was as great as intraspecific attraction. In both interspecific and intraspecific attraction, 10–15% of the males and females were actually in contact at all observed time points.

In nonbarrier design experiments which we reported previously, homosexual attraction was as strong as heterosexual attraction (Eveland et al., 1982), but when barrier designs were used homosexual attraction was much weaker (Eveland et al., 1983), suggesting that more than one type of chemoattractant may be operative. For example, it is possible that homosexual attraction in the absence of chimneys was due to a nonspecific, aggregative chemoattractant, and that expressed in the presence of chimneys was more specific. It is also

possible that the attractants responsible for interspecific and homosexual effects were the same, which differed from those responsible for the more specific effects seen in the presence of barriers. More studies are needed to test interspecific attraction using barrier designs. In any case, the studies reported here show that at least some schistosome chemoattractants cross species lines and suggest that if blocking agents can be developed against schistosome chemoattractants, they may be broadly applied.

Worm ES products, prepared by incubating single males or females for 1 hr in EBSS have also been used in our bioassay, both before and after separation in chloroform–methanol or *n*-hexane into hydrophilic and lipophilic fractions. Initially, 5 μl of one-male incubate was pipetted onto a paper disk prepared by punching holes out of Whatman No. 40 filter paper with a paper punch. A disk was then placed in a channel in lieu of a male worm, and one female was placed in the center of the starting zone. Observations on the migration of the female toward or away from the disks were made up to 0.5 hr. Two additional experiments were carried out in which 200 μl of one-male incubate was applied to paper disks by multiple applications, and observations on the migration of the female were made up to 4 hr. As seen in Table 1, female attraction to the disks was significant at both concentrations and at all time points.

One-male incubates were then extracted with chloroform–methanol, and 10 μl of lipophilic or hydrophilic fractions was pipetted onto paper disks, then the disks were put into channels to test for female attraction. No significant attraction occurred in either case. When female incubates were extracted with chloroform–methanol, and 10 μl of lipophilic fraction was pipetted onto paper disks, males appeared to be attracted, although the attraction was not statistically significant. Hydrophilic fractions did not attract males.

In an attempt to improve the bioassay using ES products, it was modified in the following way: Agar cylinders were prepared by adding 75 μl of 2.5% agar in phosphate buffer (pH 7.0) at 40°C to microtiter round-bottom 250-μl

TABLE 1. ATTRACTION OF *Schistosoma mansoni* FEMALES TO PAPER DISKS CONTAINING ES PRODUCTS FORM ONE MALE

Time (min)	ES products (μL)	Attraction (%)	Probability (*t* test)
30	5	71	<0.01
30	200	60	<0.05
60	200	64	<0.05
90	200	68	<0.01
120	200	68	<0.01
150	200	65	<0.01
180	200	62	<0.05

wells. To this was added 100 μl of male ES products, followed by another 75 μl of agar. When the agar had solidified, the cylinders were removed with a 26-gauge hypodermic needle and placed in one end of the linear channel of the bioassay chamber, and a cylinder without ES products was placed in the other end. A single female was placed equidistant between the two cylinders and was allowed to migrate either toward or away from the ES products. Using videotape methodology in a 37°C environmentally controlled room, the females were scored either attracted or unattracted at 1 min intervals for 30 min. In 40 separate trials, beginning at 3 min, attraction was significant ($P < 0.05$) at every time point. With the agar plug technique, heterosexual attraction sometimes, but not always, occurred with lipophilic fractions and did not occur with hydrophilic fractions.

We then separated lipophilic and hydrophilic fractions with *n*-hexane. As shown in Table 2, when male lipophilic fractions extracted in *n*-hexane were tested in agar cylinders, they attracted females significantly at least through the 4-hr experiment. Interestingly, chloroform–methanol extraction from females did appear to attract males, although the attraction was not statistically significant. Again, hydrophilic fractions were not attractive to females.

Further studies are needed on the effects of distance. The 15-mm distance used in our bioassay was arbitrarily selected, and more studies need to be done to test diffusion rates and concentrations as a function of distance, as well as to establish the maximum distance over which the chemoattractants can be detected.

TABLE 2. ATTRACTION OF *Schistosoma mansoni* FEMALES TO AGAR PLUGS CONTAINING LIPO- OR HYDROPHILIC ES PPRODUCTS FROM ONE MALE (SEPARATED IN *n*-HEXANE)

Time (min)	ES product	Percent Attraction (%)	Probability (*t* test)
30	Lipophilic	66	<0.05
60	Lipophilic	73	<0.05
90	Lipophilic	70	N.S.*
120	Lipophilic	70	<0.05
150	Lipophilic	66	<0.05
180	Lipophilic	63	<0.05
30	Hydrophilic	39	N.S.
60	Hydrophilic	41	N.S.
90	Hydrophilic	45	N.S.
120	Hydrophilic	42	N.S.
150	Hydrophilic	41	N.S.
180	Hydrophilic	44	N.S.

*Not significant.

We have not yet established the time of onset for the elaboration and detection of chemoattractants. Experiments are planned with 3- to 4-week-old worms which have not yet matured completely and paired in vivo, but for which sex can be determined. The worms will be placed in bioassay chambers in the absence of barriers as described above, and maintained at 37°C. For these 3- to 4-week-old worms, it will be necessary to reestablish optimal bioassay conditions, since smaller worms may not be able to attract each other over the relatively longer distances used for older worms (6 weeks and older). Worm combinations will be placed in separate channels, in a design similar to that of Eveland et al. (1982). One male and one female worm will be positioned in a channel so that each can move in either direction, toward or away from each other, and distances between worms will be measured in millimeters at various time periods. We have found that this nonbarrier design gives maximal worm attraction (Eveland et al., 1982). Worms from unisexual infections, which have never been exposed to the opposite sex will also be tested in the nonbarrier design.

Now that lipids are shown to attract schistosomes in vitro, studies should be done with "shelf lipids" to confirm results of assays using schistosome lipid fractions. If specific lipids are found to be chemoattractive, then efforts can be focused on these putative substances in the appropriate worm fractions. In summary, we have shown that schistosomes attract each other in vitro and that the lipophilic fraction of at least *S. mansoni* males attracts females. Thus, there is a chemical basis for the attraction.

The critical question of whether these events observed in vitro occur normally to worms in the blood vessels is controversial. However, we have demonstrated evidence of in situ lipid transfer from males (Haseeb et al., 1984), and it is possible that such lipids may serve as both primer and releaser pheromones. It is expected that further studies will result in knowledge of schistosome chemoattractants which may lead to the development of convenient, economical, and effective measures against schistosomiasis. For example, it may be possible to specifically block chemoattractants, as has been done in ticks (Sonenshine et al., 1982). The resulting unmated females could not reach maturity and lay eggs, thus preventing the major pathology of schistosomiasis. Alternatively, if the chemoattractants are immunogenic, they may be collected from the in vitro cultivation of schistosomes (Eveland et al., 1979; Basch, 1981a,b) and used as vaccines.

The studies using *S. mansoni*, *S. japonicum*, and *S. haematobium* demonstrate that the attraction is interspecific, and thus if blocking agents can be developed against schistosome chemoattractants, they may be broadly applied.

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CHEMICAL COMMUNICATION IN ADULT SCHISTOSOMES

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Abstract—Lipids released by *Schistosoma mansoni* adult males attract females in vitro. Lipid release is modulated by the presence of other worms. Although *S. mansoni* males release lipid when paired with females, the release is enhanced when they are separated. *S. japonicum* adults release more free sterols when incubated individually than when incubated together. Similarly, individually incubated *S. haematobium* males release more free sterols than when incubated in groups. However, *S. haematobium* females incubated in groups release more free fatty acids than do equal numbers of males or pairs incubated in groups. There is evidence that *S. mansoni* adult females concomitantly accumulate and release cholesterol in the absence of an exogenous supply, although de novo synthesis of cholesterol in schistosomes has not yet been demonstrated. Schistosomula and adult schistosomes incorporate exogenous lipids. Lipids are incorporated chiefly through the tegument. Cholesterol is transferred between males and females.

Key Words—Trematoda, Digenea, *Schistosoma mansoni*, *Schistosoma japonicum*, *Schistosoma haematobium*, behavior, chemoattraction, chemical communication, pheromones, lipids, receptors, histochemistry, electron microscopy, thin-layer chromatography.

INTRODUCTION

How male and female schistosomes first locate each other in vivo is still a matter of conjecture, but there is now evidence from in vitro studies that pairing is chemically mediated. Interest in interactions between males and females date back to the 1920s when Severinghaus (1928) and Sagawa et al. (1928) inde-

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pendently postulated hormonal dependence of females upon males for maturation. Later studies by Vogel (1941, 1942, 1947), Standen (1953), and Moore et al. (1954) provided further evidence for male-induced female maturation.

Several studies have indicated that pairing with males is a prerequisite not only for female maturation but also for migration of paired worms to mesenteric and bladder veins and for continued egg laying (Standen, 1953; Moore et al., 1954; Armstrong, 1965; Michaels, 1969; Shaw, 1977).

MATE LOCATION AND MIGRATION IN THE MAMMALIAN HOST

Because he observed homosexual male pairs, Armstrong (1965) believed that in vivo mating of *Schistosoma mansoni* occurs by trial and error and that pairing results from thigmotaxis rather than chemotaxis. He also concluded that a pheromone from males is responsible for maturation of females in heterosexual pairs and retards the development of one male in homosexual pairs.

We now know that *S. mansoni* females require continuous male contact for reproduction. When mature females from bisexual infections are deprived of males, they are reduced in size and their vitellaria and ovaries degenerate. These regressive changes are reversed if pairing is resumed with males (Clough, 1981; Popiel et al., 1984). Males in the absence of females produce normal sperm and show no degenerative changes (Floyd and Nollen, 1977; Kolzow and Nollen, 1978; see Nollen, 1983, for review).

The route of schistosome migration in the mammalian host following cercarial penetration has been studied by several workers and several hypotheses have been proposed (see Miller and Wilson, 1978, 1980; Wheater and Wilson, 1979, for review). However, none of these studies addresses the issue of initial pairing. Our information is limited to the fact that males are required to escort females to the small veins of mesentery or bladder wall (Standen, 1953).

Recent studies have demonstrated that adult *S. mansoni* attract each other in vitro, and the attraction is chemically mediated (Imperia et al., 1980; Eveland et al., 1982). Imperia et al. (1980) stated that adult males emit pheromone(s) which attract females. The in vitro studies by Eveland et al. (1983) and Shirazian and Schiller (1982) suggest worm-finding and pairing mechanisms which may operate in vivo. Autoradiographic tracking of schistosomula can also elucidate events which occur in vivo prior to, during, and following mating. These techniques have been profitably used in the schistosome–mouse model for purposes other than identified here (Georgi, 1982; Mangold and Dean, 1983).

Several studies have indicated that young schistosomes pair on arrival in the liver. No serious effort has been made to elucidate mechanisms of site selection in the mammalian host except an in vitro study by Awwad and Bell (1978) which indicated that *S. mansoni* pairs were attracted more strongly than

unpaired worms to a dialysate of human feces. Chemical substances providing site-finding cues to schistosomes may originate in the human diet and from metabolic products of normal enteric flora. Whether *S. haematobium* pairs migrate to veins of the urinary bladder because of substances absorbed from the bladder, and whether they would respond to urine in vitro, both remain to be determined.

CHEMICAL INTERACTIONS IN VITRO

Although studies on hermaphroditic trematodes have yielded considerable information on pairing behavior (Fried and Leiby, 1982; Fried and Jacobs, 1980), these digeneans are not suitable models for sex-dependent behavior studies. Schistosomes are ideally suited for studies on both heterosexual and homosexual interactions between worms. Shaw et al. (1977) showed that acetone and ether extracts of male *S. mansoni* induced development of vitelline cells with a corresponding increase in body length of females from unisexual infections. Results of another study also suggest involvement of lipids in the development of females. Unisexually developed females showed increased [^{3}H]tyrosine uptake when they were exposed to males or chloroform extracts of males (Popiel and Erasmus, 1981). These observations suggest that factors produced by males induce maturation in females and that the developmental changes are mediated by lipids. Alternatively, the active substance(s) may be factors for which lipids serve as carriers or solvents. The pheromone of the hard ticks has been shown to be dissolved in neutral lipid (Sonenshine et al., 1981).

LIPID RELEASE IN SCHISTOSOMES

Free sterols and triacylglycerols are the major neutral lipid fractions in extracts of both male and female *S. mansoni* (Smith and Brooks, 1969; Fried et al., 1981), and free sterols constitute the major lipid fraction in male excretory-secretory (ES) products (Fried et al., 1981). Cholesterol is the most abundant lipid and the major free sterol in *S. mansoni* adults (Smith and Brooks, 1969; Meyer et al., 1970). Since free sterol fraction is the major neutral lipid fraction in worm ES products (Fried et al., 1981), Fried et al. (1983) quantitated cholesterol in adult males and females and their ES products to study cholesterol accumulation and release over time (Table 1). They found that males tend to maintain their cholesterol for at least 0.5 hr following removal from the host. At recovery, males contained 1.2–1.5 μg cholesterol/worm and at 0.5 hr they had 0.8–1.5 μg. However, during 0.5 hr incubation, males released 0.012–0.028 μg cholesterol/worm into the incubation medium. Females initially contained 0.13 μg cholesterol/worm and by 0.5 hr their content had risen to 0.42 μg/worm. Females had, however, released 0.008–0.013 μg cholesterol/worm

TABLE 1. AMOUNTS OF CHOLESTEROL (μg) IN *Schistosoma mansoni* ADULTS AND THEIR WORM-FREE INCUBATES[a]

Worms	Extracts		Incubate
	0 hr	0.5 hr	
Pairs	1.9–2.8	1.2–1.5	0.003–0.013
Males	1.2–1.5	0.8–1.5	0.012–0.028
Females	0.13	0.42	0.008–0.013

[a]Data from Fried et al. (1983).

into the incubation medium during 0.5 hr incubation. Heterosexual worm pairs contained 1.9–2.8 μg cholesterol/worm pair and at 0.5 hr incubation, they contained 1.2–1.5 μg cholesterol/worm pair. Their incubation medium contained 0.003–0.013 μg cholesterol/worm pair at 0.5 hr. These data indicate that the two sexes handle lipid differently and that pairing influences lipid release. However, separate lipid analyses of males and females following incubation of pairs would more clearly delineate the influence of the sexes on each other.

Earlier studies on *S. mansoni* showed that in males lipids are localized in the parenchyma and tubercles (Gönnert, 1955; Smith et al., 1969; Fried et al., 1981) and in females they are found primarily in the vitellaria (Fried et al., 1981). Even though neutral lipid release was reported from *S. mansoni* (Fried et al., 1981, 1983), no specific structure was associated with the release. Recent histochemical and electron microscopic studies indicated that neutral lipids are released from the tegument (Haseeb et al., 1984, 1985b).

These studies confirmed previous observations on lipid distribution in adult schistosomes and demonstrated marked differences between males and females in lipid release. In *S. mansoni* males which were separated from females immediately following their recovery from the host, lipid was usually seen in the parenchyma but rarely in tubercles. However, when incubated in vitro for 0.5–1.0 hr, males accumulated lipid in both the parenchyma and tubercles with release from the latter. These studies demonstrated lipid accumulation and release from the dorsal tegument of unpaired males but not from females. Senft et al. (1978) believed that the gynecophoral canal tegument is a site of active lipid secretion, but our studies do not confirm their observations.

Males which had not been separated from females after recovery from the host contained lipid both in the parenchyma and tubercles with release from the latter. At 0.5 hr incubation in vitro, these males were not observed to release lipid, although their tubercles and parenchyma were lipid-positive. But at 1.0 hr of incubation, they were again releasing lipid and lipid droplet shift from parenchyma to the tubercles was obvious. No prominent differences could be noticed in females which had been incubated with males and those which were

TABLE 2. SUMMARY OF HISTOCHEMICAL DETECTION OF LIPIDS IN TEGUMENT OF *Schistosoma japonicum* ADULTS MAINTAINED IN VITRO[a]

	Paired worms		Separated worms					
	0 hr		0 hr		0.5 hr		1.0 hr	
	m	f	m	f	m	f	m	f
ORO	++r	−	+r	−	++r	−	+®	−
NBS (A)	+	+	+	+	−	−	+	+
(N)	+r	+	+r	−	+	−	+	−

[a]Data from Haseeb et al. (1986). ++, moderately positive; +, weakly positive; −, negative; ORO, Oil red O; NBS, Nile blue sulfate; A, acidic lipid; N, neutral lipid; r, lipid droplet release; ®, occasional release. Worms pairs were either fixed at 4°C in 10% neutral buffered formalin immediately following recovery from hosts or mechanically separated and fixed without incubation (0 hr) or after 0.5 and 1.0 hr incubation in Earle's balanced salt solution containing 0.1% glucose and 0.5% lactalbumin hydrolystate.

incubated singly. Females contained lipids in the vitellaria and at times in the lumen of intestinal ceca. However, females from unisexual infections were lipid-negative (Haseeb et al., 1984).

Similar studies on *S. japonicum* revealed a similar pattern of lipid distribution as in *S. mansoni* adults, although, patterns of lipid release are different in the two species. Although neutral lipid release was restricted to the dorsal tegument in *S. mansoni* males, in *S. japonicum* males release occurred from both the dorsal and ventral teguments. Haseeb et al. (1984) reported acidic lipid release into the ceca of *S. mansoni* males, but it was not observed in *S. japonicum*. *S. japonicum* males also released neutral lipid from the intestinal ceca. Females released neutral lipid into the intestinal ceca but not from the tegument. Acidic lipid release was not observed from worms of either sex (Haseeb et al., 1986). Results of histochemical lipid studies on *S. japonicum* are summarized in Tables 2–4.

These differences in lipid release patterns between the two schistosome species cannot be explained simply on the basis of absence of tubercles in *S. japonicum*. Although it was conceived earlier that *S. mansoni* males release lipids from their tubercles, electron microscopic studies showed that the whole dorsal tegument is involved in this process (Haseeb et al., 1985b). These studies further indicated differentiation of dorsal and ventral teguments in males of these species. Lipid distribution in *S. haematobium* remains to be determined.

Even though we have demonstrated neutral lipid release in *S. mansoni* and *S. japonicum* by both histochemical and biochemical methods, information on lipid secretion at the cellular level is needed. Further studies are also required to determine if released lipid droplets are membrane bound.

There is some evidence that schistosomes release lipid in vivo. Cryostat

TABLE 3. SUMMARY OF HISTOCHEMICAL DETECTION OF LIPIDS IN PARENCHYMA[a] OF *Schistosoma japonicum* ADULTS MAINTAINED IN VITRO[b]

	Paired worms		Separated worms					
	0 hr		0 hr		0.5 hr		1.0 hr	
	m	f	m	f	m	f	m	f
ORO	++	+	+	+	++	+	+++	+
NBS (A)	+	++	++	++	+	++	++	++
(N)	++	+	+	−	++	+	++	+

[a] Vitellaria in females.
[b] Data from Haseeb et al. (1986). +++, strongly positive; ++, moderately positive; +, weakly positive; −, negative; ORO, Oil red O; NBS, Nile blue sulfate; A, acidic lipid; N, neutral lipid. See legend to Table 2.

sections of mouse mesenteric veins harboring adult worms revealed neutral lipid droplets in parts of blood vessel walls which were in contact with male worms. These droplets had staining properties similar to those observed in schistosomes. Only parts of the male gynecophoral fold exposed to female contain lipid, suggesting an influence of females on male lipid secretion. Although males of worm pairs released neutral lipid both in vitro and in situ (Haseeb et al., 1984), the release was greater in males after they were separated from females (Haseeb et al., 1985b).

Lipid droplets released from the ventral tegument of *S. japonicum* males were observed in the gynecophoral canal and occasionally in the adjacent female tegument, suggesting possible lipid transfer from male to female. Acetone and ether extracts (presumably lipids) of *S. mansoni* males induce maturation in females (Shaw et al., 1977), and females show vitelline gland differentiation only in regions which contact males (Popiel and Basch, 1984a).

TABLE 4. SUMMARY OF HISTOCHEMICAL DETECTION OF LIPIDS IN CECAL WALLS OF *Schistosoma japonicum* ADULTS MAINTAINED IN VITRO[a]

	Paired worms		Separated worms					
	0 hr		0 hr		0.5 hr		1.0 hr	
	m	f	m	f	m	f	m	f
ORO	++r	++r	−	−	+r	−	++r	+
NBS (A)	++	+	++	++	+	++	++	++
(N)	++	+	+	−	+	−	+r	−

[a] Data from Haseeb et al. (1986). ++, moderately positive; +, weakly positive; −, negative; ORO, Oil red O; NBS, Nile blue sulfate; A, acidic lipid; N, neutral lipid; r, lipid droplet release. See legend to Table 2.

Although *S. japonicum* females released neutral lipid into the intestinal ceca only when paired with males, lipids were detected in worm-free incubates of separated females (Haseeb et al., 1986).

Densitometric thin-layer chromatographic data on neutral lipid release in *S. japonicum* and *S. haematobium* are also available. Pooled worm-free incubates of 10 singly incubated *S. japonicum* males contained 0.30 μg of free sterols, whereas 10 males incubated together failed to release enough to be detected by densitometry. Similarly, females incubated separately released more free sterols than those incubated together (Haseeb et al., 1986). *S. haematobium* males also released more free sterols when incubated singly than a similar number incubated in groups. Ten females incubated together released more free fatty acids than 10 males or 10 pairs incubated in groups (Haseeb et al., 1985c).

Our histochemical and TLC studies clearly indicate that lipid release is affected by the presence of other worms. These observations are in accord with the findings of Eveland et al. (1983), who reported that worms of either sex do not show attraction to worm pairs, two males or two females, although heterosexual attraction to single worms occurred. They proposed that either the "window effect" (Kemp and Devine, 1982) or a "shut-down" mechanism is involved in diminishing attraction. Demonstration of a dose–response relationship would help clarify this issue.

Lipid release and its modulation by the presence of other worms is one aspect of the complex chemical communication system in schistosomes. We do not know how this modulation occurs or what chemical messages are involved. Whether the messages are different from those which mediate attraction is also unknown. We do know that lipids are involved in worm attraction. Excretory–secretory products of worms incubated in vitro and their lipophilic fractions prepared in chloroform–methanol attract worms of the opposite sex (Eveland et al., 1984). Our recent studies indicate that lipophilic fractions of worm-free incubates prepared in *n*-hexane attract worms of the opposite sex better than those prepared in chloroform–methanol (Eveland and Haseeb, 1986). Because all animal pheromones do not belong to a single group of chemical substances (Law and Regnier, 1971; Silverstein, 1981), we must consider that chemicals used in separation procedures may interfere with biologic activities of potential chemoattractants. For example, lipid and protein moieties of most lipoproteins are separated in procedures using chloroform–methanol since methanol precipitates proteins. In separation procedures employing *n*-hexane, such precipitates are not observed.

METABOLIC DEPENDENCE

Metabolic interdependence of male and female schistosomes has been the subject of several studies. Transfer of [^{14}C]glucose from males to females has been demonstrated in *S. mansoni, S. haematobium*, and *S. japonicum* in vitro (Cornford and Huot, 1981). These workers further demonstrated that the rate

TABLE 5. [4-^{14}C]CHOLESTEROL (ng/WORM ± SEM) TRANSFER BETWEEN *Schistosoma mansoni* ADULTS

0 hr	3.0 hr	
Males	Males	Females
0.722 ± 0.083–0.792 ± 0.063	0.346 ± 0.025	0.090 ± 0.003
Females	Females	Males
0.245 ± 0.020–0.298 ± 0.031	0.167 ± 0.012	0.086 ± 0.004

[a] Data from Haseeb et al. (1985a).

of glucose assimilation by worms was significantly greater in paired than in separated worms and that unpaired males contained more glycogen than did paired males.

Atkinson and Atkinson (1980) reported that "The male worm retains little of the protein it produces in greatest abundance, and this protein is electrophoretically identical to the most abundant protein found in, but not synthesized by, the female." This 66-kd polypeptide occurs naturally in males and females from both unisexual and bisexual infections and, when such worms are exposed to [^{14}C]leucine, it is incorporated into 66-kd bands of mature and unisexually developed females as well as mature males (Popiel and Basch, 1984b).

Our recent studies have shown that cholesterol is transferred in both directions between males and females (Table 5). In these experiments single worms removed from pairs were incubated for 3.0 hr in RPMI 1640 containing 0.16 μCi [4-^{14}C]cholesterol/ml. After initial labeling, these worms were incubated with unlabeled, freshly recovered and separated worms of the opposite sex for another 3.0 hr. Females incorporated 0.090 ± 0.003 ng cholesterol/worm when incubated with males containing 0.722 ± 0.083 ng cholesterol/worm. Males contained 0.346 ± 0.025 ng cholesterol/worm following incubation. Males incorporated 0.086 ± 0.004 ng cholesterol/worm when incubated with females containing 0.245 ± 0.02 ng cholesterol/worm. At the end of the experiment, females contained 0.167 ± 0.012 ng cholesterol/worm (Haseeb et al., 1985a).

Light-level autoradiography revealed the tegument to be the major site of cholesterol uptake. In males, more transtegumental uptake occurred on the dorsal than the ventral surface and label was heavier in the tubercles than the rest of the dorsal surface. The tubercles on the mid-dorsal surface were more heavily tagged than those near the periphery. The cecal walls were tagged in both males and females. Dorsal subtegumental parenchyma was more heavily tagged than the ventral. Vitellaria accumulated most of the label in females and the tegument was not as heavily labeled as in males (Haseeb et al., 1985a).

S. mansoni females accumulate labeled cholesterol in the vitellaria but radioproline is rather sparingly incorporated into the vitellaria (Senft, 1968). *S.*

mansoni adults incorporate proline through the gut and tegument (Senft, 1968), but Fripp (1967) did not detect any radioactivity in *S. haematobium* gut after providing [1-^{14}C]glucose in vitro. In our experiments in which the worms were labeled individually, dense labeling was on the dorsal surface. Therefore, it appears that receptors for different biologically active molecules are located at different sites in schistosomes.

If the worms were incorporating cholesterol through the cecal route, heavier tagging might be expected in the anterior than the posterior half of the gut. Since the tagging in cecal walls was uniform throughout the length of worms of both sexes, it seems unlikely that cholesterol uptake occurs through the ceca, but suggests that excess cholesterol is released into the ceca following transtegumental uptake.

Catabolism of lipids as an alternative source of energy probably does not occur in schistosomes since they do not consume O_2 for their energy-yielding metabolic reactions (Bueding, 1950). Moreover, lipids absorbed by schistosomes are mainly utilized as constituents of parasite membranes (Rumjanek and Simpson, 1980). Cholesterol is incorporated into the outer half of the outer lipid bilayer (Torpier and Capron, 1980). A recent study has shown de novo synthesis of cholesterol in *Fasciola hepatica* (Gerasimova and Leutskaya, 1983). In contrast, schistosomes do not appear to synthesize cholesterol de novo (Smith et al., 1970; Meyer et al., 1970).

Cholesterol metabolism in schistosomes appears to be in a state of dynamic equilibrium. Both the incorporation (Rumjanek and Simpson, 1980; Haseeb et al., 1985a) and release (Fried et al., 1983) of cholesterol are obviously governed by the physiologic state of the worm, but the precise regulatory mechanism is not known. Lipid incorporation in schistosomula is modulated by the amount of lipid present in the incubation medium (Rumjanek and McLaren, 1981). Such data are not available for adult worms in which the presence or absence of worms of the opposite sex may affect lipid uptake. As mentioned above, the presence of worms of the opposite sex affects lipid release (Haseeb et al., 1985c, 1986).

Biologically active ecdysteroids are known to occur in certain helminths, such as *Dirofilaria immitis* (Mendis et al., 1983) and *Moniezia expansa* (Mendis et al., 1984). Ecdysone and 20-hydroxyecdysone have also been detected in *S. mansoni* (Torpier et al., 1982; Nirde et al., 1983). These molecules have been shown to be produced by the worms and have been detected in serum and urine of patients (Nirde et al., 1984). Their role in the biology of the parasite is unclear. The function of ecdysteroids in insects has been reviewed by Loof et al. (1984).

RECEPTORS

From her studies on the mating of transected worms in vitro, Michaels (1969) postulated that the mating position is determined by linear receptors on

S. mansoni males and females. She used several enzymes, including ribonucleases and deoxyribonucleases, and snake venoms in an attempt to determine the nature of postulated receptors, but these attempts remained unsuccessful. Results of her studies are difficult to interpret since normal and abnormal mating positions of transected worms are not clearly defined. She further reported that only one in 10 males mated with frozen and thawed females, which argues against the occurrence of receptors that determine mating position.

Lectin-binding sites have been demonstrated on both schistosomula and adult schistosomes (Stein and Lumsden, 1973; Bennett and Seed, 1977; Murrell et al., 1978). Several oligosaccharide residues, notably alpha-methyl-D-mannoside, D-galactose, D-mannose and/or D-glucose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine and sialic acid occur on the schistosome surface (Simpson and Smithers, 1980; Simpson and McLaren, 1982). In these studies, however, distinction between male and female worms was not made. Bennett and Seed (1977) believed that these lectin-binding carbohydrates occur as glycoproteins. Results of other studies did not support this notion (Murrell et al., 1978). A recent study of the interaction of *Salmonella* and schistosomes suggest that at least the mannose-containing receptors are glycolipids (Melhem and Lo-Verde, 1984).

Although these studies have provided information about lectin-binding receptors in schistosomes, there has been no attempt to determine the role of receptors in chemoattraction and/or pairing and mating. Such information is available for other systems, e.g., lectin-mediated functional impairment and inhibition of chemotaxis have been demonstrated in *Caenorhabditis elegans* by Jeyaprakash et al. (1985). Enzyme-mediated inhibition of chemotaxis in this worm has also been demonstrated by Jansson et al. (1984). These workers reported that mannosidase and sialidase caused 100% inhibition and trypsin caused a 50% reduction of chemotactic behavior in *C. elegans*.

Host immunoglobulin binding to schistosomes has been shown to be mediated by Fc receptors present on the worm surface. In addition, receptors for C3 and C1q have also been demonstrated on schistosomula (Smithers and Doenhoff, 1982) but differences between males and females were not examined. At present, there is no evidence of involvement of these receptors in interactions between male and female worms.

Although electron microscopy has revealed structures on schistosome surfaces which may serve as sensory receptors, there is still no information on their function (Hockley, 1973; McLaren, 1980).

ULTRASTRUCTURAL INTEGRITY OF WORMS MAINTAINED IN VITRO

Smith et al. (1969) described degenerative changes in adults following 90 min incubation in either isotonic phosphate-buffered saline, normal human serum, hyperimmune serum, or horseradish peroxidase. Hockley (1970) reported de-

generative changes in worms maintained in hypotonic and hypertonic media (Hockley, 1973). Simpson and McLaren (1982) observed more degenerative changes in worms maintained in RPMI 1640 than in those maintained in Earle's medium supplemented with lactalbumin hydrolysate (LAH). Other studies have reported morphologic changes in worms maintained in vitro in a variety of media (Carlisle et al., 1983; Weisberg et al., 1983). In our laboratory, scanning and transmission electron microscopy following in vitro incubation for 1.0 hr did not reveal any morphologic damage (Haseeb et al., 1984, 1985b). Since Carlisle et al. (1983) and Weisberg et al. (1983) used suction to collect worms, it is possible that they damaged worm surfaces. They also reported that LAH, a constituent of EBSS, appeared to adversely affect the morphology of adult worms. We have not observed damage in the presence of LAH. In other studies LAH has been reported to stimulate egg maturation and production in *S. mansoni* females maintained in vitro over a period of 14 days (Newport and Weller, 1982), which argues against deleterious effects.

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MATING PREFERENCE IN *Schistosoma mansoni*

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Abstract—We investigated the suitability of an in vitro culture system for measurement of mating behavior of *Schistosoma mansoni*. The criteria used to evaluate this system were the level of phosphorylated nucleotides, egg production, and mating status of parasites. The level of ATP, ADP, AMP, and G6-P was measured at different time intervals during cultivation of worm pairs and remained essentially the same as that of control worms for up to 6 days. Egg production was observed in this system during 19 days of cultivation. Peak egg production occurred on day 4 with 72% of the total eggs being laid during the first week of cultivation. The variability in the number of eggs produced by different pairs of *S. mansoni* necessitated the selection and matching of tubes with the same number of eggs after 48 hr. This permitted the detection of small changes in egg production by decreasing intertube variation. Mating recognition between male and female *S. mansoni* was evaluated by culturing separated adult worms with their original partner or with a different partner. During the first 24 hr, mating occurred among a greater percentage of worm pairs comprised of their original partner than among worm pairs comprised of different partners ($P < 0.001$). After 48 and 72 hr of cultivation, these differences were not statistically significant. Similar results were obtained with a culture of mixed males and females. Two drugs were studied for their effects on the mating of *S. mansoni* in vitro. Aminoglutethimide (AG) at a concentration of 1×10^{-4} had no effect on the frequency of mating whereas diethyldithiocarbamate (DDC) completely inhibited mating at a concentration of 3×10^{-6} M and reduced the level of ATP in these worms.

Key Words—*Schistosoma mansoni*, in vitro cultivation, mating, egg production, phosphorylated compounds.

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INTRODUCTION

One of the distinctive features of the genus *Schistosoma* is the sexual dimorphism exhibited by the parasite. Consequently, the reproduction of the parasite is governed by several important requirements. In the first instance, contact between individuals of separate sexes must be achieved, and, second, this must be followed by successful pairing and insemination. It is also difficult to determine precisely, under in vivo conditions, the spatial relationship between male and female worms and, thus, to assess the relative importance of the various components of the relationship which are essential for the development of the female. The in vitro technique overcomes many of these difficulties, enables more precise observation to be made under relatively defined conditions, and affords an opportunity to isolate or modify aspects of the relationship between the sexes.

In early attempts at in vitro cultivation of schistosomes, Lee and Chu (1935) maintained adult *S. japonicum* for several weeks in horse, sheep, and rabbit sera changed every one to two weeks. Rabbit serum in Carrel flasks gave a maximum survival of $2\frac{1}{2}$ months. Chu (1938) reported egg production and copulation by *S. japonicum* in diluted horse serum. In later studies, successful survival of *S. mansoni* in various sera was reported by several workers. Newsome and Robinson (1962) was able to maintain pairs of *S. japonicum* in an egg-laying state in a medium containing human serum (diluted 3:10 with tyrode solution), glucose, and antibiotics. Eggs produced in this system hatched into miracidia but never successfully established an infection in the intermediate snail host. Michaels and Prata (1968) used a medium consisting of 90% medium 199 plus 10% calf serum to which penicillin and streptomycin were added. In this medium, maximum egg production occurred after two to five days of cultivation and oviposition stopped after 10 days. Senft and Senft (1962) cultured *S. mansoni* in a defined medium which promoted oviposition but the eggs were nonviable. To culture *S. mansoni*, Schiller et al. (1975) used a diphasic medium which had been originally designed for cultivation of the rat tapeworm. *Hymenoplepis diminuta*. In this system under anaerobic conditions egg production was minimal, whereas under aerobic conditions large numbers of eggs were produced with a peak occurring at four to six days cultivation. The average number of eggs laid per day was five times greater than that observed by Michaels and Prata (1968). Schiller et al. (1975) found that adult *S. mansoni* survive under anaerobic as well as when under aerobic conditions. Thus, they suggested that for egg production oxygen is utilized by some non-ATP-yielding reaction such as the oxidative tanning of eggshells or the oxidation of a sterol to a hormone-like compound controlling reproductive physiology.

Short (1952) observed that in *S. douthitti* the males and females may migrate independently from liver to mesenteric venules, and the females proceed to lay eggs without mating. But in *S. mansoni*, mating is considered a prereq-

uisite to this migration, although according to Standen (1953) a proportion of the worms in all-male infections moved toward the mesenteric veins. Vogel (1947) reported that unmated females lack the copious black material that fills the cecae of mated females.. He proposed that the female is too weak to feed normally until held by the stronger male. He also hypothesized a hormone-like substance produced by the male and transferred to the female in copula either with the sex products or through the integument. Michaels (1969) reported that the influence of the male on the female worm was not confined to an initial developmental stimulus but the male also influenced the fecundity of the mature female. He also showed that under in vitro conditions the reassociation of separate males and females takes place rapidly, and within the period of 18–24 hr, 85–100% of the worms become paired. Michaels was unable to determine whether chemical factors were involved, but was able to show that successful pairing was related to features resident in the posterior half of the male worm.

From cross-mating experiments between different species of schistosomes, Armstrong (1965) concluded that the adult members of both sexes are active in the search for mates, and that mating is accomplished by means of thigmotaxis and by trial and error rather than by chemotactic response. Armstrong (1965) and Michaels (1969) both agree that mating is active on the part of both sexes. Armstrong also hypothesized that the male controls the attainment of full body size of the female, and this control is dependent on a pheromone. Voge et al. (1978) reported that the tegumental surface of immature males is indistinguishable from the female, and the differentiation of the adult surface begins several days after copulation. The studies of Shirazian and Schiller (1982) indicated that mating recognition exists between separated pairs of *S. mansoni* in vitro, and there is a preference for mating with original partners at least for 24 hr after separation. Eveland et al. (1982) showed both heterosexual and homosexual attraction with and without perforated dialysis sac chimney barriers. Recently Eveland et al. (1983) demonstrated conclusively that the opposite sexes of *S. mansoni* attract each other in vitro and that dialyzable substances probably are involved in chemoattraction.

The experiments reported below were designed to further investigate aspects of pairing, egg production, and mating behavior of *S. mansoni* maintained in vitro.

METHODS AND MATERIALS

Recovery of Adult Schistosoma mansoni. Female mice (CD_1, Charles River) 4–5 weeks old were infected by tail immersion with 100 cercariae of *S. mansoni* (Puerto Rican strain) which had been obtained from *Biomphalaria glabrata*, as described previously by Bourgeois and Bueding (1971). At 55 days postinfection, *S. mansoni* were removed aseptically with the aid of a dissecting needle

from the mesenteric and portal veins of mice, and placed in a Petri dish containing Hanks' balanced salt solution (HBS) plus 200 units of penicillin and 200 μg of streptomycin (P/S) per milliliter. Worms were observed with a dissecting microscope for their gross morphology, motor activity, and sexual pairing. Worm pairs were placed in 10-cm Petri dishes in an ice bath, and they were separated from their sexual partners after 5 min.

Recovery of Schistosomulae. At 28 days postinfection, each mouse was injected with 0.1 ml of a solution containing 30 μg/ml Na heparin. After 30 min, they were sacrificed by cervical fracture, and the abdomen was painted with 2% iodine in alcohol. The iodine was removed, the abdominal skin deflected, and the hepatic vein, inferior vena cava, and portal vein were tied and cut distal to the ties. After cutting the esophagus and freeing the diaphragm from its insertion, the liver and diaphragm were transferred to a sterile Petri dish. The tie was removed from the portal vein, and a 22-gauge needle was inserted into the hepatic venous sinus. Thirty to 60 ml of HBS and P/S containing 10 μg/ml sodium heparin was forced through the liver at a rate sufficient to keep it slightly distended. The worms were located in the fluid with a dissecting microscope and transferred with the aid of a dissecting needle to the culture media.

In vitro Cultivation. The separated worms were cultured by Schiller's method (Schiller et al., 1975), except that (1) blood agar was dispensed into test tubes (13 × 80 Borosilicate glass, Belco Company) in the amount of 1 ml/tube and stoppered with cotton; and (2) 1 ml of HBS and P/S containing 1 mg glucose was added 24 hr before inoculation of *S. mansoni*. One pair of adult worms was cultured in each test tube, and egg production was recorded.

The eggs were studied to determine the presence of various developmental stages, as well as the type of egg abnormalities recognizable under the microscope as previously described by Belding (1965) and Edungbola (1978). The viability and the development of eggs produced in vitro were further studied by incubating the eggs for seven more days at 37°C in a Dubnoff incubator. The number of viable miracidia obtained after diluting the culture media with dechlorinated water was determined as the criterion for viability and development.

Determination of Phosphorylated Compounds. Commercially prepared hexokinase was used for determination of ATP and G6-P. The method used for the determination of the level of AMP and ADP was similar to the one described by Adam (1963), modified primarily by methods listed as follows: (1) 3% perchloric acid (PCA, Mallinkrodt) was used for extraction instead of potassium carbonate; (2) potassium glycylglycine (Sigma, pH = 7.4, 0.5M) was used as buffer instead of EDTA. Pyruvate kinase (110 mg/10 ml) and myokinase (10 mg/2 ml) were purchased from Boehringer/Mannheim. The levels of phosphorylated compounds were determined by changes in optical density of substrates after the addition of appropriate enzymes, as described by Adam (1963).

Effect of Drugs on Mating Behavior. Prior to cultivation of one male and one female worm, 0.02 ml of drug were added to each culture tube. Control test tubes received 0.02 ml of 8.5% saline. All cultures were incubated in a Dubnoff metabolic incubator under conditions previously described. The drugs utilized were: aminoglutethimide (AG, Aldrich Chemical Co., Milwaukee, Wisconsin) and diethyldithiocarbamate (DDC, sodium salt crystalline, Sigma Chemical Co., St. Louis, Missouri).

RESULTS

The suitability of the in vitro culture system for *S. mansoni* was investigated by measuring the level of some phosphorylated compounds such as ATP, AMP, and G6-P at different time intervals during cultivation beginning with 56-day-old worm pairs in vitro. The level of ATP of paired worms cultured in vitro at day 6 was comparable to freshly recovered parasites. After nine days of cultivation, ATP levels declined 30% and then remained at that level until the end of the 12-day experiment. This decrease in ATP after six days of cultivation was not accompanied by an increase in the level of ADP or AMP, nor was there any significant change in the level of G6-P during the cultivation period (Table 1). Supernatants of culture media did not have any significant amount of phosphorylated compounds. Since a large number of parasites were needed for each determination of phosphorylated compounds, no measurements were made beyond the 12th day in vitro. When ATP was measured six times in parasites freshly obtained from mice and after six days of in vitro cultivation, it was found that the concentration of this compound remained essentially the same.

TABLE 1. LEVELS OF PHOSPHORYLATED COMPOUNDS IN *Schistosoma mansoni* CULTURED IN VITRO
(mg/g OF TISSUE, WET WT)

Sample[a]	Time in culture	G6-P	ATP	ADP	AMP
I	0	0.067	3.29	0.71	
II	2 hours	0.34	3.27	0.87	0.22
III	3 days	0.34	3.11	0.63	0.09
IV	6 days	0.44	3.25	0.64	0.12
V	9 days	0.33	2.31	0.77	0.13
VI	12 days	0.44	2.41		
Control (media without parasite)	3 days		0	0	0.17

[a] Each sample consists of 20 test tubes with a single pair of worms.

Separate pairs of adult worms find each other and mate in this in vitro system. Pairing rarely occurred during the first 6 hr, but thereafter the frequency of mating events increased rapidly. A maximum of pairing was reached on the third day, and then gradually declined until no new matings were observed after the seventh day of cultivation. When separated pairs of worms were cultivated with their original partners, the frequency of mating events was significantly greater than when the worms were cultured with nonoriginal partners. The difference remained statistically significant even after the worms had been separated from their original partners for as long as 72 hr.

In another series of experiments, individual adult worms that had no previous association with each other were cultured in groups. One group consisted of three females and one male, and the other was comprised of three males and one female. After 24 hr of cultivation, the frequency of mating events in both groups was approximately the same as that observed in the preceding experiments among worm pairs comprised of nonoriginal partners. However, when either of these groups contained a male and a female that had been partners originally, the frequency of mating events increased significantly. Pairing between males was not observed in any of the cultures, as previously reported by Shirazian and Schiller (1984).

Egg production was observed daily in 100 test tubes, each containing one pair of parasites, during 19 days of cultivation. Peak egg production occurred on day 4, and 72% of the eggs were laid during the first week of cultivation. Overall, 80% of the parasites produced eggs. Acetocarmine staining of the worms which produced no eggs showed that 60% had no eggs in the reproductive tract, 20% had one abnormal egg, and 20% had only empty eggshells in the uterus.

All stages of egg maturation were observed when eggs were kept for an additional 10 days in the in vitro culture system. Additionally, when the contents of the culture tubes containing mature eggs were diluted with distilled water, the eggs hatched, and living, vigorously active miracidia were released.

In our in vitro system the number of eggs produced per pair of worms is variable. To determine if better reproductive uniformity could be achieved by selecting those pairs that had produced approximately the same number of eggs, 164 worm pairs that had produced 36 $\pm$ 1 ($\bar{X} \pm$ SD) during the first 48 hr in culture were transferred to fresh media and cultivated for an additional six days. At the end of this period, each of the worm pairs had produced eggs ranging in number from 299 to 317 ($\bar{X}$ = 306, SD $\pm$ 7.8). According to these results, it is evident that this selection technique decreases the range of variability in egg production that occurs among worms taken at random, thus providing a means for standardizing samples of *S. mansoni*, (Shirazian and Schiller, 1984).

Attempts were made to inhibit the mating of parasites by adding certain drugs to the in vitro system. Because aminoglutethimide is known to be an inhibitor of all sex hormones in mammals and is nontoxic to living cells at a

concentration of 1×10^{-4} M (Howal, 1967), it was selected for this purpose. In these experiments, separated adult males and females were cultured individually in media containing 1×10^{-4} M of this compound for 48 hr. The parasites then were placed in fresh media, either with a drug-treated or a non-drug-treated worm of the opposite sex. There was no significant difference between the frequency of mating in these experiments.

The copper-containing enzyme, phenoloxidase, plays an important role in egg production by *S. mansoni* (Lees, 1946; Mansour, 1958). Its activity can be inhibited by DDC in vivo (Bennett and Gianutsos, 1977) as well as in vitro (Seed et al., 1978); thus it was of interest to determine if this drug had any effect on the mating behavior of these parasites. Our results indicate that concentration of 3×10^{-6} M of DDC can inhibit mating of *S. mansoni*. However, this effect was accompanied by reduction of the level of ATP. It is thought that inhibition of mating is mainly due to the toxicity of DDC for *S. mansoni* rather than a direct inhibitory effect on mating.

DISCUSSION

Until recently, most of the in vitro systems for *S. mansoni* have involved the use of balanced salt solutions to which various mammalian sera and/or embryonic extracts, amniotic fluids, or ascitic fluids were added (Lee and Chu, 1935; Hoeffli, et al., 1938; Newsome and Robinson, 1954, 1962, 1963; Senft and Weller, 1956). Although such fluid culture media allowed survival of worms and, in some cases, growth of young adults, only limited egg production has been reported (Cheever and Weller, 1958; Robinson, 1960). Several investigators have employed chemically defined media for the in vitro culture of parasites without success, except for that of Ross and Bueding (1950), who found that *S. mansoni* survived for 18 hr in a defined medium. The addition of muscle extract derivatives (largely unidentified substances called protogens) considerably enhanced survival time in the defined medium. Mayo and Lyu (1957) found that *S. japonicum* could survive about 16 days in a Tyrode–glucose–vitamin mixture. Such short-term survival is, however, quite inadequate for growth, regeneration, or egg production studies. Fu et al. (1976) reported that media 199 and NCTC 135 were less than satisfactory for maintenance of adult *S. japonicum*. After a time in these media, body lengths decreased and genitals degenerated. Considering these reports, it appears that a more successful system for the culture of adult *S. mansoni* with production of viable eggs may best be achieved in a diphasic medium prepared with animal blood. Schiller et al. (1975) used a diphasic medium for in vitro cultivation of adult *S. mansoni*. In the current studies, a modified version of this system was used to maintain mated parasites for extended periods of time with the production of viable eggs.

In the present studies, the levels of phosphorylated compounds such as G6-P, ATP, ADP, and AMP were measured during the 12 days of cultivation. These measurements allowed further investigation of the suitability of the in

vitro system for the maintenance of *S. mansoni*. The ATP level in mated parasites slightly decreased for the first three days of culture. This decrease may be interpreted as an adaptation period for the parasite. There is some evidence that can be used to support this hypothesis. As noted earlier, schistosome egg production in vitro is inhibited by physiological changes due to the removal of the worm from its host. Egg production remains suppressed for at least 48 hr. However, when parasites are in the stage of maximum egg production (on day six), the level of ATP reaches its normal control level. After eight days of cultivation, the level of ATP in *S. mansoni* declined 30%. It is interesting to note that this drop in ATP was not accompanied by changes in the levels of ADP or AMP. The reason for this may be due to decreased glucose utilization during this time, as observed by Schiller et al. (1975). The most important finding is that the level of ATP returns to its original level on day six of cultivation. This observation provides additional evidence of the suitability of this system for the in vitro mating and egg production by this parasite.

Edungbola and Schiller (1979) indicated that maximum egg production occurred after three to six days of cultivation. Our observations on 80% of the parasites which produced eggs are in accord with this report and show that the peak of egg production is on day 4 and that more than half of all the eggs are produced between three and six days of cultivation. The reason for the peak of egg production on day 4 may be due to the fact that parasites at this time have adapted themselves to the in vitro system. Since the parasites were transferred every three days, day 4 of cultivation would be the first day in the fresh culture media when there is more nutritional material and fewer waste products in the system.

Eggs which had been produced during the first six days of cultivation were kept for an additional seven days in vitro. These eggs hatched, and active, live miracidia were released when the supernatant of culture media was diluted with distilled water. This result is in agreement with Edungbola and Schiller (1979) that *S. mansoni* eggs from the in vitro system of Schiller (1975) were viable and, since they reached the miracidial stage, induced typical granulomatous response in the livers of recipient mice. Although parasites can survive in this in vitro system for at least two months, egg production was never observed after nineteen days of cultivation.

In the control of schistosomiasis an advantageous development would be an agent which interfered with the reproductive capacity of the worm. Many investigators have reported that the reproductive system of the female worm is a delicately coordinated system which is easily disturbed by a variety of factors, for example, unisexual infection, drugs, host diet, and hormonal imbalances of the host animal. This sensitivity has been utilized by Pellegrino and Katz (1968) to develop the oogram method of assessing the effect of antihelmintics on the parasite. However, *S. mansoni* produce different numbers of eggs in different

in vitro culture systems (Michaels and Prata, 1968; Senft and Senft, 1962; Schiller et al., 1975), and there is a significant variation in the number of eggs produced by individual parasites in the same culture medium. Our methods provide an accurate means of standardizing egg production by these parasites, so that the effect of different drugs on this process can be quantitatively determined.

These results are in agreement with previous work that established that separated pairs of adult worms find each other, mate, and produce eggs in vitro (Eveland et al. 1982, 1983, Shirazian and Schiller, 1982, 1984). Pairing rarely occurred during the first 6 hr, but thereafter the frequency of mating events increased rapidly, reaching a maximum on the third day, and then gradually declined until no new matings were observed after the seventh day of cultivation. As previously reported, when separated pairs of worms were cultured with their original partners, the frequency of mating was significantly greater than when the worms were cultured with nonoriginal partners ($P < 0.001$) (Shirazian and Schiller, 1982). This difference remained statistically significant even after the worms had been separated from their original partners for as long as 72 hr. Even in insect systems in which mating behavior has been studied extensively, the phenomenon of individual recognition seems to be controversial (Shorey, 1976). For this reason and also because the deduction made herein is inferential, it must be regarded with some caution until more direct evidence can be obtained through the use of some technique, not yet available, for marking individual worms.

In an attempt to prevent mating in vitro, we used two different drugs, in this study. Aminoglutethimide, which is known to inhibit the formation of steroids, had no effect on the mating behavior of the parasite. The percentage of mating was not reduced, although the possibility still exists that AG could have been incorporated into the culture medium, thus precluding any effect. It is also possible that the worm's receptors and/or the mediators are not very sensitive to this drug.

DDC was used to inhibit the schistosome copper-containing enzymes which are similar to that described for the phenol oxidase of *F. hepatica* (Mansour, 1958). Exposure to a concentration of 3×10^{-6} M for three days completely inhibited the mating of *S. mansoni*, and significantly reduced the level of phosphorylated compounds of *S. mansoni*.

In general, our results support the conclusion that our in vitro culture system provides the conditions necessary to study the physiology, mating behavior, and egg production of *S. mansoni*. The levels of phosphorylated compounds remain stable for six days, and the number of eggs produced during this time period is comparable to that recorded for the same strain of parasite in vivo. This system is adequate for the short term study of mating behavior and the effects of pharmacological substances on related events.

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EXTRACTION OF INTERSEXUAL CHEMOATTRACTANTS FROM *Schistosoma mansoni*

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Abstract—Female *Schistosoma mansoni* and their excretory–secretory (ES) products were extracted with a series of solvents to provide fractions of varying polarity. These fractions were assayed for chemoattractivity to males in vitro. One major component of these mixtures was found to be nonattractive and was identified as cholesterol. *n*-Pentane- and ether-soluble fractions derived from ES products exhibited chemoattractive activity comparable to that possessed by whole-worm extracts, but appeared to be simpler mixtures.

Key Words—*Schistosoma mansoni*, intersexual chemoattractivity, cholesterol, excretory–secretory products

INTRODUCTION

Evidence for chemoattraction between the sexes of various species of nematodes and trematodes has been available for some time (Bone et al., 1977; Bone and Shorey, 1977; Fried et al., 1980; Fried and Robinson, 1981). Host reactions to eggs resulting from mating of one such organism, the parasitic trematode *Schistosoma mansoni*, are the primary cause of the debilitating effects of schistosomiasis. Shirazian and Schiller (1982) have shown that each sex of *S. mansoni* shows a tendency to be attracted to the opposite sex in vitro in the

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absence of worm tactile behavior. This observation suggests that sexually specific chemoattractive agents may be produced by each sex of *S. mansoni*. If such agents could be isolated and identified, knowledge of their chemical structures could be used to develop ways of interfering with the mating process. This approach may be useful in combating schistosomiasis. We describe here procedures for obtaining extracts with intersexual chemoattractivity from *S. mansoni*.

METHODS AND MATERIALS

Samples of *S. mansoni* were obtained and prepared for study as described elsewhere in this issue (Shirazian et al., 1986). All solvents employed were distilled in glass. Thin-layer chromatography (TLC) was performed using glass plates which were precoated with a 250-μm layer of silica gel 60 F-254. Chloroform–methanol (9 : 1) was employed as the mobile phase. Compounds were visualized with a vanillin–50% sulfuric acid spray reagent or by exposure to iodine vapor. Gas chromatography (GC) was carried out using a 6-ft glass column of 3% OV-1 on 100–120 Gas-chrom Q, a temperature program from 40 to 280°C at 10 C/min, and a Varian model 2100 gas chromatograph. The mass spectrum was obtained using a Finnigan model 3000 mass spectrometer. The proton NMR spectrum was recorded at 300 MHz on a Bruker WM-300 spectrometer.

Fresh, freeze-dried female *S. mansoni* (150 individuals; 7.5 mg dry weight) were ground with a small mortar and pestle to give a powdery solid. This solid was extracted progressively with solvents of increasing polarity to fractionate the soluble components. Two milliliters of *n*-pentane were added, and the solids were triturated and allowed to soak for 15 min. The solids were centrifuged, and the solution was decanted, filtered, and evaporated under a stream of dry nitrogen to give a colorless oily residue. This procedure was repeated twice more, and all three extracts were combined to afford an *n*-pentane fraction (0.14 mg). This sequence was repeated using diethyl ether in place of *n*-pentane to extract the worm solids. All of the ether-soluble material was combined to give an ether fraction (0.40 mg), and the procedure was repeated consecutively with ethyl acetate, methanol, and water to provide three more fractions (0.75, 1.83, and 2.41 mg, respectively).

Two different types of worm-free incubates were also prepared which contained *S. mansoni* excretory–secretory (ES) products (Shirazian et al., 1986). Groups of 100 female worms were incubated in Hanks' balanced salt solution (HBSS) for 72 hr, or cultured in biphasic medium (Schiller et al., 1975) for 48–72 hr. In each instance, the supernatant solution was collected, freeze-dried, and the resulting residue was subjected to the extraction procedure described above

using only *n*-pentane and ether. The HBSS supernatant gave a 0.38-mg *n*-pentane fraction and a 0.33-mg ether fraction, while the biphasic supernatant gave a 0.14-mg *n*-pentane fraction and a 0.02-mg ether fraction.

Ten percent of each of the fractions described above was set aside for chromatographic analysis, while the remaining material was assayed for chemoattractivity to males using the procedures described elsewhere in this issue (Childs et al., 1986). Extracts of males were prepared in an analogous fashion, but studies were focused on the female extracts because preliminary assays showed that males were more strongly attracted to female extracts than females were to male extracts.

RESULTS

Results of assays for chemoattractivity are reported elsewhere in this issue (Childs et al., 1986). In all cases, attractive activity could be found in the least polar (*n*-pentane- and ether-soluble) fractions. Analysis of the *n*-pentane-soluble whole-female extract by TLC showed that a total of five major components were present (R_f: 0.07, 0.19, 0.75, 0.82, and 0.90), along with an indefinite number of minor constituents. The ether-soluble fraction contained the same five major components, although in different concentrations. Analysis of the *n*-pentane fraction by proton NMR spectroscopy strongly suggested that at least some of the major components of the mixture were steroids. Chromatographic comparison with an authentic sample indicated that the most abundant component of the mixture (R_f 0.75) might be cholesterol. Analysis of the mixture by GC showed a single major peak which coeluted with cholesterol upon coinjection. Analysis by GC–MS showed that this component had a molecular weight of 386 and the expected fragmentation pattern, thus confirming its identity as cholesterol. Comparison with male and ES extracts showed that cholesterol was a major component of each. Cholesterol seems very unlikely to be a sexual chemoattractant for *S. mansoni*. A sample of cholesterol was tested for chemoattractivity in our assays and was found to be unattractive to both sexes (Childs et al., 1986).

The major components of the whole-worm extracts appeared to be identical (by TLC) for males and females even though extracts of each sex were attractive only to the other. This result suggests that the chemoattractants are not major components of these fractions. The *n*-pentane- and ether-soluble fractions obtained from the female worm-free incubates described above exhibited a degree of attractivity comparable to that observed in the whole-worm extracts (Childs, et al., 1986). TLC analysis of these fractions gave results which were very similar to those of the corresponding whole-worm extracts, except that the more polar major components (R_f 0.07, 0.19) were clearly much less abundant in the

worm-free incubate extracts. Since the ES product mixtures are apparently less complex than the whole worm fractions, isolation of the attractive component(s) may be simplified by focusing studies on ES products.

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IN VITRO ORIENTATION OF MALE *Schistosoma mansoni* TO EXTRACTS DERIVED FROM FEMALE SCHISTOSOMES

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Abstract—Chemical orientation of adult male *Schistosoma mansoni* was studied during cultivation in vitro. Directional preference was assessed in culture vessels marked with compass coordinates by the application of circular statistics for determining clustering and orientation to a predicted direction. Organic solvent extracts of fresh female schistosomes and supernatant fluids from 72-hr cultures of female parasites were tested for potential chemotactic activity. Analysis showed significant ($P < 0.05$) directional preference and clustering of male worms towards test compounds at all time periods (24, 48, and 72 hr) with one mixture of female extracts and at one of three time periods with a second female extract. Male worms did not respond to ecdysone, cholesterol, or solvent controls by orienting in predicted direction, although clustering was observed on two of 12 occasions. These results indicate the presence of a chemoattractant compound(s) in female extracts.

Key Words—*Schistosoma mansoni*, chemotaxis, attraction, primer pheromone, releaser pheromone, in vitro maintenance, circular statistics, ecdysone, cholesterol.

INTRODUCTION

Numerous studies have documented the existence of a reproductive stimulus (or stimuli), provided by male schistosomes, that mediates complete development of the reproductive system of females. These stimuli have been attributed to

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hormones (Moore et al., 1954; Severinghaus, 1928), nutritional influences (Vogel, 1941), tactile contact (Armstrong, 1965), and pheromones (Armstrong, 1965). Substances or stimuli that induce or maintain sexual maturity (e.g., "primer" pheromones) may act independently of chemical substances that act at a distance to aggregate or attract parasites to each other or members of the opposite sex (e.g., "releaser" pheromones).

Chemical orientation of adult schistosomes to substances likely to be significant as mating pheromones is an area of study that has received little attention. Armstrong (1965), in a series of experiments on the mating behavior and development of schistosomes in mice, concluded that both sexes are active in the search for mates and that mating probably occurs via thigmotaxis and trial and error rather than chemotactic response. Imperia et al. (1980) demonstrated in vitro that female *Schistosoma mansoni* were attracted to single males isolated in chambers which allowed release of excretory–secretory (ES) products; however, females failed to respond significantly to chambers containing three males. Eveland et al. (1982) used a linear chamber bioassay to show chemoattraction in adult *S. mansoni* tested in both heterosexual and homosexual combinations. These same investigators implicated a chemoattractant of greater than 1000 molecular weight (M_r) by isolating target worms in dialysis sac barriers with varying molecular exclusions (Eveland et al., 1983). Shirazian and Schiller (1982) inferred the presence of chemical compounds mediating mating recognition on the basis of preferential responses of male and female *S. mansoni* cultivated in vitro for original over nonoriginal partners.

Chemoattractants are known to be released by nematodes, and in vitro demonstration of attractant activity has been reported from several species including *Trichinella spiralis* (Bonner and Etges, 1967) and *Nippostrongylus brasiliensis* (Bone et al., 1977).

This study was designed to use a modified version of an in vitro culture system (Schiller et al., 1975) to investigate the chemoattractant properties of substances derived from extracts of female schistosomes, or culture supernatant fluids, on the behavior of males. Two authentic steroid substances, B-ecdysone and cholesterol, were also tested for chemoattractiveness. Ecdysone, an insect moulting hormone, has been detected in adult *S. mansoni* and their ES products (Nirde et al., 1983), and substances similar to cholesterol, by thin-layer chromatography (TLC), have been implicated as pheromones in other helminths (Fried and Gioscia, 1976; Roberts and Thorson, 1977).

METHODS AND MATERIALS

Adult *S. mansoni* (Puerto Rican strain) were obtained from female CD1 Charles River Mice infected with 100 cercariae 50–60 days prior to dissection as described by Shirazian and Schiller (1982). Paired worms were separated

over crushed ice, sorted by sex, and placed individually in the experimental apparatus for testing of compounds. Single sex groups of 70–150 worms were placed either in saline or in culture for the future collection of supernatant fluids or worms for lyophilization and subsequent extraction of test substances. Each potential chemoattractant compound was tested on 7–10 previously mated, active, male worms, obtained from a single mouse. Replicate experiments were conducted on all test compounds.

Test compounds were prepared from the worms placed in saline and from the supernatant fluids from cultures, maintained for 72 hr, containing the single-sex groups of worms. These materials were lyophilized and lipids were extracted with organic solvents (*n*-pentane, ether, methanol, or ethyl acetate) as described by Gloer et al. (1986). Extracts were dried under nitrogen and reconstituted with solvent prior to analysis by TLC or use in behavioral studies.

Extracted fractions from female parasites were combined into two pools, called female extract pool 1 and female extract pool 2, in order to allow testing for the presence of any chemoattractant activity among the large number of concentrated extracts obtained. An impregnated test agar was made by mixing pooled extracts with 5 ml of 1.6% agar to give total concentrations of 0.14 mg extract/ml agar in female extract pool 1 (five lipid fractions pooled), and 0.19 mg extract/ml agar in female extract pool 2 (same as pool 1, plus three additional nonpolar lipid fractions). Two plugs (0.053 ± 0.004 ml) were cut from this impregnated test agar for use in each experimental vessel. Total amounts of extracts used per individual worm experiment ranged from approximately 15 to 20 μg. This amount of extract was approximately equal to 1.4–3.2 worm equivalents. Ecdysone and cholesterol (Sigma Chemical Company, St. Louis, Missouri) were tested at concentrations similar to those of female extracts. Control substances consisted of appropriate solvents mixed with agar.

A circular culture vessel was used to text experimental compounds. Sterile plastic culture vessels, 5 cm in diameter, were inoculated with 5 ml blood agar (Schiller et al., 1975), and two circular plugs (2–3 mm in diameter; 0.053 ml) were cut and removed by suction from the periphery of the agar sheet approximately 1 mm from the vessel rim. The plugs cut from agar impregnated with worm extracts were placed in the complementary holes cut in the experimental vessels and cemented in place with warm agar. Experimental vessels were left overnight at 4°C to allow some diffusion to test compounds from the plugs to the surrounding agar, although the final concentration gradients were not determined. Culture vessels were then overlayed with the liquid portion of the diphasic medium (Schiller et al., 1975) and placed on clear acetate sheets marked with individual compass coordinates and distance grids corresponding to each test vessel. Single male worms were then placed at the center of test vessels.

Acetate sheets and culture vessels were enclosed in a humidified incubator at 37.5°C under a Plexiglas cover equipped with gas ports and charged with

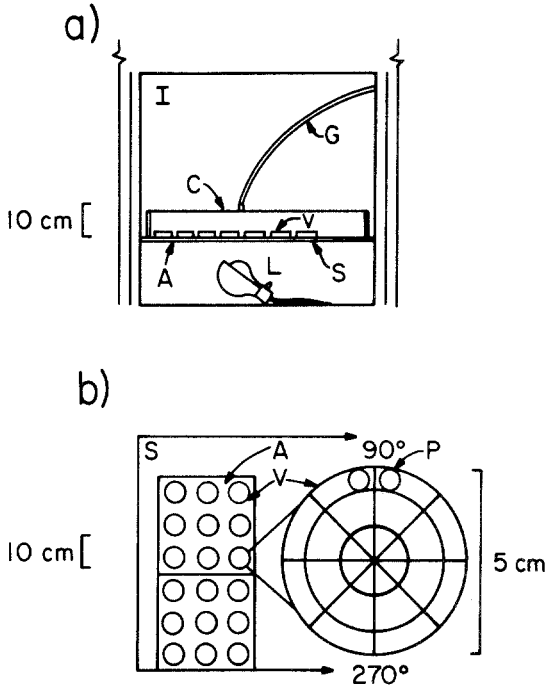

FIG. 1. Experimental apparatus for testing in vitro chemical orientation of *S. mansoni*. (a) Side view; A = acetate sheet marked with grid coordinates; C = Plexiglas cover; G = gas line; I = incubator; L = light; S = Plexiglas shelf; V = vessel; (b) top view showing enlarged view of vessel with grid coordinates; P = agar plug position. Parasites were placed in the center of the vessel.

5% CO_2, 5% O_2, and 90% N_2. The entire testing apparatus rested on a 1.2-cm-thick shelf of Plexigas raised 0.2 m from the bottom of the incubator (Figure 1). Parasite locations were recorded by viewing grid coordinates through the agar, by placing a 50-W light bulb directly below individual culture dishes. Parasites were maintained in the dark, except when location recordings were made at 24-, 48-, and 72-hr intervals. This procedure allowed data collection with minimal disruption of normal parasite activity.

Parasite orientation to each test substance or extract pool was tested by calculation of a mean vector, obtained from summing individual coordinates of parasite locations. Data from replicate tests of compounds were pooled prior to calculation of test statistics. A modified Rayleigh test (MRT) was used to compare mean directions (average of summed sines and cosines) of parasite vectors to the expected preferred direction towards the agar plugs containing the test compounds (Batschelet, 1972). The Rayleigh test (RT) was also applied to test for significant directional preferences (clustering) which could have occurred

independently of any orientation towards test compounds (Batschelet, 1972). In reporting data, two test statistics were used: r = the length of the mean vector and v = the direction of the mean vector. The predicted direction, towards the agar plugs, was standardized to 90° for ease of comparison with experimentally derived mean directions, although during actual tests the location of plugs was randomized with regard to compass bearing.

RESULTS

The results of TLC analyses are presented in detail elsewhere (Gloer et al., 1986). Briefly, the extracts obtained from both female *S. mansoni* and their supernatant fluids contained three nonpolar fractions. The presence of these substances in both whole worms and culture supernatant fluids indicates they were probably ES substances released by worms in culture. One of these nonpolar extracts had a chromatographic mobility similar to cholesterol.

Male *S. mansoni* showed significant clustering and directional preference towards female extract pool 1 at all three time periods (Figure 2). Males showed significant clustering and directional preference at one of three time periods (48

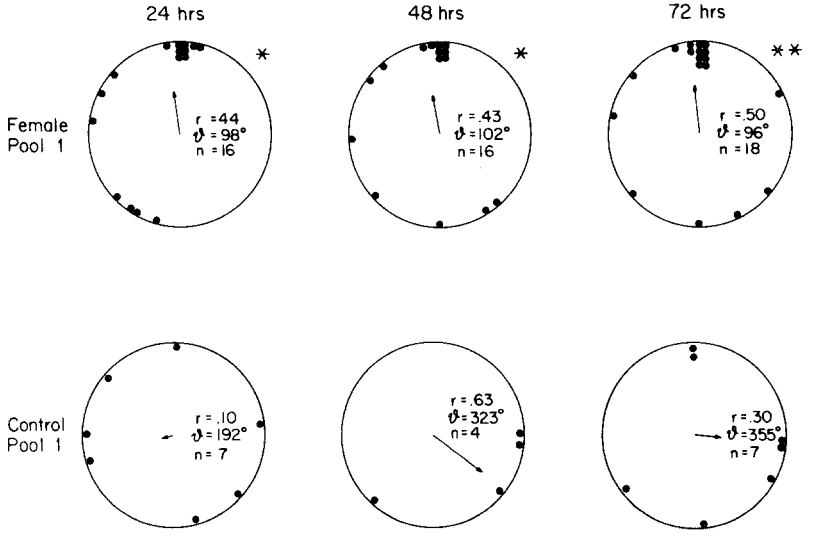

FIG. 2. Individual orientation angles (•), mean vector length (r), and preferred directions of orientation (v) of male *S. mansoni* to agar plugs impregnated with female extract 1. Location of plugs is always 90°, towards the top of the page. Radius of circle = 1. Values shown for p (* = $P < 0.05$; ** = $P < 0.01$) were the least significant obtained by either the Rayleigh test or modified Rayleigh test. Values of P for all time points are shown in Table 1.

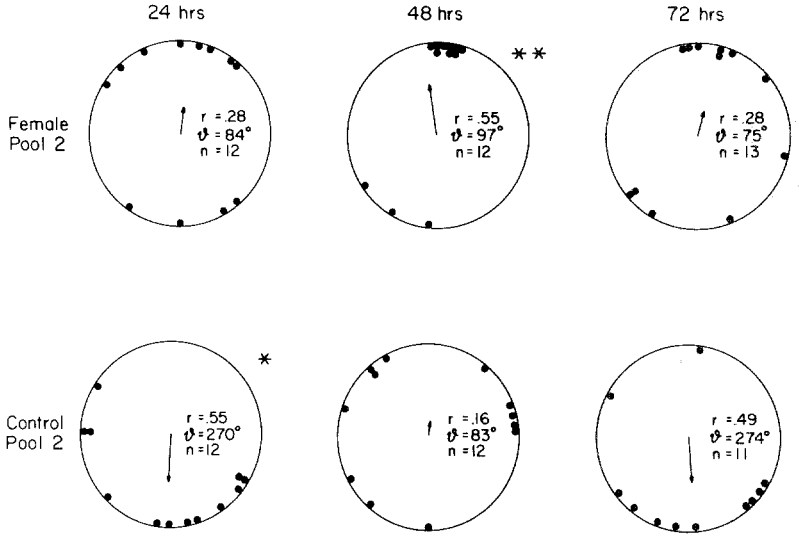

FIG. 3. Individual orientation angles, mean vector length, and preferred directions of orientation of male *S. mansoni* to agar plugs impregnated with female extract 2. Notation as in Figure 2.

hr) for female extract pool 2 (Figure 3). Mean directions in both sets of experiments clustered around the predicted value of 90° even in cases when statistical analyses were insignificant (Table 1). Male schistosomes were significantly clustered in one of six control periods (Figure 3, 24 hr), however, the mean direction was 270°, directly opposite the agar plugs impregnated with control substances. It is possible that solvents used in control plugs are repellent when offered in the absence of female extracts. The number of worms counted per time interval varied (Figures 2 and 3) because occasionally worms would crawl under the agar medium and could not be visualized without disrupting the experiments.

Male schistosomes failed to orient and move in predicted directions when presented with plugs impregnated with ecdysone or cholesterol (Table 1). At one time point, there was significant clustering with ecdysone at a mean direction of 350°.

DISCUSSION

These results, although based on a limited number of experiments, strongly suggest that male *S. mansoni* were responding to a substance(s) in female extracts that was chemoattractive. This substance, presumably a lipid, was present in both whole female schistosomes and in culture supernatant fluids, indicating it may have been an ES product.

TABLE 1. SUMMARY OF CHEMOATTRACTANT EXPERIMENTS WITH DIFFERENT SUBSTANCES ON MALE *S. mansoni*

Test substances	No. replicates (No. worms)	v range (degrees)[a]	r, range[b]	RT[c]	MRT[d]
♀ *S. mansoni* Extract 1	2 (16–18)	96–102°	0.43–0.50	$P < 0.05$[e] all	$P < 0.01$ all
Extract 2	2 (12–13)	75–97°	0.28–0.55	$P < 0.05$ 48 hr	$P < 0.01$ 48 hr
Ecdysone	2 (18)	197–350°	0.15–0.44	$P < 0.05$ 72 hr	N.S.
Cholesterol	1 (8)	258–304°	0.15–0.43	N.S.	N.S.

[a] Direction of mean vector, 90° = direction to test substances.
[b] Length of mean vector, maximum = 1.
[c] Rayleigh test.
[d] Modified Rayleigh test.
[e] P values are for test interval shown (all = 24, 48, 72 hr).

Steroids that can act as chemoattractants have been identified in other in vitro experiments with helminths. Fried and Gioscia (1976) demonstrated that metacercaria of *Leucochlorlidiomorpha constantiae* were attracted to a compound with the identical chromatographic mobility as cholesterol. Roberts and Thorson (1977) reported that adult *Nippostrongylus brasiliensis* were attracted to a lipid fraction of ES products that cochromatographed, by TLC, with colesterol and B-sitosterol; however, neither authentic sterol was attractive when tested in vitro. Our results show that a single nonpolar fraction of *S. mansoni* extract comigrated with cholesterol, but cholesterol alone was not chemoattractive in a single test. The individual substance, or group of substances, that is active as a chemoattractant remains to be identified.

Recent observations on *S. mansoni* (Haseeb et al., 1985a) and *S. haematobium* (Haseeb et al., 1985b) indicate that both species of parasite release lipids when maintained in vitro. Fried et al. (1983) demonstrated that *S. mansoni* release cholesterol in vitro and that male parasites released considerably more than females or pairs.

The potential importance of lipophilic substances in helminthic sexual processes has been stressed (Imperia et al., 1980). Lipids may serve a function as chemoattractants as well as to stimulate sexual development of unmated female *S. mansoni* (Shaw et al., 1977). Shorey (1976) has pointed out that pheromones that elicit aggregation at a distance can stimulate additional close-range behavior. Ecdysone and related hormones, known to stimulate moulting in insects,

have recently been found in *S. mansoni* and are known to be released by these parasites in vivo (Nirde et al., 1983, 1984). The exact function of these hormones in trematodes is still unclear. A suggestion has been made that the predilection of invading miracidia of schistosomes for the ovotestis of snail hosts is due to the presence of morphogenetic substances needed by the worms (Muftic, 1969). Muftic (1969) suggests that these substances may be steroids and reports on the significance of ecdysones in miracidial transformation. The implications of these observations are that this life stage of the worm is capable of locating and aggregating in tissues of a host containing high concentrations of a steroid that may be ecdysone. In our experiments, male adult schistosomes did not respond to ecdysone, but it would be interesting to test miracidia of *S. mansoni* in varying concentrations of ecdysone, as Roberts et al. (1979) have done with snail-conditioned water.

The use of circular statistics has allowed the comparison of experimentally derived measures of response (clustering and orientation to preferred direction) against predicted values. In making these comparisons the magnitude of movement has been intentionally ignored. These measures were chosen because of the nature of the test apparatus and limitations of the sampling procedure (enclosed vessels of limited size, examined at discrete intervals). Ideally, detailed, continuous measurements of the direction and extent of worm movements would have been preferred, but this would have involved greater interruption of culture conditions such as continuous dark lighting and constant temperature. The individual movements of schistosomes has been monitored at shorter time intervals with some success (Schiller et al., 1986).

Increasing evidence now supports the conclusion that chemoattractive substances are present in adult schistosomes and are released in vitro (Imperia et al., 1980; Eveland et al., 1982, 1983). These substances may function as pheromones to help schistosomes become aggregated and mated, although their exact chemical nature and significance in vivo are unclear. Such substances, in other helminths, can be targeted for disruption by chemical, and potentially immunological, means (Bone and Shorey, 1977). Interruption of intraspecific communication would be extremely beneficial in controlling schistosomiasis because the major cause of pathology is due to the production and dissemination of eggs which occurs after mating.

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TIME-LAPSE VIDEO TAPE DOCUMENTATION OF CHEMICAL ORIENTATION BY *Schistosoma mansoni* IN VITRO

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Abstract—In vitro, the opposite sexes of *S. mansoni* are attracted to each other by some means of premating communication which culminates in aggregation and copulation of sexual pairs within 24 hr. The system used for time-lapse video tape documentation of worm sexual behavior in vitro is described and evidence of sexual chemoattraction is presented.

Key Words—*Schistosoma mansoni*, sexual chemoattraction, mating pheromone, videotechnology.

INTRODUCTION

Although the literature suggests that chemical communication by primer and releaser pheromones occurs extensively within the helminths, detailed knowledge of these events is sparse (Bone, 1982; Eveland et al., 1982, 1983; Haseeb et al., 1985). In vitro culture systems that support metabolic and synthetic processes of these parasites for at least 72 hr can be used to bioassay chemically mediated sexual attraction in dioecious species. The basic culture method described by Schiller et al. (1975) has proven useful for such studies of *Schistosoma mansoni* (Childs et al., 1986). Several criteria have been fulfilled in establishing the suitability of this system for investigations of this kind. It supports egg production by *S. mansoni* for at least 96 hr at a rate comparable to that recorded for the same strain in vivo (Schiller et al., 1975). These eggs are viable when laid in culture, complete their development to miracidia after 10 days of cultivation, and are capable of inducing the typical granulomatous response

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when injected into laboratory rodents (Edungbola and Schiller, 1979). Furthermore, the system provides physiologic conditions under which the ATP level, glucose utilization, and lactate production by these worms are constant for at least six days (Schiller et al., 1975; Shirazian, 1979; Shirazian et al., 1986). When coupled with videotechnology, this system has enabled the documentation and analyses of important reproductive events. The purpose of this report is to describe the time-lapse video system used in our studies of worm sexual behavior and to illustrate some aspects of chemical attraction exhibited by them.

METHODS AND MATERIALS

The source of worms and procedures of in vitro culture were as described by Childs et al. (1986). The system used for recording worm behavior consisted of a Panasonic VHS video camera enclosed in a specially constructed plastic, waterproof housing (Photo-Tech, Baltimore, Maryland). This unit was placed inside the environmental chamber of a Wedco incubator so that it rested on a clear plastic shelf with the camera lens directed downwards, permitting it to be focused on the parasites in a culture vessel underneath. The camera and lighting were operated by remote control through cables leading to the exterior where they were attached to a Panasonic tape recorder connected to a model CT-110 MA color video monitor (Figure 1).

The incubator was maintained at a temperature of 37 C with a gas atmosphere of 90% N2, 5% CO2 and 5% O2. Exposures were made for periods of 15 sec at predetermined intervals of time (usually at 0:15, 0:30, 0:45, 1:00, 2:00, 3:00, 4:00, 5:00, 6:00, and 24 hr). Camera focusing, operation, lighting, and recording were all controlled remotely, thus precluding the need to open the incubator door or to disturb the cultures in any way. A transparent plastic sheet, with circular coordinates drawn upon it, was placed over the video screen of the monitor (Figure 1). Upon completion of a taping sequence, the tape was viewed on the monitor and the location of the worm(s) at each time interval was recorded on work sheets bearing corresponding compass direction and distance grids drawn to scale. Thus, the location of the worms, their direction, and distance traveled in a given period of time could be measured. The resultant data were analyzed statistically as described by Childs et al. (1986).

For heterosexual attraction experiments, adult worms surgically removed from mice were inoculated into culture vessels with four males close together in a group at one side and, similarly, four females at the opposite side. In homosexual experiments, the procedures were the same, except that the two groups of four worms were either all males or all females. Only unpaired worms from the same host animal were used in each test. The experimental design for recording worm response to test compounds was as described by Childs et al. (1986).

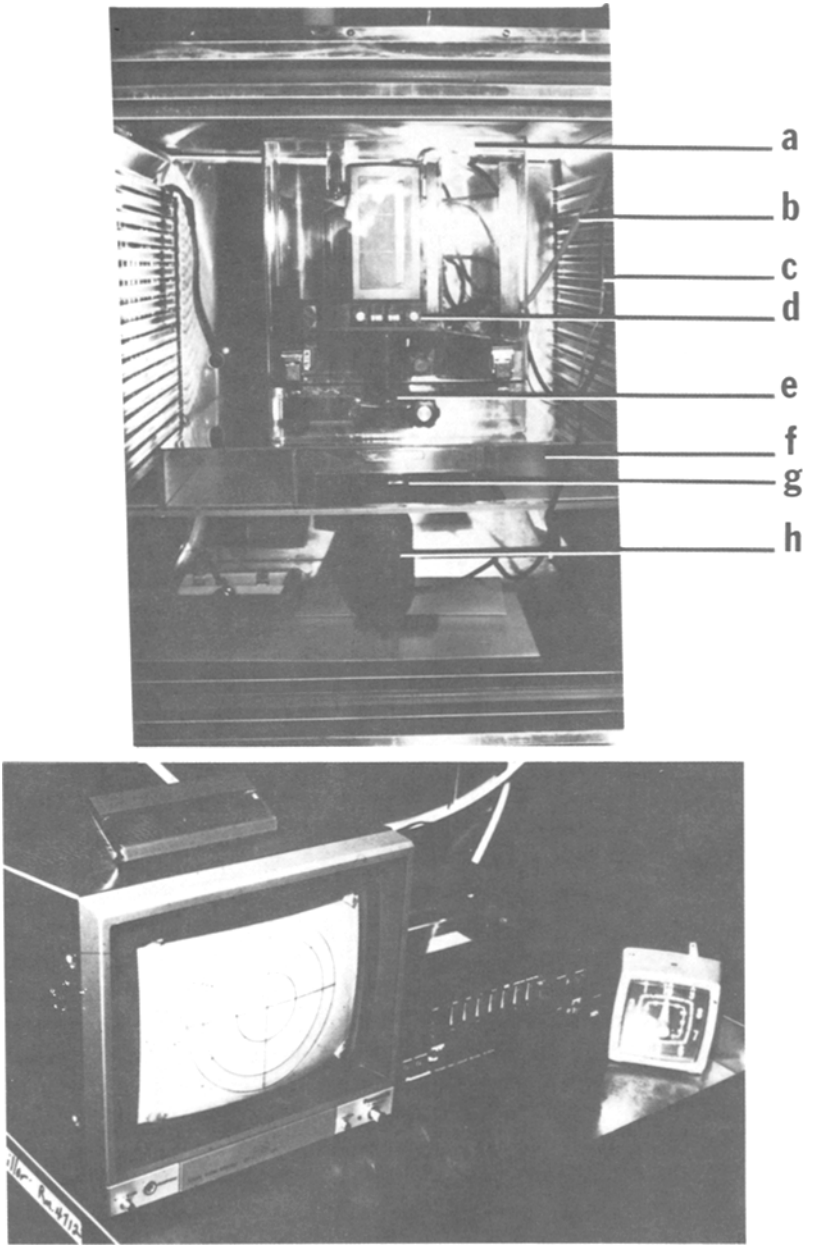

FIG. 1. System employed for time-lapse video tape recording of the responses of schistosomes to chemoattractants during cultivation in vitro. Upper photograph: (a) plastic waterproof camera housing; (b) mixed-gas line; (c) camera remote control cable; (d) camera; (e) lens system; (f) culture chamber; (g) culture vessel; and (h) light source. Lower photograph: monitor, video tape recorder, and timer.

RESULTS

Videotapes made during each of six experiments were selected as being representative for purposes of illustrating some aspects of worm sexual behavior. The first three experiments were designed to record heterosexual behavior. The significant events revealed by these three video sequences were that: (1) The worms of each sex tended to remain aggregated in groups which moved toward each other, but movement of the male group towards that of the opposite sex was consistently more rapid and more vigorous; (2) Intersexual clustering, with some male to female contact, occurred within 1 hr; (3) Mating, defined herein as being in copula, with the female enclosed within the gynecophoric canal of the male, occurred as early as 5 hr postinoculation; (4) Six of the 12 potential pairs (50%) in these three experiments had mated by the end of the first 12 hr in culture. (5) Paired worms, after mating, tended to disperse at random from the original intersexual cluster.

Experiments 4 and 5 were designed to record homosexual behavior. The video sequences made during these experiments revealed little evidence of sexual attraction, either between males or between females. In both male-to-male and female-to-female situations, worm dispersal was gradual and remained random throughout the 24-hr period of cultivation.

Experiment 6 was designed to record an example of male orientation to chemical fractions of female substances (for details of the experimental design, see Childs et al., 1986). The video sequence made during this experiment revealed considerable randomness of worm directional movement during the first 5 hr, but strong attraction to the test compound (*n*-pentane extract of female worms, see Gloer et al., 1986) was exhibited thereafter. At the end of 24 hr, the worm was located on top of the chemically impregnated agar plug where it remained actively motile throughout the next 24 hr of cultivation.

DISCUSSION

In the system employed in these studies for the in vitro cultivation of *S. mansoni*, the opposite sexes are attracted to each other by some means of premating communication which culminates in intersexual aggregation and copulation of sexual pairs within 24 hr. Male worms respond more vigorously to females than do females to males. As documented by time-lapse video tape photography, the migratory patterns and directional preferences exhibited by male worms in response to female extracts indicate the presence of chemical substances in the latter that serve as a mating pheromone(s). Other studies (Imperia et al., 1980; Eveland et al., 1982, Shirazian and Schiller, 1982) also clearly demonstrate that adult *S. mansoni* attract each other in vitro and that there is a chemical basis for this attraction. Eveland et al. (1982) similarly found the greatest attraction to be heterosexual, with males moving toward females in linear chambers provided with perforated barriers. Male homosexual attraction was not seen in their system but homosexual female attraction was said to be

approximately equal to heterosexual attraction involving female movement towards males.

As demonstrated by this presentation, videotechnology, when coupled with an appropriate method of in vitro cultivation, provides a powerful tool, not only for visualizing worm sexual behavior, but also for generating the analytical data necessary for meaningful bioassays.

New approaches to the control of schistosomiasis are needed. A knowledge of the pheromones that regulate sexual attraction and reproduction in schistosomes could lead to the development of effective pharmacologic and/or immunologic means of interfering with them. Preventing worms from mating would preclude egg production, thereby eliminating the main cause of pathology in this disease. Interference with the processes of sexual reproduction also would eliminate the excretion of eggs into the external environment, thereby significantly reducing the transmission potential of the parasite.

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MALE-STIMULATED FEMALE MATURATION IN *Schistosoma:* A Review

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Abstract—In *Schistosoma mansoni* and other schistosome species, pairing of the female with a male partner is necessary for the completion of reproductive morphogenesis and growth. Permanent contact with a male is also necessary for the maintenance of reproductivity in the sexually mature female. This phenomenon appears to be unique within the animal kingdom. The mechanism of male-stimulated female reproductive development in schistosomes remains unknown however. In this paper, the theories for the nature of the process are reviewed briefly, recent findings are added, and the biological and technical problems associated with its study are highlighted.

Key Words—*Schistosoma mansoni*, male-derived cholesterol, vitellogenesis, female maturation.

In schistosomes, male stimulation is not species specific, and in a few cases, neither is it genus specific. Interspecific pairing between *S. mansoni, S. haematobium*, and *S. japonicum* results in female maturation and egg production (Vogel, 1941, 1942), as does intergeneric pairing between *S. mansoni* and *Schistosomatium douthitti* (Short, 1948) and between *S. mansoni* and *Heterobilharzia americana* (Armstrong, 1965).

The stimuli for reproductive morphogenesis and growth appear to be different and can act independently of each other. When the female reproductive system is irreversibly damaged by irradiation (Armstrong, 1965) or drug action (Cioli, personal communication), the worms will still grow to normal adult size if paired with males. Conversely, when females of *S. mansoni* pair with males of *Schistosomatium douthitti*, reproductive development occurs but the worms do not grow to normal adult size (Short, 1948; Armstrong, 1965).

Male stimulation is also required for continuous reproductive function in sexually mature females. When mature pairs of *S. mansoni* are separated,

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regression of the female reproductive system takes place, and the worms become reduced in size (Popiel et al., 1981, 1984; Clough, 1981). If such regressed females are allowed to pair again with males, they can grow back to full size and attain sexual maturity (Clough, 1981). Female *S. mattheei* is exceptional within the genus *Schistosoma* in that reproductive maturation can take place in the absence of males (Taylor et al., 1969; Taylor, 1970).

Explanations for the influence of the male worm on the maturation of the female were reviewed by Michaels (1969); she categorized them into four areas: (1) the presence of sperm or sperm products in the female oviduct; (2) nutritional supplementation; (3) hormonal stimulation; and (4) tactile stimulation. It has been established that sperm is not the stimulus for female maturation (Erasmus, 1973; Shaw, 1977). Investigations within the last ten years have been based on the theory that a chemical factor is involved.

Atkinson and Atkinson (1980) reported that the most abundant female protein of 66 kd is synthesized exclusively by males and is transferred from males to females. They proposed that this is the biochemical basis for continuous copulation in schistosomes, although no evidence for this was provided. More recently, Popiel and Basch (1984a) found no synthetic deficiency in females for a protein of 66 kd. Furthermore, no specific male to female protein transfer was detected.

In a study of glucose uptake in worm pairs, Cornford and Huot (1981) demonstrated a transfer of [^{14}C]glucose from males to females. They suggested that a considerable proportion of the energy requirements of female reproductive activity may be supplied by the male.

The most direct evidence that the male-derived stimulus for female development is of a chemical nature was provided by Shaw et al. (1977). They reported that acetone and ether extracts of male worms stimulated vitellogenesis and growth in unisexual females in vitro and suggested that a male-derived lipophilic factor is responsible. Using identical procedures and the same PR strain of *S. mansoni*, this experiment was repeated in our laboratory at Stanford. A comparison of the results obtained in the two laboratories is given in Table 1. In culture medium plus male extract, vitellogenesis was observed in 56% of the "Cardiff unisexual females" of Shaw et al. Interestingly, 6% of these females, freshly recovered from mice, already exhibited some vitelline cell development, and this percentage increased to 16% and 23% after in vitro maintenance in basic culture medium and NCTC 35, respectively. "Stanford unisexual females" freshly recovered from mice did not show any vitelline cell development, and no maturation took place in either culture medium alone or culture medium plus male extract.

Realizing that the stage of development of the vitelline gland in unisexual females was not as precisely defined as previous studies had suggested, Shaw and Erasmus (1981) performed a systematic study of the reproductive status of females from single sex infections in Tuck strain mice. A similar study has

TABLE 1. PERCENTAGE OF UNISEXUAL FEMALE *S. mansoni* EXHIBITING VITELLINE GLAND DEVELOPMENT FOLLOWING EXPOSURE TO EXTRACTS IN VITRO[a]

Culture medium	Mean number of worms per extract (range)		Total number females exposed		Percentage females exhibiting vitelline gland development (range per experiment)	
	C	S	C	S	C	S
Basic medium[b] + acetone or ether extracts of male worms	150 (18–289)	150 (100–200)	135	105	56 (25–80)	0
Basic medium + chloroform extracts of male worms	ND[c]	150 (100–200)		90		0
Medium 169[d] + acetone or ether extracts of male worms	ND	150 (100–200)		150		0
Basic medium + acetone or ether extracts of female worms	88 (15–274)	150 (100–200)	48	80	17 (0–38)	0
Basic medium + fresh male extract[e]	110 (50–170)	ND	30		6 (0–30)	
Medium 169 + fresh male extract	ND	150 (100–200)		80		0
Basic medium only	0	0	171	80	16 (0–30)	0
NCTC 135 only		ND	26		23 (0–36)	
Medium 169 only	ND			80		0
Noncultured females 70–100 days old[f]			390	521	6 (0–26)	0

[a] A comparison between the results of Shaw et al (1977) using Cardiff females (C) and Popiel and Basch using Stanford females (S).
[b] Basic medium of Shaw et al. (1977).
[c] Not done.
[d] Medium 169 of Basch (1981).
[e] Males homogenized in culture medium.
[f] Unisexual females recovered from mice and assessed immediately for vitelline gland development.

TABLE 2. RELATIONSHIP BETWEEN AGE, SIZE, AND REPRODUCTIVE STATUS OF FEMALE *S. mansoni* FROM SINGLE-SEX INFECTIONS[a]

Age postinfection (days)	Number females examined		Mean worm length (mm)		Percentage of females with vitelline cells (range per mouse)	
	C	S	C	S	C	S
30–39	456	105	3.8	3.3	5.0 (0–20)	0
40–49	522	121	4.1	3.5	7.5 (0–28)	0
50–59	383	101	4.3	3.5	2.6 (2–8)	0
60–69	439	258	3.9	3.3	9.8 (2–12)	0
70–79	1277	389	4.9	3.5	5.9 (2–15)	0
80–89	426	296	5.1	3.4	10.6 (12–17)	0
90–99	151	304	4.9	3.6	13.2 (2–34)	0
100–109	560	211	5.1	3.4	29.8 (4–60)	0
110–119	158	94	4.7	3.2	21.5 (20–25)	0
120–129	286	83	5.5	3.3	15.0 (8–18)	0
130–139	137	61	4.7	3.4	8.0 (3–16)	0
140–149	216	75	5.2	3.5	26.4 (16–20)	0
150–159	1199	87	5.1	3.1	30.7 (8–91)	0
160–169	174	66	5.5	3.0	29.9 (10–20)	0
170–179	104	79	5.7	3.4	9.6 (15–28)	0
180–189	114	85	5.6	3.5	17.5 (15–43)	0
190–200	628	216	5.6	3.3	14.6 (4–72)	0

[a] A comparison of the results of Shaw & Erasmus (1981) using Cardiff females (C) and Popiel and Basch using Stanford females (S).

been performed on Stanford unisexual females in Swiss Webster mice. A comparison of the results is given in Table 2. In the Cardiff females there was an increase in length and in the number of worms exhibiting vitellogenesis with age. In the Stanford females no growth took place after days 30–39 postinfection and no vitellogenesis had taken place in any females as old as 200 days. The clear difference in the extent of maturation attained by these two populations of unisexual females could be the result of a genetic difference or a differential effect of the two strains of mouse host.

From a further comparative experiment, it was found that Stanford unisexual females respond more slowly to stimulation when paired with males in vitro than did Cardiff females. Shaw (1977) demonstrated that in a medium comprising human serum and Earle's balanced salts (50:50), extensive vitellogenesis took place in unisexual females within four days of pairing with males. Stanford females, in the same medium or culture medium 169 of Basch (1981), need to be paired with males for a minimum of six days before even the beginning of vitellogenesis can be detected. It is possible that the reduced respon-

siveness of Stanford females is the basis for their lack of response to male extract.

Despite these conflicting results, the possibility that a male-derived lipophilic compound is responsible for the induction of female maturation cannot be ruled out. Fried et al. (1983) demonstrated that the major neutral lipid component of adult *S. mansoni* excretory–secretory products was cholesterol and that males released considerably more cholesterol in vitro than either females or worm pairs. They implicated cholesterol as a factor influencing growth, development, and behavior of adult worms. We have recently investigated the transfer of [^{3}H]cholesterol in adult *S. mansoni* following labeling of worms in vitro and pairing with unlabeled partners in vitro (Popiel and Basch, 1986). Scintillation counting of thin-layer chromatographic separations of lipid extracts of worm tissues and the culture medium was performed (Figure 1). During the period of time allowed for pairing, labeled worms lost up to 65% of their [^{3}H]cholesterol. In both unlabeled males and females which had paired with labeled partners, levels of [^{3}H]cholesterol were higher than in unpaired controls. We can assume that normal cholesterol transfer in worm pairs is bidirectional, and it is likely that it takes place, at least in part, by a process involving physical contact between adjacent membranes. At this stage we cannot assign a specific function to the process, and it remains to be determined to what extent cholesterol exchange is a by-product of normal tegumental turnover of the molecule.

In Michaels' final category of explanations, she suggested that the male stimulus for female development could be tactile and that a neurosecretory process within the female might convey the message from the tegumentary sense organs to the target organs of the reproductive system. Tactile stimulation was eliminated as a possible mechanism when the results of Shaw et al. (1977) implicated a chemical stimulus. However, even if tactile stimulation per se is not involved, the most efficient pathway for chemical communication would be through direct membrane-to-membrane contact between male and female.

Without knowing the nature of the stimulus, one can only speculate on its localization in the male and on the biochemical and physiological sequellae within the female which result from stimulation and lead to reproductive morphogenesis. The possible regional localization of the stimulatory factor in the male and its target in the female have been investigated (Popiel and Basch, 1984b). Unisexual female and mature male worms were transected into segments and put together in various combinations. Pairing took place between intact worms of each sex and segments of the other, and between segments of both sexes. Vitellogenesis occurred in the majority of female worms and segments so paired. In intact unisexual females paired with small male segments, vitellogenesis was limited to that portion of the worm that had been held by the male. It was concluded that (1) worm pairing, male stimulation, and female developmental response are independent of central nervous control by the ce-

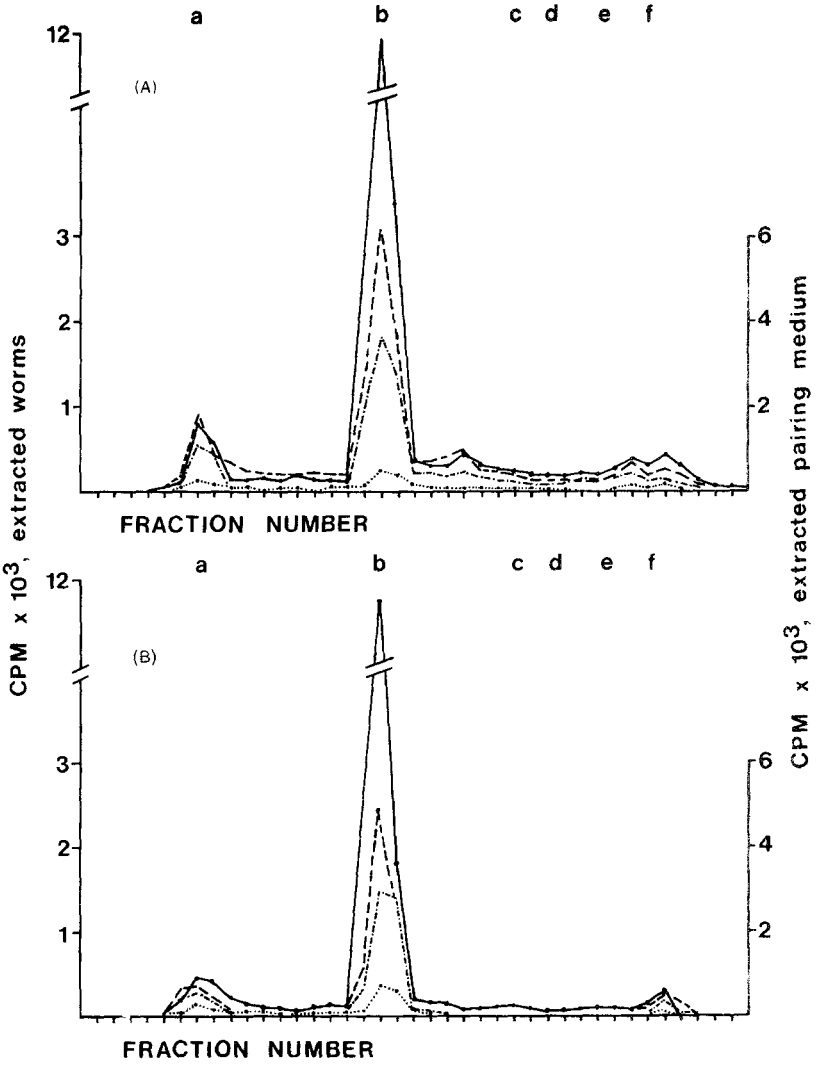

FIG. 1. The distribution of ^{3}H in TLC-separated lipid extracts of [^{3}H]cholesterol-labeled males (•——•) (A) and females (•——•) (B), unlabeled partners (-·--·-), unpaired controls (· · · · · ·), and the pairing medium (---) after a 24-hr period of in vitro pairing. a = origin, b = cholesterol, c = oleic acid, d = triolein, e = methyl oleate, f = cholesterol oleate.

rebral ganglia; (2) males have no centralized location for the female-stimulating factor; (3) vitelline gland differentiation in the female requires local stimulation through male contact, and this is not propagated throughout the worm. Systemic conveyance of the "developmental message" from the tegumental surface of the female to all parts of the reproductive system can, therefore, be ruled out. Neurosecretion, first suggested by Michaels as a mediating process, is still a possibility. However, in higher forms neurosecretory cells are typically morphologically discrete and often operate independently of the nervous system, whereas in schistosomes neurosecretory material has been reported only within nerve cells (Silk and Spence, 1969).

The above review covers results of investigations which have most directly addressed the process of male-stimulated female reproductive morphogenesis in schistosomes. It is useful at this point to highlight a number of factors which, to varying extents, compound progress towards a better understanding of this developmental process.

1. Worm pairing and subsequent female development in vivo is not synchronous (Table 3). Females recovered from mice on a given day will comprise a variable population of worms at different developmental stages. This is especially so on days 30–38 postinfection when reproductive morphogenesis is taking place. Variations in biochemical data obtained from samples of such populations are inevitable. If unisexual females and mature males are transplanted together to recipient hosts, pairing and subsequent female maturation are more synchronized (Table 4). This is a time-consuming and involved method for obtaining females at specific stages of reproductive development.

2. Viable schistosome eggs have yet to be produced by females reared in vitro. The in vitro culture conditions at our disposal are far from satisfactory for the study of reproductive development, a process so dependent on optimal conditions for normal function.

3. The first visible indication that sexually immature females are responding to male stimulation is the appearance of differentiating vitelline cells in the vitelline gland region. Fast red B staining of these cells is the presently used assay for the induction of female maturation. However, it takes three to four days after pairing in vivo and six days in vitro for vitelline cells to appear. Additional knowledge of the biochemical changes which take place in females immediately after pairing would provide a more rapid assay for the stimulation of female maturation and lead to a better understanding of the developmental process.

4. The first step in establishing that the male stimulus for female maturation is chemical in nature is the unequivocal demonstration of the stimulatory effect of whole-male extract or a fraction thereof. The conflicting results in this area are disappointing and require further investigation. The presence of the

TABLE 3. PERCENTAGE INCIDENCE OF FEMALES AT STAGES 0–5 OF VITELLINE GLAND DEVELOPMENT ON SUCCESSIVE DAYS OF BISEXUAL MICE INFECTIONS AND DISTRIBUTION BETWEEN MESENTERIC AND HEPATIC PORTAL VEINS[a]

	Percentage incidence of females at stage 0–5 of vitelline gland development											
	Mesenteric veins						Hepatic portal vein					
Days postinfection	0	1	2	3	4	5	0	1	2	3	4	5
25							100					
26	2						98					
27	2						98					
28	3						97					
29	3	1					92	2	2			
30	5	5	3	1			84	2				
31	11	9	5	5	1		51	5	7		6	
32	10	8	4	4	5		50	8	6	5		
33	14	17	4	4	5	6	33	4	5	2	3	3
34		4	4	4	19	9	28	6	8	8	7	3
35	2	4	6	6	14	6	13	10	14	10	9	6
36	1	4	13	12	14	12	4	6	12	7	8	2
37		3	4	14	14	16	13	9	5	8	4	10
38			1	4	22	19		4	2	18	12	18
39			2	4	9	21		2	3	10	19	30
40				1	9	27		1	1	5	17	39
42				2	11	29			1	2	10	45
44				3	9	34			2	1	4	47
46				1	8	40		1		1	5	44
48					2	48			1	2	1	46
50						43					1	56

[a]Mice were killed by cervical dislocation, worm pairs were recovered by dissection, separated, and the females stained with fast red B.

insect hormones, ecdysone and 20-hydroxyecdysone (Nirde et al, 1983), lends weight to the idea that hormones are involved in schistosome maturation.

As schistosomes evolved form hermaphroditic ancestors, the phenomenon of male-stimulated female maturation is probably a relic of this ancestry. It was suggested (Popiel et al., 1984; Popiel, 1986) that schistosomes may have evolved from a protandrous ancestor in which maturation of the female reproductive system was dependent on complete development of the male system. Although immature males are unable to stimulate female maturation (Standen, 1953), it has been shown that both mature males lacking testes (Armstrong, 1965) and transected somatic tissue segments of mature males (Popiel and Basch, 1984b) are able to do so. Female development is thus dependent on

TABLE 4. EXTENT OF VITELLINE GLAND DEVELOPMENT IN UNISEXUAL FEMALE *S. mansoni* FOLLOWING TRANSPLANTATION TO NILE RATS AND PAIRING WITH MATURE MALES

Days postinfection	Number females examined	Percentage females at stages 0–5 of vitelline development[a]					
		0	1	2	3	4	5
0	15	100					
2	10	80	20				
3	16	6	84	10			
4	15	30	38	8	8	16	
5	18	7	19	50	16	4	4
7	16		16	25	8	16	35
11	18						100
16	15						100
21	4						100
28	4						100
35	4						100

[a] 70-day-old unisexual females were transferred to Nile rats together with mature males. Worm pairs were recovered at various times posttransfer and the extent of vitelline gland development was assessed on a scale of 0–5 using fast red B-stained whole mounts.

stimulation from the somatic tissue of mature males rather than the reproductive system. In most invertebrate species which depend for existence on a very high reproductive output, females are larger than males. Why are schistosomes atypical in that males are larger than females? In the male the testes are anteriorly situated, and the rest of the body, comprising approximately 80% of the worm, is devoid of additional organ systems. It is interesting to speculate that the size of the male schistosome is, for the most part, a reflection of its role in the stimulation and maintenance of female reproductivity and in the conveyance of the female around the portal system.

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STRUCTURAL AND METABOLIC CHANGES IN FEMALE *Schistosoma mansoni* FOLLOWING MALE STIMULATION

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Abstract—Pairing of males and females from single-sex infections results in the multiplication and differentiation of undifferentiated cells of the vitelline lobule culminating in the production of mature vitelline cells involved in egg shell formation. These changes are accompanied by increases in the rate of uptake of tyrosine, thymidine, and an increased accumulation of calcium.

Key Words—*Schistosoma mansoni*, vitelline gland, differentiation, tyrosine, thymidine, calcium, rates of uptake, male stimulation.

INTRODUCTION

It is well established that the sexual maturation of the female schistosome is dependent upon pairing with male worms. The precise nature of this developmental stimulus is still under debate. However, the gross physical changes in the female consequent to male stimulation are easily recognized and consist of an increase in length, maturation of the ovary, and development and maturation of the vitelline gland. As the protection of the fertilized ovum and the results of its differentiation are dependent on an egg shell produced from the synthetic products of the vitelline cell, this paper will largely be concerned with various aspects of vitelline cell development.

METHODS AND MATERIALS

All the experiments conducted in the author's laboratory were based on a Puerto Rican strain of *Schistosoma mansoni* maintained in Tuck Swiss albino

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mice and *Biomphalaria glabrata*. Details of all procedures, as well as those of other authors, are described in the relevant publications.

OBSERVATIONS AND DISCUSSION

Vitelline Gland of Mature Female. In mixed infections (male and female worms present) sexual dimorphism is not clearly apparent until approximately 25 days postinfection (DPI). The ovary, oviduct, uterus, and ootype are the first structures to appear, followed by the vitelline gland. This begins to differentiate at about 29–31 DPI, and there exists considerable variation in the degree of development until about 40 DPI when presumably most worms have paired and the developmental sequence has stabilized (J.E. Roberts, personal communication).

The vitelline gland consists of a large number of lobules extending on either side of the cecum from the posterior end of the ovary to the terminal portion of the body. The developmental sequence occurring within each lobule was first described by Erasmus (1975b) and later semiquantified by Erasmus and Popiel (1980). (Figures 1 and 2). The continuous developmental sequence was categorized into four stages: S1, representing the undifferentiated cell, proceeding through S2 and S3 to S4, the fully differentiated cell which ultimately is released into the vitelline duct. Mechanisms controlling vitelline cell turnover are unknown, although Nollen et al. (1976) suggested a turnover time of six days. On the basis that a mature female schistosome deposits 300 eggs per day and each egg capsule contains an ovum plus 38 vitelline cells (Gönnert, 1955), then the vitelline gland must produce approximately 11,000 cells in 24 hr. Furthermore, as recent data (Harris *et al.*, 1984) suggests that *S.mansoni* may still be egg-laying after 32 years postinfection, then the productivity of the gland must be enormous. Further analysis of the composition of the lobules did not reveal any differences between the composition of anterior and posterior lobules nor between lobules from worms recovered at 50 and 100 DPI.

A series of experiments to determine the effects of drugs on the vitelline lobule (Erasmus, 1975b; Erasmus and Popiel 1980; Popiel and Erasmus, 1981, 1982, 1984; Popiel et al., 1984) revealed a differing sensitivity between the developmental stages in the lobule to drugs. In the case of Astiban (Erasmus, 1975b; Erasmus and Popiel, 1980) (Figure 3) and Oxamniquine, the undifferentiated cells survived treatment and eventually began to differentiate and repopulate the lobule (Erasmus, 1975b; Erasmus and Popiel 1980; Popiel et al., 1984). The success of in vitro culture methods may be partly dependent on the ability of the S1 vitelline cell to survive and differentiate. It is also likely that it is this cell which, as far as the vitelline gland is concerned, makes the initial response to male stimulation by dividing and differentiating. The complete differentiation, coordination, and functioning of the female reproductive system may, of course, depend on a sequence of other stimuli.

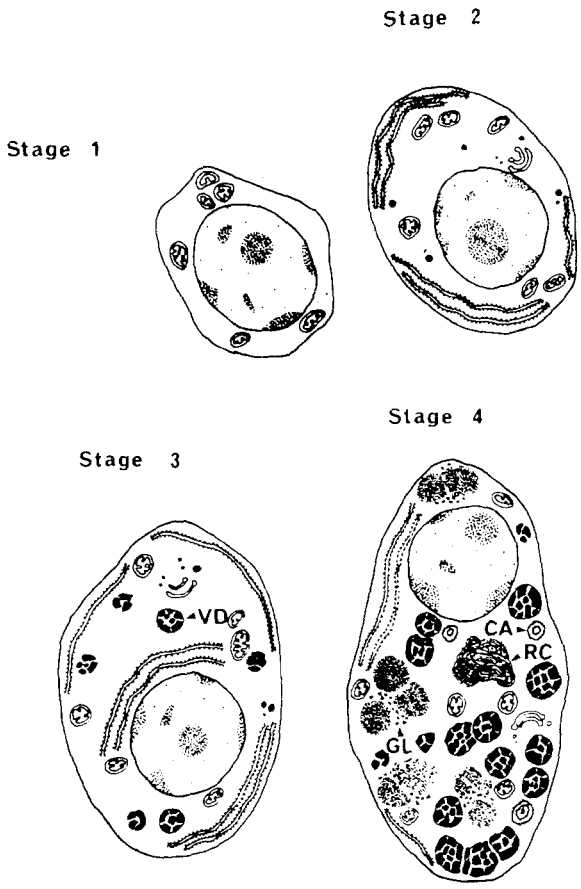

FIG. 1. The four main stages in the differentiation of the vitelline cell in the vitelline lobule of *S. mansoni*. Stage 1 (S1) is the undifferentiated cell; stage 2 (S2) shows a change in nuclear/cytoplasmic ratio together with the appearance of Golgi complexes and granular endoplasmic reticulum; stage 3 (S3) at which the vitelline droplets (VD) begin to appear; and stage 4 (S4), the mature vitelline cell containing vitelline droplets, calcareous concretions (CA), ribosomal complexes (RC), and glycogen (GL) associated with lipid/droplets.

There is some evidence which suggests that the lobules of the gland may develop and function independently. Treatment with Niridazole (Popiel and Erasmus, 1981) results in initial damage occurring at the posterior end of the gland. Exposure in vivo to Oxamniquine results in a patchy disappearance of the lobules (Popiel and Erasmus, 1982). During sexual maturation, the vitelline gland starts to appear as isolated lobules nearest the ovary. The comparison of vitelline lobule composition from anterior and posterior ends of the worm also suggests there is no linear differentiation of development. The most elegant

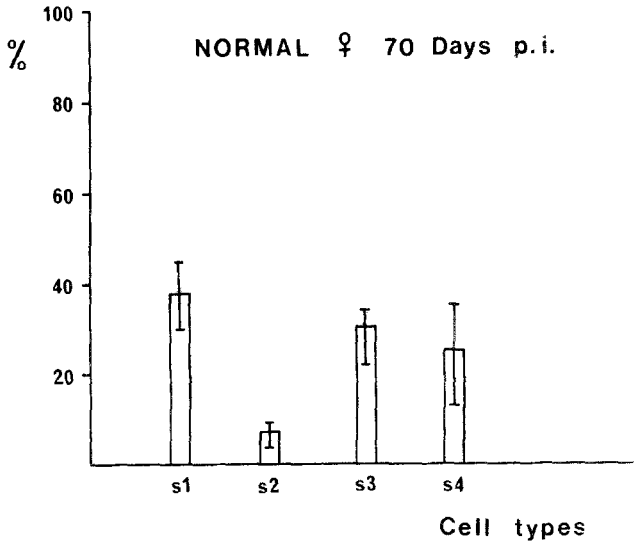

FIG. 2. The relative proportions of the four developmental stages of the vitelline cell in lobules from a normal, 70-day-old worm. Taken from Erasmus and Popiel (1980).

demonstration of isolated lobule development was made by Popiel and Basch (1984) who showed, using unisexual females (with no previous exposure to males) and male worm segments, that differentiation of the vitelline lobules only occurred in those regions enveloped by male segments. This limited evidence suggests that stimulation and activation of S1 cell differentiation and development might occur quite locally.

Unisexual Female. Because of the heterogamety exhibited by schistosomes (Short, 1983), it is possible, using cercariae derived from single miracidial infections, to obtain experimentally a schistosome population consisting of a single sex. Unisexual females are those derived by such techniques and have had no previous exposure to males. The classical picture of such females at 50 DPI and later is a stunted worm (Moore et al., 1954, provide comparative figures) which is sexually immature. An ultrastructural study by Erasmus (1973) revealed that in such worms oviduct, ootype, and uterus were well developed; although the ovary was present, its cells did not produce cortical granules, and the vitelline gland was represented by patches of undifferentiated cells corresponding to the S1 cells of later studies (Erasmus, 1975b).

One of the routine tests used to demonstrate the presence of fully differentiated vitelline cells is the fast red B technique (Johri and Smyth, 1956) which reveals the presence of phenolic substances within the cell. Using this technique Shaw et al. (1977) showed that a small percentage (6%) of unisexual females immediately upon removal from the host showed positively stained cells in the

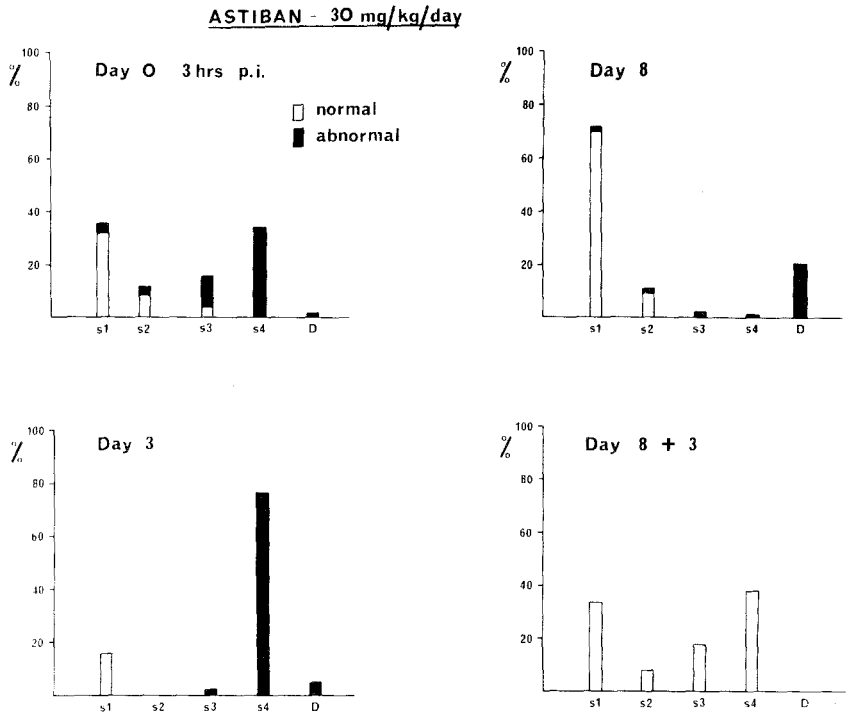

FIG. 3. The effect of Astiban on the cell population of the vitelline lobules of *S. mansoni*. Day 0 = 3 hr after single injection; D 3 = 3 daily injections; Day 8 = after 8 daily injections. Note that 70% of the lobule population consists of S1 cells. Day 8 + 3 = eight daily injections, followed by withdrawal of drug for 3 days and then sampling. D = "dead cells." Taken from Erasmus and Popiel (1980).

region of the vitelline gland. Furthermore, after four days in vitro culture this proportion increased to >16%. In a later study (Shaw and Erasmus, 1981), it was shown that the proportion of a population of unisexual females exhibiting positive fast red B reactions increased with time postinfection. However, the degree of development of the vitelline gland did not increase between worms recovered 30 DPI and those at 200 DPI. At the ultrastructural level the vitelline lobules corresponded to previous descriptions: Mehlis' gland was present in some worms and not in others, but the cells of the ovary remained in a state characteristic of the initial description (Erasmus, 1973). Worm length also increased with time postinfection.

These observations suggest that, in the host/parasite model used, some development of unisexual females occurred in the absence of males. Consequently it may be argued that male stimulation simply accelerates the process of female maturation rather than inducing a de novo response.

Male Worm. It is not anomalous to include the male worm in this consideration of female development as (reviewed by Shaw and Erasmus, 1982), the occurrence of pseudohermaphroditism in the male has been reported by several workers. Using the fast red B technique and ultrastructural studies, it was shown that mature S4 cells, similar in most respects to those in the vitelline lobule from the mature female, occurred in male worms recovered 30–100 DPI. These mature cells were scattered, not incorporated into lobules, nor were prior stages of development observed. A characteristic feature was that they were closely associated with the cecum.

In a study of [^{3}H]thymidine uptake by male and female worms, Den Hollander and Erasmus (1984) showed by autoradiography that undifferentiated cells closely associated with the gut in the male possessed labeled nuclei. It is possible that these cells represented the precursors of the mature cells revealed by histological staining.

These results may indicate that the heterogametic dimorphism associated with the genus may not be absolute but may be influenced by particular host–parasite strain relationships. These anomalies may also influence our consideration of those factors stimulating vitelline cell maturation in the female.

Biochemical Aspects of Vitelline Cell Development. In light of what has been said previously regarding numbers of eggs produced, it is obvious that the vitelline lobule is a highly productive unit. The egg shell or capsule is regarded as consisting of a quinone-tanned protein, and Clegg and Smyth (1968) have identified the presence of proteins rich in $-NH_2$ groups, phenolase, and phenolic materials in the vitelline droplets. Stimulation of the unisexual female by male worms, therefore, not only results in multiplication and differentiation of the S1 cells but also, at the S4 stage, the production of the various precursors of the egg shell components. Both these changes, morphological and synthetic, must involve considerable metabolic changes in the female following stimulation.

As mentioned earlier, one of the standard techniques used to demonstrate vitelline cell differentiation is the fast red B test for the presence of phenolic substances (Johri and Smyth, 1956). The disadvantage of this technique is that it only identifies the final stages of differentiation, i.e., the mature S4 cell. In order to determine the initial events of female development following stimulation by the male, more sensitive methods are required. Erasmus (1975a) demonstrated the uptake of labeled L-tyrosine by mature females of *S. mansoni* and its localization in the mature vitelline cells. Later, Popiel and Erasmus (1981) showed that after unisexual females had paired with males in vitro for five days, tyrosine uptake had risen by 7% on day 5 and by 32% on day 6 over a baseline shown by unpaired unisexual females. These changes must represent the changes in protein synthesis by S4 cells.

Although undetected in an initial study (Erasmus, 1973), further work

revealed the presence of calcareous concretions approximately 0.3 μm in diameter in the cytoplasm of the mature S4 cells of *S. mansoni*. These concretions originated in dilations of the GER, and X-ray probe analysis revealed the presence of calcium, phosphorus, and magnesium (Erasmus and Davies, 1979). In a study of the changes in elemental composition of male, unisexual female, and paired female *S.mansoni* in relation to age and sexual status, Shaw and Erasmus (1983) showed that in paired female worms the calcium content increased significantly over that of males and unisexual females after 30 DPI. This change must reflect the gradual appearance of S4 cells in the vitelline lobules. The possible function of these calcium stores was extensively discussed by Shaw and Erasmus (1983), but no specific conclusion was reached. In a comparative study of vitelline cell structure in *S. mansoni*, *S. japonicum*, *S.haematobium*, and *S.mattheei* (Erasmus et al., 1982), calcareous deposits were found in the vitelline cells of all species with the exception of *S. japonicum*. This anomaly is difficult to explain but may be related to the higher rate of egg production (Moore and Sandground, 1956) and the slightly different chemical composition of the egg capsule (Byram and Senft, 1979).

As has been mentioned, male stimulation results in division and differentiation of the S1 cells of the vitelline gland. This process must involve large-scale DNA synthesis, and Den Hollander and Erasmus (1984) determined the pattern of DNA synthesis in male and female *S. mansoni* from mixed and single-sex infections in mice. [^{3}H]Thymidine was used as a specific marker for DNA synthesis and biochemical tests showed that up to 35 DPI males and females from mixed infections had comparable activity. After this period the activity in males decreased, whereas that of females persisted at a high level, presumably reflecting the continued cell division in the lobules of the vitelline gland.

In females from single-sex infections, the activity was significantly lower than that of single-sex males and females from mixed infection. Thus stimulation of unisexual females by males results in a significant increase in DNA synthesis. Autoradiography at both light and electron microscope levels revealed that in mature females the label localized in the S1 cells of the vitelline lobule and also in small, undifferentiated cells in the parenchyma. In unisexual females after 45 DPI very few labeled cells were observed other than a few cells in the ovary. In mature cells, cells of the testes were labeled as were scattered undifferentiated cells. The general pattern was that in both males and females there was an overall labeling, but as the reproductive systems matured the labeling became concentrated in these organs. The ability of males to stimulate a change in the rate of DNA synthesis of females was also studied by Den Hollander and Erasmus (1986). Under in vitro conditions most unisexual females had paired by 18 hr. By 24 hr after culture, the uptake of labeled thymidine by paired females was significantly different from that of unpaired fe-

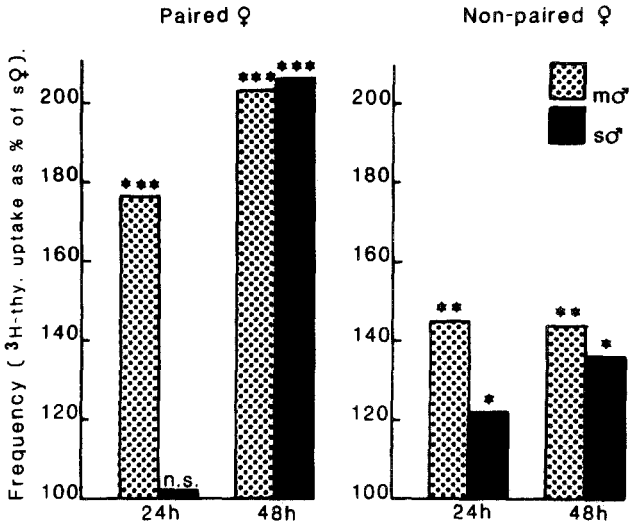

FIG. 4. Mean [^{3}H]thymidine uptake (dpm) by individual unisexual female *S. mansoni* after 24 or 48 hr stimulation with males from either mixed (mσ) or unisexual (sσ) infections expressed as a percentage of the uptake by unstimulated (no males) unisexual females. *** significant, $P < 0.001$; ** significant, $P < 0.01$; * significant, $P < 0.1$; n.s., not significant. Taken from Den Hollander and Erasmus (1986).

males. By 48 hr, the uptake was approximately double that of unpaired females. These experiments therefore indicate a very rapid change in rates of DNA synthesis by the female following male stimulation (Figure 4).

Stimulating Male. It is generally assumed that once males have reached maturity, they possess the ability to stimulate females to develop. However, Den Hollander and Erasmus (1985) showed that females paired with males from mixed infections had a significantly higher rate of uptake of labeled thymidine than females paired with males from unisexual infections (Figure 4). After 48 hr in vitro this difference had disappeared. These observations suggest that some form of maturation of the male is required before efficient stimulation of the female can occur. Popiel and Erasmus (1981) also observed that male extract resulted in a much greater increase in tyrosine uptake by females compared to females paired with males for four days (Figure 5).

Thus the pairing of unisexual females with males results in considerable morphogenesis, changes in the rates of uptake of tyrosine and thymidine, and in the accumulation of calcium. These changes occur rapidly and probably in periods considerably less than the 24 hr demonstrated for thymidine. It is possible that male stimulation produces changes in the physicochemical characteristics of the female tegument which affects the uptake of substances essential to growth and the maturation of the female reproductive system. It seems pos-

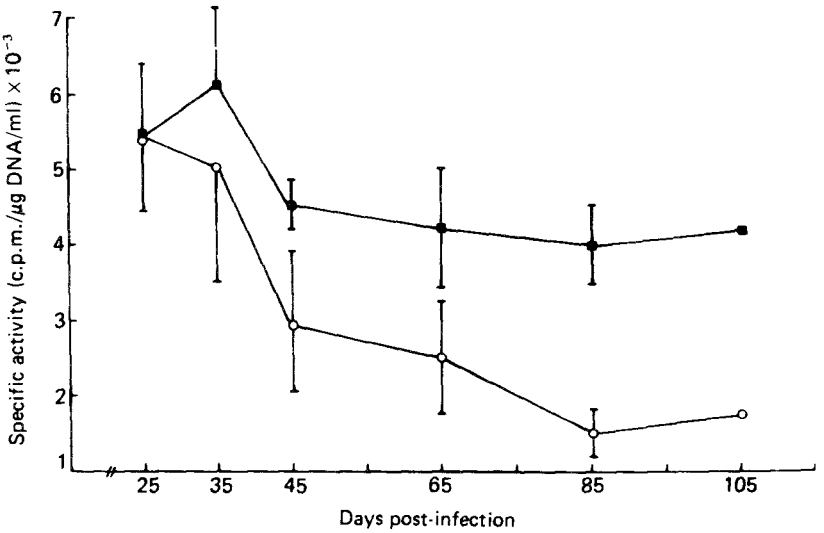

FIG. 5. DNA synthesis in male (○) and female (■) *S. mansoni* from mixed infections. The worms were labeled in vitro with 20 μCi/ml methyl [^{3}H]thymidine, the DNA was extracted, measured, and presented as a specific activity (cpm/μg DNA/ml). Each point is the mean of three experiments with the standard deviation shown. Taken from Den Hollander and Erasmus (1984).

sible that this relationship between stage 1 cell differentiation and changes in tegumental characteristics may provide a suitable target for intervention by drugs.

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ELEMENTAL CHANGES DURING SEXUAL MATURATION IN *Schistosoma mansoni*

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Abstract—Changes in the elemental composition of *Schistosoma mansoni* in relation to age and sexual status are described, and studies on the structural localization of calcium in adult worms and the elemental composition of the tegument-subtegumental tissues are reviewed. Some preliminary results on the distribution of Ca^{2+}-dependent ATPase activity in the tegument of worms from both mixed and single-sex infections are also presented.

Key Words—*Schistosoma mansoni*, inorganic metabolism, elemental composition, sexual status, calcium localization, X-ray probe microanalysis, cryosections, tegument, subtegumental tissues, Ca^{2+}-dependent ATPase activity.

INTRODUCTION

Despite significant advances in our understanding of the biology of *Schistosoma mansoni*, information about inorganic metabolism and ionic regulation in both *S. mansoni* and digeneans in general is very limited (see Brand, 1966, 1979; Barrett, 1981; Coles, 1973, 1975, 1984). Until very recently, apart from reports on the influence of salinity on the survival of schistosome cercariae (e.g., Donnelly et al., 1984), our knowledge of the inorganic metabolism of schistosomes has been largely confined to reports of the presence of calcium in the cercarial penetration glands (Dresden and Edlin, 1975; Dorsey and Stirewalt, 1977), and the presence of calcium in the mature vitelline cells of *S. mansoni, S. haematobium*, and *S. mattheei* (Erasmus and Davies, 1979; Erasmus et al., 1982; Shaw and Erasmus, 1982).

For a number of years we have been interested in the biology and metabolism of the male–female relationship of *S. mansoni*. Our aim has been to define

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more precisely the changes occurring during sexual development and the factors necessary for the maintenance of sexual maturity, particularly in female worms (see review by Erasmus, 1986). Following the initial work of Erasmus and Davies (1979) we have begun to look at various aspects of the inorganic metabolism of *S. mansoni*, particularly in relation to age and sexual status of the worms. The present article reviews our previous work and briefly describes the results from a number of current investigations.

OBSERVATIONS AND DISCUSSION

In our initial study (Shaw and Erasmus, 1983) changes in the elemental contents (Ca, P, K, Mg, and S) of whole male and female *S. mansoni* from both mixed and single-sex infections were measured at various times postinfection, using a quantitative method of X-ray analysis (Davies and Morgan, 1976; Morgan, 1983). From this study the most obvious difference between mixed infection male and female worms and worms from single-sex infections was the significantly higher levels of calcium in females from mixed infections, particularly after 35 days postinfection (DPI) (i.e., after sexual maturation) (Figure 1). Slightly higher levels of phosphorus were also found in female worms from mixed infections as compared to male worms or females from single-sex infections. The levels of the other elements measured (Mg, S, and K) were, in general, similar in male and female worms from both types of infection.

In *S. mansoni*, the main anatomical difference between male and female worms from mixed infections, and between female worms from mixed and single-sex infections, is the presence in sexually mature females of an extensive vitelline gland system (Erasmus, 1973). The mature, stage 4, vitelline cells of *S. mansoni* contain discrete Ca- and P-containing granules (Erasmus and Davies, 1979), and the presence of these granules presumably accounts for the significantly higher levels of calcium found in the sexually mature females. Also, in female worms from mixed infections there is a sharp rise in calcium (Figure 1) and, to a lesser extent, phosphorus levels, between 30 and 35 DPI. This coincides with the rapid development of the vitelline gland and the beginning of egg production. In contrast, females from single-sex infections do not show any dramatic increase in calcium content during the same period. Over the long term (up to 250 DPI) there is a very slight, although variable, increase in calcium content with increased age. This may reflect the low level of differentiation, maturation, and turnover of vitelline cells reported in females from single-sex infections (Shaw and Erasmus, 1981).

From this initial study, it was obvious that there are significant differences in the calcium metabolism of *S. mansoni* particularly in relation to the degree of sexual development of the female worms. This study, however, only provided data on the total calcium content of individual worms and, apart from the

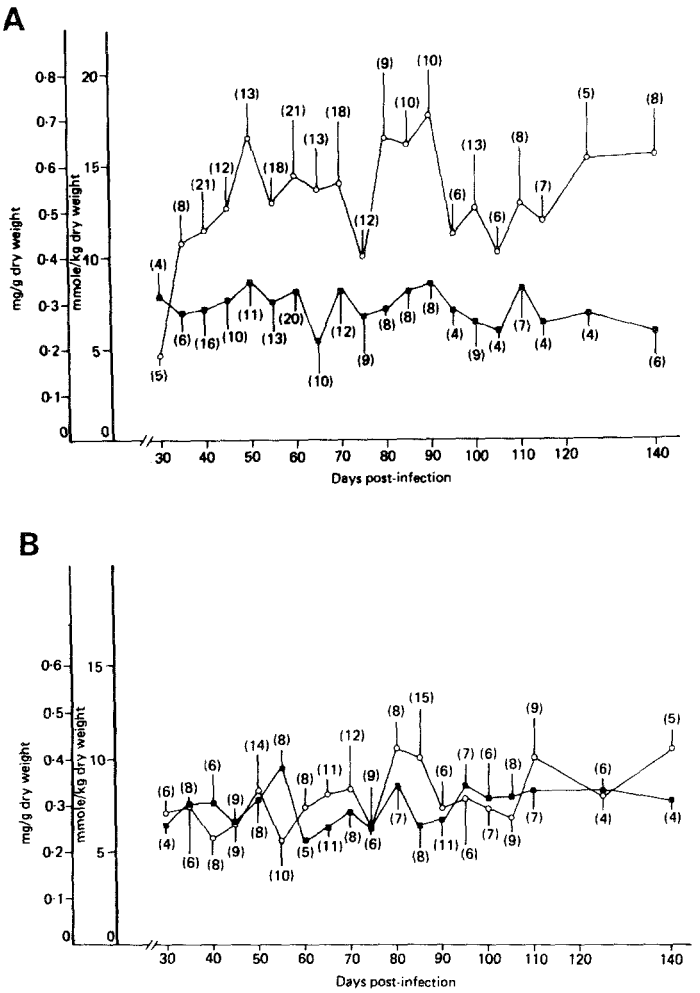

FIG. 1. Changes in the calcium content of male (●) and female (○) *Schistosoma mansoni* from (A) mixed-sex infections and (B) single-sex infections. All values are the mean ± SD. Figures in parentheses are the number of samples analyzed. (From Shaw and Erasmus, 1983.)

study of Erasmus and Davies (1979), little information was available regarding the structural localization of calcium within adult worms.

Initial attempts at localizing calcium within adult worms using either the pyroantimonate or oxalate precipitation methods were not very successful (unpublished observations). These techniques, which involve precipitating reactions and the subsequent analysis of the precipitates have many serious disad-

vantages (Chandler, 1978; Morgan et al., 1978; Morgan, 1980), and the use of freeze-dried cryosections with electron probe X-ray microanalysis has provided a more reliable and accurate method for examining the in vivo distribution of elements within adult *S. mansoni*.

In general, unstained freeze-dried cryosections of the tegument and subtegumental tissues of adult *S. mansoni* have sufficient mass contrast to enable ultrastructural details to be readily correlated with structures recognizable in conventionally fixed and embedded material (see for example Shaw and Erasmus, 1984). We have employed this technique to determine the distribution of calcium within adult *S. mansoni*, and the elemental composition of the tegument and subtegumental tissues of adult worms.

Sites of Calcium Localization in Adult Worms

Males. A conspicuous feature of freeze-dried cryosections of male worms is the presence within the parenchymal tissues of groups of electron-dense granules. These granules, which measure 50–130 nm in diameter, consist of an inner dense core separated from the enclosing membrane by a thin layer of less dense material (Figure 2). X-ray analysis of these granules has shown that they contain, in comparison with the surrounding cytoplasm, significantly larger amounts of calcium and sodium (Figure 2) (Shaw et al., 1982).

These granules have been correlated with the electron-dense, membrane-bounded granules found throughout the peripheral nervous system in sections of glutaraldehyde-fixed material.

Some of the dense granules in *S. mansoni* have been shown to contain the neurotransmitter 5-hydroxytryptamine (5HT) (Dei-Cas et al., 1979), and *S. mansoni* is known, in toto, to contain relatively high levels of 5HT (Bennett et al., 1969). Therefore, a significant proportion of the total calcium in adult male worms may be localized within parts of the nervous system. In comparison with the neuronal granules, the general cytoplasm also contains calcium, albeit at a very much lower level (between 10 and 100 times less), although these low levels of calcium may in total contribute significantly to the total calcium content. What proportion of the total calcium in adult male worms is "parenchymal" as opposed to neuronal is not known. The only other major site(s) of calcium localization in male worms is within the small number of mature vitelline cells present in some worms (Shaw and Erasmus, 1982).

Females. The mature, stage 4 vitelline cells in both *S. mansoni* and *S. haematobium* contain conspicuous inclusions which have been shown to contain calcium, phosphorus, and magnesium (Erasmus and Davies, 1979). Comparison of the calcium contents of female *S. mansoni* from mixed and single-sex infections would suggest that between 50 and 75% of the total calcium in sexually mature, egg-laying female worms is located within the vitelline gland and more specifically within the mature, stage 4 vitelline cells.

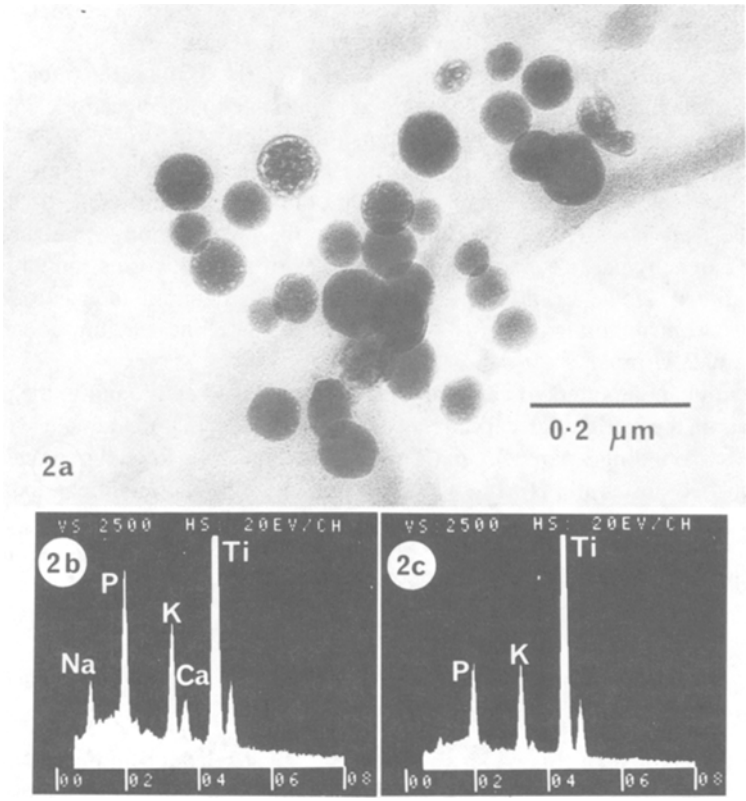

FIG. 2. (a) High-power electron micrograph of the dense granules from the peripheral nervous system of adult male *Schistosoma mansoni*. Freeze-dried cryosection. (b) X-ray spectrum of a group of neuronal granules from a cryosection of *S. mansoni*. Note sodium and calcium peaks. (c) X-ray spectrum of the cytoplasm adjacent to neuronal granules analyzed in b. Note absence of sodium and calcium peaks. (From Shaw et al., 1982).

If it is assumed that each female *S. mansoni* produces between 200–300 eggs per day and that each egg contains, on average, 38 vitelline cells (Gönnert, 1955), then the productivity of the vitelline gland as a whole must be large, producing and releasing up to 11,000 cells/24 hr. Consequently, the total calcium turnover of sexually mature female *S. mansoni* must be relatively large. The physiological significance of relatively large amounts of calcium within the vitelline cells of sexually mature female *S. mansoni* is not known (for a discussion of the possible roles of this calcium, see Erasmus and Davies, 1979; Shaw and Erasmus, 1983), although the presence of calcium-containing corpuscles within the mature vitelline cells present in male worms (Shaw and Erasmus,

1982) would suggest that calcium sequestration was, in itself, a normal part of the developmental biology of the vitelline cells of *S. mansoni*.

Sexually mature female *S. mansoni* feed at nearly 10 times the rate of male worms (Lawrence, 1973), and it was thought possible that the gastrodermis and gut lumen contain relatively large amounts of calcium. Analysis of freeze-dried cryosections of the posterior regions of the gut have shown that, although calcium is present in both the lumen and within the gastrodermis itself, the levels observed were not significantly different from the levels found in the subtegumental/parenchymal (i.e., non-vitelline) tissues of female worms (unpublished observations). However, it is again possible that the calcium in the gastrodermis may also contribute significantly to the total non-vitelline calcium content of adult female worms.

Significant amounts of calcium (and sodium) have been found in the dense granules in the peripheral nervous system of sexually mature female worms, and the surrounding "parenchymal" cytoplasm contains a low level of calcium (unpublished observations). In contrast to male worms, the tegument and, to a lesser extent, the subtegumental tissues of adult female *S. mansoni* contain relatively higher levels of calcium and, in relatively small areas of the tegument, isolated calcium-containing granules have been found (Shaw and Erasmus, 1984; see below).

Elemental Composition of Tegument and Subtegumental Tissues of Adult S. mansoni

In the context of this paper, probably the most relevant finding from this study was that there are statistically significant differences in the elemental composition of the tegument and subtegumental tissues between adult male and female worms, particularly in relation to the levels of calcium (see Shaw and Erasmus, 1984). In general, both the tegument and subtegumental tissues of female worms contain significantly higher elemental levels than the equivalent tissues in male worms. In particular, the female tegumental cytoplasm was found to contain significantly higher levels of both calcium and sodium and, in a number of relatively small areas, discrete calcium-rich granules.

Sexually mature female *S. mansoni* have been shown to contain significantly higher levels of calcium than either male worms or females from single-sex infections (Figure 1) (Shaw and Erasmus, 1983). This higher calcium content is due primarily to discrete calcium deposits within the mature vitelline cells (Erasmus and Davies, 1979). As sexually mature females may produce up to 300 eggs/female/day with each egg containing, on average, 38 vitelline cells (Gönnert, 1955), the demand and turnover of calcium within female worms must be considerable. As yet the physiological significance of this vitelline cell calcium is not known. However, the results of this study, taken in conjunction with the fact that the gut lumen and gastrodermis contain relatively smaller

amounts of calcium (unpublished observations), would suggest that this calcium demand is met, in part at least, by calcium uptake across the tegument.

The schistosome tegument has long been recognized as an important site for nutrient uptake (Pappas and Read, 1975), and there are a number of sex-specific differences in the uptake of certain amino acids (Cornford and Oldendorf, 1979) and glucose (Cornford and Huot, 1981). For a number of amino acids and hexoses in particular, this transtegumental uptake appears to be sodium-dependent (Barrett, 1981; Podesta and Dean, 1982). The presence of significantly higher levels of sodium in the female tegument would therefore be consistent with higher sodium-dependent nutrient uptake in sexually mature female worms.

Tegumental ATPase

The tegument plays a very important role in schistosome biology, and recently there has been a considerable amount of interest in the biochemistry of the tegument (see review by Coles, 1984). In particular, a Ca^{2+}-stimulated ATPase has been found associated with the tegumental membranes in adult *S. mansoni* (Podesta and McDiarmid, 1982), and as adult male worms are able to maintain a lower Ca^{2+} concentration within the tegument than occurs in the surrounding medium (Fetterer et al., 1980), it has been suggested that this enzyme may be involved in moving Ca^{2+} out of the worm (Cesari et al., 1981). In view of the differences in the calcium content of *S. mansoni*, particularly in relation to the sexual status of the female worms, studies are now in progress looking at the distribution of ATPases within the tegument of worms from both mixed and single sex infections.

Both Ca^{2+}-dependent (EC 3.6.1.3) and nonspecific (Na-K-Mg) ATPase activity were localized in whole worms using a modified one-step lead citrate method (Ando et al., 1981).

In female worms from mixed-sex infections Ca^{2+}-dependent ATPase activity was observed primarily in the tegument and in the cytoplasmic processes connecting the tegument with nucleated tegumental cytons (Figure 3a). Some reaction product was occasionally seen within the cytoplasm surrounding the tegumental nuclei. In the tegument the Ca^{2+}-dependent ATPase activity was associated with the cytoplasmic faces of both the outer and inner basal tegumental membranes, although in many regions high concentrations of reaction product also occurred over the tegumental cytoplasm in general. No marked Ca^{2+}-dependent ATPase activity was observed in association with either the tegumental spines or within the tegumental sense organs.

In sexually mature female worms Ca^{2+}-dependent ATPase activity was also localized around/over both the subtegumental muscles and the musculature beneath the gastrodermis. Membrane-associated activity was observed in parts of the excretory system. In comparison, female worms from single-sex infec-

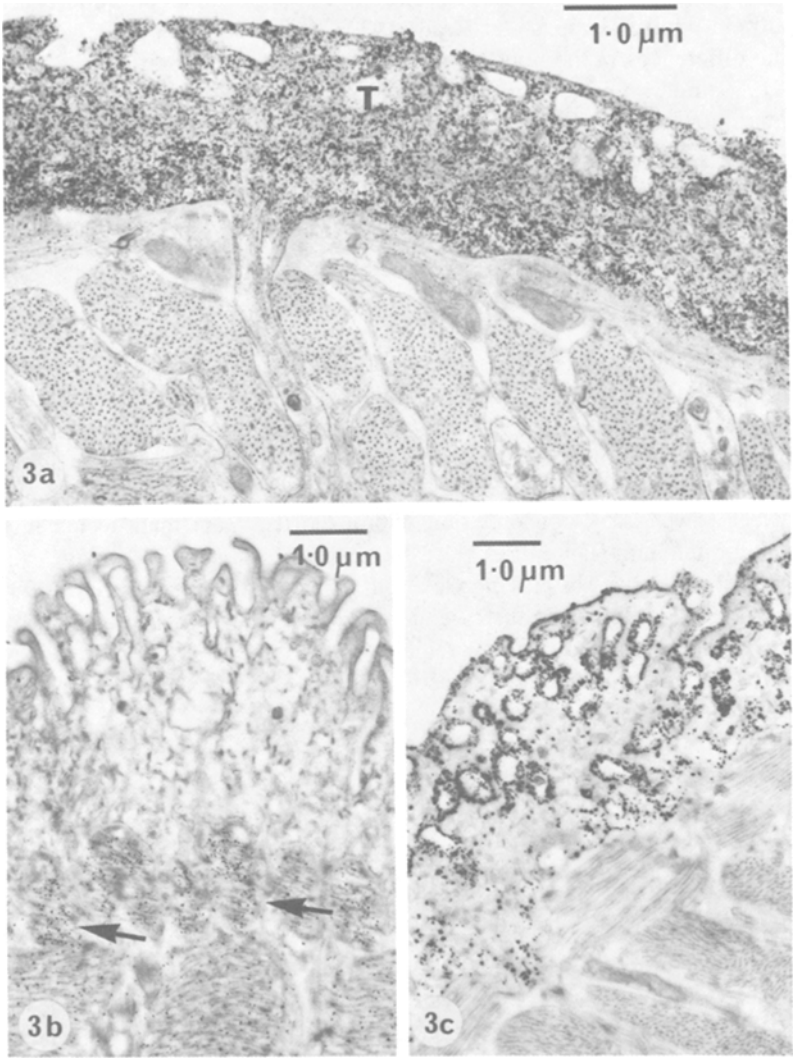

FIG. 3. (a) Electron micrograph showing the localization of Ca^{2+}-dependent ATPase in the tegument (T) of a sexually mature female *Schistosoma mansoni*. (b) Tegument of a female *Schistosoma mansoni* from a single-sex infection showing the absence of Ca^{2+}-dependent ATPase activity. Note some activity in the subtegumental muscles (arrows). (c) (Na-K-Mg)-dependent ATPase activity in the tegument of female *S. mansoni* from single-sex infection.

tions showed little or no Ca^{2+}-dependent ATPase activity in either the tegument or tegumental connections (Figure 3b), although reaction product was present around/over the subtegumental musculature and in parts of the excretory system. In male worms Ca^{2+}-dependent ATPase activity was localized in the tegument, in the tegumental connections, around/over the subtegumental muscles, and in parts of the excretory system.

In complete contrast to the sexual-status-dependent distribution of Ca^{2+}-dependent ATPase activity in female worms, females from both mixed and single-sex infections showed tegumental staining for (Na-K-Mg)-dependent ATPase (Figure 3c).

In control worms, no marked ATPase activity was observed in specimens incubated in substrate-free medium, although some small areas of the tegument and tegumental connections showed very slight membrane-associated reaction product, suggesting the presence of endogenous substrate. Further control experiments using a number of inhibitors, e.g., bromotetramisole, *p*-hydroxymercuribenzoate, and ouabain, are in progress.

These preliminary results on Ca^{2+}-dependent ATPase activity, taken in association with our initial studies on changes in calcium levels in relation to the sexual status of the worms, suggest that significant and fundamental changes also occur in the physiology of the tegument of female worms concomitant with the previously described morphological and overall biochemical changes that occur during sexual maturation.

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INFLUENCE OF MATING ON SURFACE NUTRIENT EXCHANGE IN SCHISTOSOMES

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Abstract—In schistosomes, the mating process influences male–female transfer and gender-specific exchange of nutrients. The paired male schistosome provides glucose to the female partner. Male-to-female intertegumental transfer of ^{14}C-labeled glucose, ^{14}C-labeled 3-*O*-methylglucose, [^{14}C]2-deoxyglucose and 2-fluorodeoxyglucose has been demonstrated in schistosomes. This phenomenon has been studied extensively in *Schistosoma mansoni*, and confirmed in *Schistosoma japonicum*, as well as *S. haematobium*, using radioactive pulsing methods. Male schistosomes contain significantly greater quantities (nmol/mg worm water) of glucose than do females. The transfer of glucose is apparently not an energy-dependent process, but occurs along this concentration gradient. Most, if not all, of the glucose utilized by the female is transferred from the male partner via tegumentary-facilitated diffusion mechanisms, free diffusion, or some combination of these two components. Unpaired male schistosomes contain greater quantities of glycogen than do comparable paired schistosomes, indicating that the presence of a female in the gynecophoral canal depletes the reserves of the male partner; this is additional indirect evidence for male-to-female transfer of glucose. Tegumentary surface uptake of acidic amino acids has been compared in paired and separated male and female schistosomes. In *S. mansoni*, a saturable carrier-mediated mechanism has been defined which operates only in unpaired male and unpaired female teguments. In contrast, the uptake of aspartate and glutamate is not seen in paired worms of this species. Tegumental uptake of acidic amino acids is not observed in paired or unpaired male or female *S. japonicum*. However, in *S. haematobium*, significant quantities of aspartate are taken up by both paired and unpaired male schistosomes. Measurable aspartate uptake is seen in paired female *S. haematobium*, but in the separated female, there is minimal uptake of this acidic amino acid. Thus the permeability of the teguments of human schistosome species to acidic

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amino acids is modified in response to the paired state in *S. mansoni* and *S. haematobium*, but these characteristics are not shared by *S. japonicum*.

Key Words—*Schistosoma mansoni, Schistosoma haematobium, Schistosoma japonicum*, glucose transfer, glycogen, glutamate, [^{14}C]aspartate, mating.

INTRODUCTION

It is now well established that many nutrients required by schistosomes can be assimilated across the tegumental surfaces (Fripp, 1967; Rogers and Bueding, 1975; Senft, 1968; Cornford and Oldendorf, 1979) and that a functioning gut is not essential to in vitro survival (Popiel and Basch, 1984b). The present discussion will focus upon male–female exchange of materials between the schistosome pair. A second area of focus will be tegumental exchange between the culture medium and schistosome tissues and specific consideration of the influence of mating upon nutrient uptake in male and female schistosomes.

More than 40 years ago, the possibility of male-to-female transfer of materials was suggested by Vogel (1941). The transfer of nutrients as well as hormones or pheromones was again suggested by Senft (1968, 1969). He suggested that ventralization of nutrients by the male may play a significant role in the nutrition of the female. Lennox and Schiller (1972) noted that fluctuations in glycogen content of male schistosomes were reflected in their female partners. They also indicated that in vitro studies had similarly shown a parallel depletion of histochemically demonstrable glycogen from the tissues of paired male and female schistosomes. It was suggested that the carbohydrate metabolism of the female in vivo was in some way dependent on the male partner. Furthermore, a nutritional relationship was given as the primary mechanism responsible for the growth of female worms subsequent to pairing (Lennox and Schiller, 1972). In a comparative study of glucose uptake by way of either the oral sucker or the tegumental surfaces, Rogers and Bueding (1975) reported that the tegument was probably the only site of glucose absorption by *S. mansoni*. The demonstration of tegumental glucose uptake by female worms was considered to support a hypothesis that, in schistosome pairs, the male provided glucose to the female.

We developed a triple-isotope technique for the study of nutrient transfer across the schistosomal tegument (Cornford and Oldendorf, 1979). This method (Figure 1) involves immersion of the isolated schistosomes (Duvall and DeWitt, 1967) in a buffered saline medium which contains tritiated water, a ^{14}C test substance such as glucose or an amino acid, and indium-113m chelated to EDTA. As the schistosomes are immersed in the isotopic medium, nearly all the tritiated water penetrates rapidly. An unknown quantity of the ^{14}C test substance penetrates, and, because the indium–EDTA chelate is excluded by intact

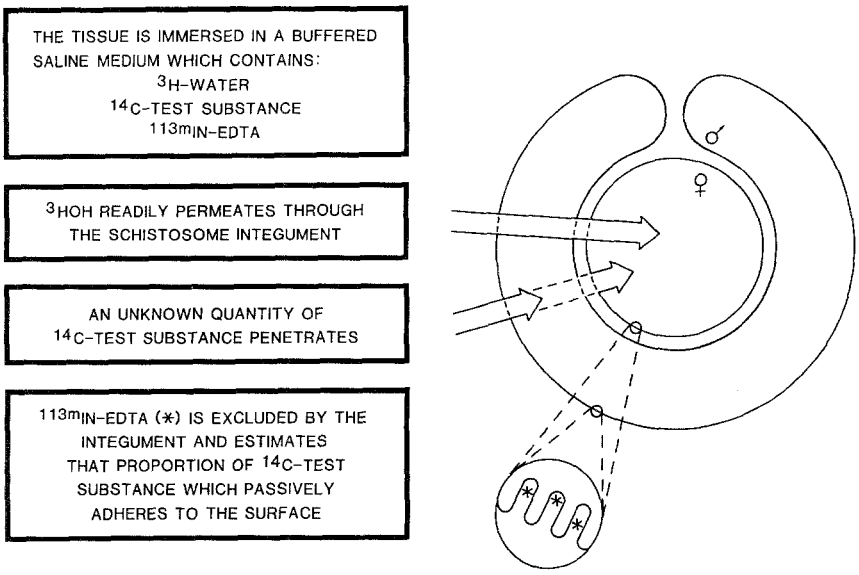

FIG. 1. The triple-isotope method involves immersion of tissue in a buffered saline medium which contains tritiated water, a ^{14}C test substance (e.g., glucose or amino acid), and indium-113m chelated to EDTA. Nearly all the tritiated water rapidly penetrates through the schistosome tegument. An unknown fraction of the ^{14}C test substance penetrates, and the ^{113m}In–EDTA chelate, which is excluded by intact membranes, is used to estimate that proportion of the ^{14}C test substance which passively adheres to the external surfaces of the schistosome. The incubation is halted by removing the schistosomes from the radioactive medium and flooding them with ice-cold silicone oil. This oil rinse not only removes excess surface-adherent isotopes by a sheeting action, but also minimizes evaporative loss of tritiated water. In addition, separation of paired male and female schistosomes is facilitated in this silicone oil environment. Isotopic content of the schistosomes, and of a known volume of the saline medium, is determined routinely in a liquid scintillation counter. The schistosomal tissue uptake index (TUI) equals (^{14}C DV − ^{113m}In DV)/(^{3}H DV) where DV = the distribution volume of each isotope, and the TUI is typically expressed as a percentage. Other studies (Cornford et al., 1982) have demonstrated that the distribution volume of water (= ^{3}H DV) can be correlated with traditional estimators of mass such as wet weight or protein content.

living membranes (such as the schistosome tegument), the third isotope is used to estimate that proportion of the ^{14}C test substance which passively adheres to the schistosomal surfaces. The isotopic content of the schistosomes, and also the isotopic content of the medium, are then measured, and the distribution volume of each isotope defined precisely. The schistosomal tissue uptake index (TUI) is a ratio of the distribution volumes of ^{14}C and ^{3}H, and it has been established that water content in both male and female schistosomes can be correlated with mass measurements (schistosome wet weight and protein con-

tent; Cornford et al., 1982). The distribution volume of water has also been correlated with protein content in *Schistosoma haematobium* and in *Schistosoma japonicum* to establish a broad base for the use of this technique. Estimation of that fraction of test substance which is passively carried on the folded tegumental surfaces of schistosomes is achieved by measuring the distribution volume of ^{113}m In–EDTA chelate. This isotope has a short half-life ($t1/2$ = 100 min), and its emissions are both gamma and beta conversion electrons. Thus the indium content of tissues can be determined without digestion of the schistosome in a gamma counter, or the high-energy beta conversion electrons may be easily distinguished from ^{14}C in a scintillation counter. We typically measure indium emissions immediately upon conclusion of an experiment, and wait two days (when all the indium has decayed away) to measure tritium and carbon contents in the absence of ^{113}m In.

We had just begun to apply this technique to the study of nutrient uptake in schistosomes when we obtained a group of infected mice which had apparently been exposed to an excess number of male cercariae. In addition to the paired schistosomes, we also perfused a large number of unpaired males and proceeded to examine glucose uptake in these unpaired males as well as the paired worms (Figure 2). Over a wide range of hexose concentrations, we observed higher TUIs in the paired males than in comparable unpaired male schistosomes (Figure 2). Thus, the observation that a paired male schistosome had a higher glucose requirement than an unpaired worm seemed to indirectly support the hypothesis that, in paired schistosomes, males supplied glucose to the females.

Further study of this phenomenon utilized an experimental design in which paired schistosomes were separated prior to incubation in isotopic medium and then analyzed. Additional comparisons were made with results obtained where the paired worms were pulsed in isotopic medium and separated immediately after exposure to the isotopes for independent analysis. Consistently, it was observed that paired schistosomes incorporated more glucose than comparable unpaired schistosomes (Figure 3). Thus for both the male and female schistosomes, the paired state is characterized by an increased requirement for hexose. A more interesting trend observed, however, was that when unpaired male and unpaired female schistosomes were compared, the male uptake was generally greater than that of the female. In contrast, when we compared paired schistosomes, the female TUI was higher than that of the comparable paired male (Cornford, 1982; Cornford and Huot, 1981). These data further supported the possibility that the male schistosome could be transferring glucose, or glucose-derived metabolites, to the female partner.

Investigations of male-to-female transfer using isotopic methods are complicated by the fact that, if the male is rapidly pulsed with radiolabeled glucose and removed to a glucose-free medium, the radioactive glucose could equili-

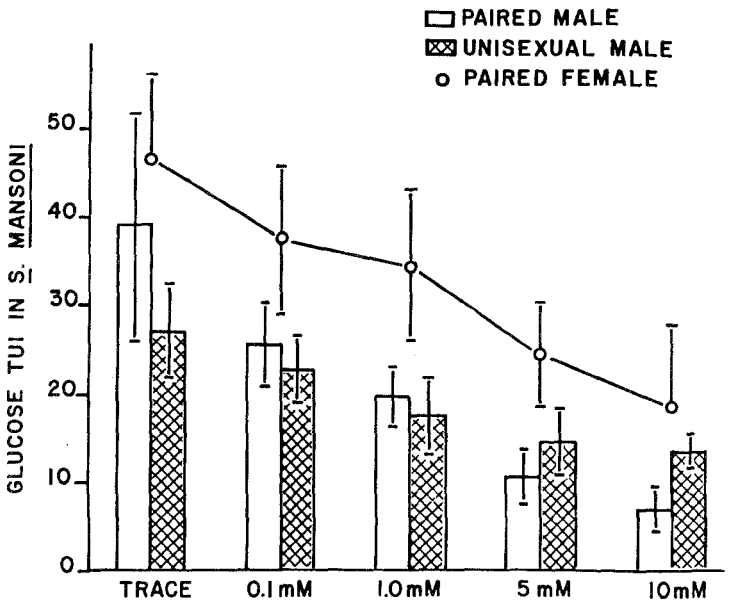

FIG. 2. A comparison of glucose uptake in paired male and female schistosomes as compared with TUIs in male schistosomes which were unpaired at the time of perfusion from mouse hosts. Over a wide range of glucose concentrations, higher glucose uptake was observed in paired males than in comparable unpaired male schistosomes, indicating that paired males apparently have a higher glucose requirement than seen in unpaired worms. The incubation period in isotopic medium was 15 sec, and each vertical bar represents ± 1 SD.

brate either into the female or into the external medium. The triple-isotope technique was consequently modified. Typically, perfused schistosomes are collected and maintained in vitro in culture medium for relatively short time periods. These schistosomes are then pulsed in radioisotopic medium, and the reactions terminated by immediately covering the schistosomes with silicone oil. This oil rinse removes excess adherent isotopic medium by a sheeting action. In addition, the use of ice-cold silicone oil depresses metabolic processes and exchange in the schistosomes. To minimize any possible evaporative loss of tritiated water, schistosome pairs are maintained under ice-cold oil while the male is mechanically separated from the female for subsequent digestion and analysis. To test for transfer of hexose isotopes from the male to the female, schistosome pairs were pulsed in isotopic medium, and then, instead of being covered with cold silicone oil, they were covered with warm (37°C) silicone oil and maintained in the warm oil for varying lengths of time (0.5–4.0 min). In mansonian female schistosomes which have been pulsed for 5 sec in radio-

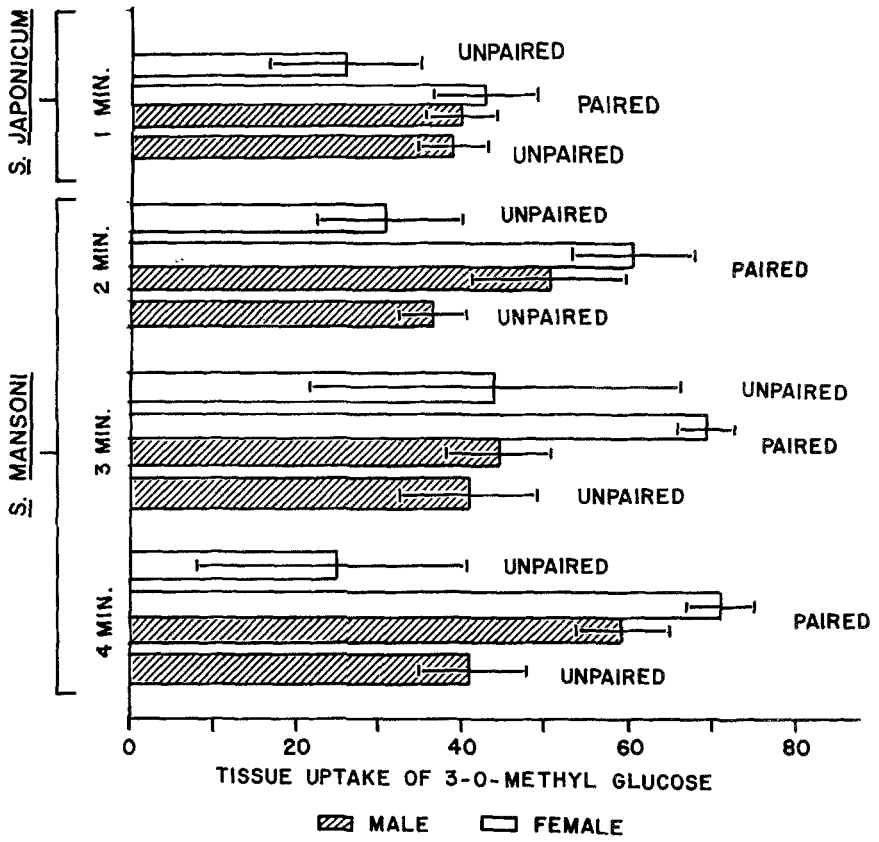

FIG. 3. Comparison of 3-*O*-methylglucose uptake in paired and separated male and female schistosomes. Note that, as in Figure 2, paired schistosomes incorporate more hexose than comparable unpaired schistosomes. Note that for each of the 1- to 4-min incubation periods studied, when schistosomes have been unpaired, the uptake measured in males is typically greater than that seen in females. In contrast, gender comparisons of paired schistosomes indicate that the female uptake is higher than that of the comparable paired male. Since it has been established that 3-*O*-methylglucose is transported, but not further metabolized in schistosomes (Isseroff et al., 1972), these data reflect the distribution of free hexose, rather than some glucose metabolite. Furthermore, the dramatically higher uptakes observed in paired (vs. unpaired) female schistosomes further support the possibility that the paired male schistosome could be transferring glucose to the female partner. Each horizontal bar represents ± 1 SD; $N > 5$ for each mean. (Adapted from Cornford, 1982.)

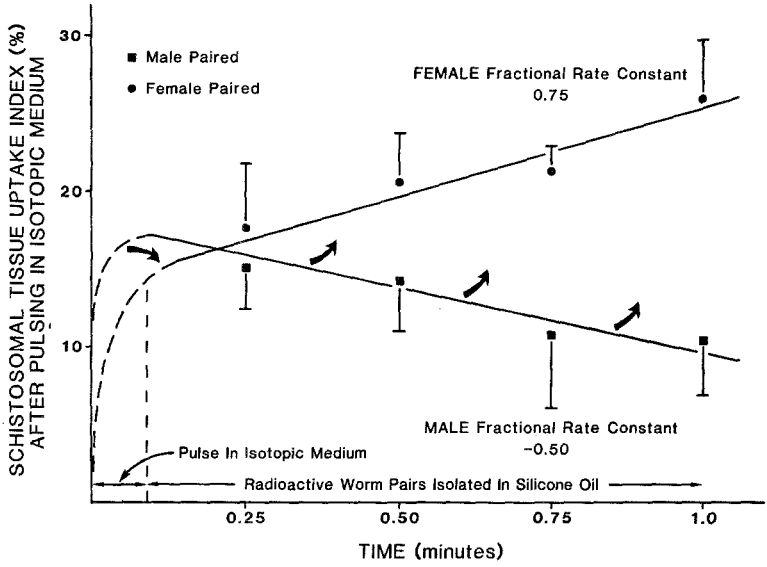

FIG. 4. Transfer of ^{14}C hexoses from the male to the female schistosome partner. (Adapted from Cornford and Huot, 1981; Cornford and Fitzpatrick, 1985.) When paired schistosomes are rapidly pulsed in radioactive glucose medium, and subsequently allowed to equilibrate in a silicone oil environment for varying lengths of time (0.25–4 min), a time-dependent increase in glucose content of the female schistosomes is observed. Concomitantly, a time-dependent decrease in glucose content of the male schistosome partners is observed. Thus, as indicated by the arrows above, [^{14}C]glucose is transferred from the male to the female schistosome partner. In the studies of Cornford and Fitzpatrick (1985), the amount of glucose gained by the female partner each minute was equal to 75% of the total free glucose present in the female. Male schistosomes lose an amount equal to 50% of the total free glucose present, as indicated by the fractional rate constants.

active glucose, chilled with cold oil, and analyzed, we typically observed a mean TUI of about 20%. Females which had been pulsed for 5 sec, and subsequently maintained in warm oil for varying periods of time prior to analysis, consistently showed a time-dependent increase in glucose content (Figure 4). In the male schistosome partners, a concomitant time-dependent decrease in glucose content was typically observed (Figure 4).

To insure that the phenomenon observed was not an artifact of the TUI technique, the distribution volume of tritiated water was measured in both male and female schistosomes. No difference was observed for water content of males pulsed for 5 sec, chilled, and examined, as compared to males which had been pulsed for 5 sec and maintained in warm oil for periods of 2 or 3 min. Similarly, the distribution volumes of water in the comparable female schistosomes showed no significant changes in the experimental time periods employed (Cornford

and Huot, 1981). These studies established that, after pulsing in [^{14}C] glucose, the transfer of ^{14}C from the male to the female schistosome did indeed occur. This phenomenon was observed using D-[^{14}C]glucose and confirmed using [^{14}C]2-deoxy-D-glucose. This hexose analog may be phosphorylated in most biological systems, but it is not further degraded, and the 2-deoxy-D-glucose-6-phosphate thus formed remains trapped intracellularly for extended periods of time (Sokoloff et al., 1977). Furthermore, the transfer of [^{14}C]3-*O*-methylglucose from male to female schistosomes has been established (Cornford and Huot, 1981). 3-*O*-Methylglucose is transported across the schistosome tegument by the glucose transport (facilitated diffusion) system, but not metabolized (Isseroff et al., 1972). Thus the molecular species that is exchanged in the hexose, rather than some glucose metabolite (Cornford and Huot, 1981).

It has also been demonstrated that both [^{14}C]glucose and [^{14}C]3-*O*-methylglucose can be transferred from male to female *S. japonicum*. In addition, transfer of glucose has been established in the Egyptian strain of *S. haematobium*. In the latter species, not only is a time-dependent increase in the glucose content of females seen, but a time-dependent decrease in the glucose content of male schistosomes was concomitantly observed in every instance. The physical appearance of *S. haematobium* pairs is characterized by the fact that the female is most consistently retained within the male gynecophoral canal, and the only portion of the female surface exposed to culture medium is anterior of the acetabulum. Thus this species is particularly suited to the study of male-to-female transfer phenomena, and the most unequivocal demonstrations of transfer are observed in *S. haematobium*. Curiously, it has also been demonstrated that the most striking examples of gender-specific polypeptides were observed in *S. haematobium* (Aronstein and Strand, 1983), and the authors also suggested that because *S. haematobium* males had eight unique polypeptides, this species was the appropriate model for study of gender-specific phenomena. To summarize, male-to-female intertegumental transport of ^{14}C-labeled glucose, ^{14}C-labeled 3-*O*-methylglucose, [^{14}C]2-deoxy-D-glucose (Cornford and Huot, 1981; Cornford, 1982), and 2-fluorodeoxy-D-glucose (Cornford and Fitzpatrick, 1985) has been demonstrated in schistosomes. In addition, this phenomenon has been studied extensively in *S. mansoni* and confirmed in *S. japonicum*, as well as *S. haematobium*.

Once the transfer of glucose had been established, it became necessary to estimate how much glucose was transferred. If the quantity exchanged from the male to the female was comparable to the rate at which glucose was metabolized, this would be presumptive evidence for male-derived glucose as the primary or sole source of hexoses for the female partner. Alternatively, the female gut could be an additional source of glucose, if only a relatively small quantity is transferred from the male to the female in paired worms. Definition of the mechanism by which glucose is transferred also is of importance. It was reasoned that either an active mechanism pumped glucose between the male and

the female, or glucose was transferred along a concentration gradient. If a concentration gradient existed between the male and female schistosome, then the free glucose level in males would be anticipated to be greater than that of female schistosomes. Alternatively, if glucose levels were similar in the paired male and female schistosomes, then presumably an energy-consuming active mechanism must exist and be responsible for the transfer of glucose. Since active transport mechanisms are typically inhibited by ouabain, Cornford and Fitzpatrick (1985) compared glucose transfer in schistosomes exposed to ouabain (preincubated 2 min in 1 mM ouabain) with that of untreated paired worms. Their initial studies demonstrated that the transfer of glucose from males to females was not sensitive to ouabain, and they thus focused their attention on the alternate possibility that a concentration gradient of glucose existed between the male and female schistosomes. Certain problems exist in measuring free glucose levels. Glucose utilization rates are extremely high in schistosomes (Bueding, 1950), and thus schistosomes must be rapidly fixed. In addition, it is important to discriminate between intraworm free glucose and free glucose which may be carried on the amplified external surfaces of the schistosome tegument.

As indicated in Figure 5, it was established that in mansonian male schistosomes, the free glucose level represented 20–30% of the external medium, and levels in the female partners were 40–50% less. Studies of pairs which had been separated and maintained in vitro indicated that unpaired males also had slightly greater free glucose concentrations than paired mansonian males. Cornford and Fitzpatrick (1985) further established that in *S. haematobium* and *S. japonicum*, male schistosomes had significantly higher free glucose contents than seen in females of these two species (Figure 5). Other work demonstrated that, over the range of glucose concentrations anticipated to exceed minimal and maximal portal blood glucose levels (5–20 mM), the free glucose content of mansonian males was always significantly higher than that of female schistosomes. It was concluded that glucose was not actively transported between the paired worms, but exchanged along this concentration gradient. The glucose gradient transfer is accomplished by way of the facilitated diffusion (transport) mechanism, by free diffusion, or by some combination of these two components.

The quantity of glucose transferred between mansonian pairs of schistosomes has also been estimated. In a silicone oil environment, devoid of exogenous glucose, *S. mansoni* females receive 0.1 μmol/mg dry wt/hr (Cornford and Fitzpatrick, 1985). It was conservatively estimated that at least 40% of the glucose utilized by the female schistosome was obtained from the male partner. However, this estimate was based upon a glucose utilization rate of 0.38 μmoles/mg hr of intact worm pairs in a medium containing 8–11 mM glucose (Shapiro and Talalay, 1982). These authors further indicated that the glucose consumption rate was a saturable function of glucose concentration; at a medium con-

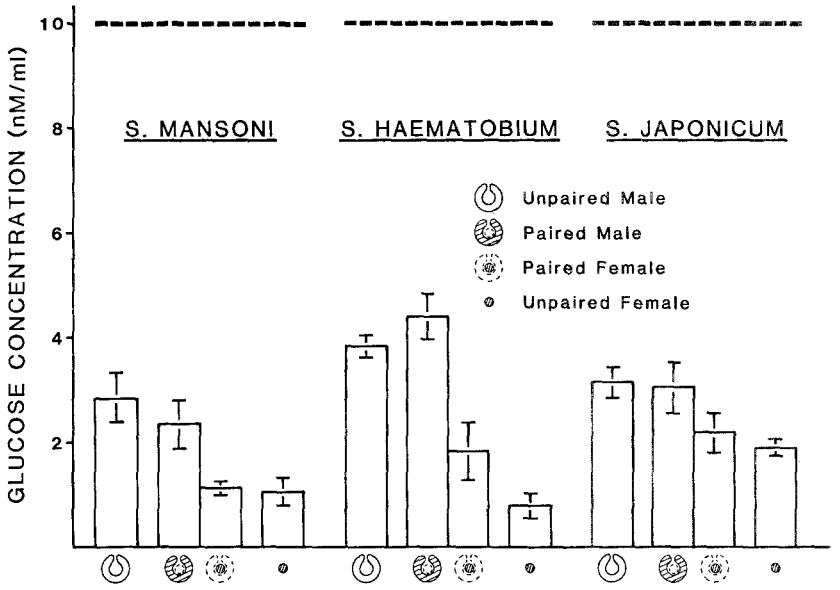

FIG. 5. Free glucose content in separated and paired male and female schistosomes after in vitro incubation in a medium containing 10 mM glucose. Note above that male schistosomes have significantly higher free glucose concentrations (nmol/ml worm water) than seen in females of the same species. In mansonian schistosomes, a gradient exists such that the free glucose content of male schistosomes is 20–25% that of the medium, and females contain only about half the concentrations seen in their male partners. In all three species examined, the presence of a distinct gradient (medium > male > female), together with the observation that male-to-female transfer of glucose was not ouabain-sensitive (Cornford and Fitzpatrick, 1985), indicates that glucose is not actively transported between the paired worms, but rather exchanges along this concentration gradient. Presumably this gradient transfer involves a saturable facilitated diffusion mechanism, free diffusion, or some combination of these two components. (Adapted from Cornford and Fitzpatrick, 1985; each vertical bar = ±1 SD.)

centration of 11 mM glucose, this represented 95% of the calculated maximum rate. Other workers have also reported that glucose utilization rate may be a function of the glucose concentration in the medium. For example, Watts (1978) indicated that when schistosomes were maintained in 0.4 mM glucose, the glucose utilization rate was one fourth to one fifth of that measured for pairs maintained in 5 mM glucose. Since the tegument is believed to be the only site of glucose uptake by *Schistosoma mansoni* (Rogers and Bueding, 1975), it seems reasonable to assume that most, if not all, of the glucose utilized by the paired female schistosome is obtained from the male partner. Any portion of the female surface exposed in vitro to the plasma, as would occur during egg deposition, can directly assimilate glucose.

One other report suggested that male *S. mansoni* synthesize a 66,000-molecular-weight protein which is not synthesized by the female (Atkinson and Atkinson, 1980). It was further suggested that this labeled polypeptide could be transferred from the male to the female over a 20-hr time period. In a subsequent study, some 75 polypeptides were resolved in two-dimensional gels of [^{14}C]leucine-labeled male and female *S. mansoni* (Atkinson and Atkinson, 1982). The extensive studies of Popiel and Basch (1984a) failed to demonstrate transfer of any polypeptides from male to female mansonian schistosomes. Thus the contemporary understanding of intertegumental exchange between the paired male and female schistosome can be succinctly summarized. Transfer of polypeptides does not occur (Popiel and Basch, 1984a). The stimulation of vitelline development in unisexually reared female *S. mansoni* by a lipid-soluble extract obtained from male worms (Shaw et al., 1977) was believed to be indirect evidence for male-to-female transfer of materials. However, this topic is currently under reinvestigation (Popiel, personal communication). Although the transfer of hexoses from male to female schistosomes has been established and confirmed in three different studies (Cornford and Huot, 1981; Cornford, 1982; Cornford and Fitzpatrick, 1985), it has yet to be confirmed outside our laboratory.

Analyses of glycogen contents in unpaired and paired male schistosomes, as well as paired females, indicate that unpaired males are distinctly different from paired males (Figure 6). In three different human species, it has been demonstrated that glycogen content was significantly lower in female than male schistosomes, and significantly higher in unpaired males than in paired males (Cornford and Huot, 1981). This observation has been confirmed in other laboratories (Bueding, personal communication). Thus the presence of a female in the gynecophoral canal of the male has the ability to diminish or deplete glycogen stores in the male partner, and thus represents indirect evidence supporting glucose transfer from male to female schistosomes. When radiolabeled schistosome pairs are transferred into hosts which subsequently receive a second transfer of unlabeled, unpaired male schistosomes, Cioli (personal communication) has observed that a small but significant number of radiolabeled females are recovered two weeks later paired with nonradioactive males. Thus female mansonian schistosomes are apparently able to change male partners in vivo. However, the elevated glycogen content distinctive in unpaired males can be attributed to the fact that schistosomal glycogen levels change rapidly in response to nutritional stress (Cornford et al., 1983), in addition to the low frequency with which female schistosomes change partners.

Most parasitic platyhelminths are hermaphroditic. Sexual dimorphism is a unique characteristic of schistosomes, and complete maturation of female worms is dependent upon a close association with the male partner. Three to six decades ago, the influence of mating upon female somatic development in *S. japonicum* was described (Severinghaus, 1928; Moore et al., 1954). The lack of

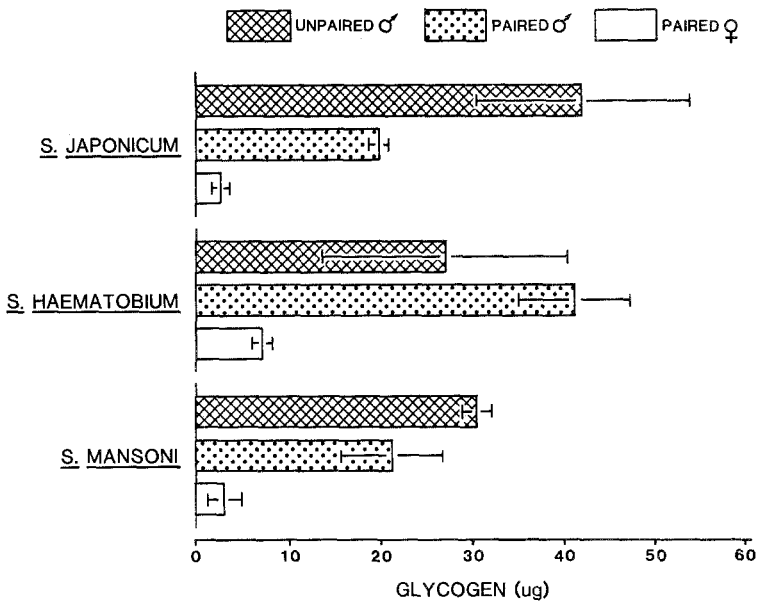

FIG. 6. Comparison of glycogen contents in paired male and female schistosomes, as well as unpaired males perfused from the same infections. In the three human schistosome species, unpaired males have significantly greater quantities of glycogen than do paired male schistosomes (adapted from Cornford and Huot, 1981). These observations suggest that the presence of a female within the gynecophoral canal of the male partner has the ability to diminish or deplete glycogens reserved in the male partner. These observations represent indirect evidence supporting glucose transfer from male to female schistosome partners. (Each vertical bar = ±1 SD; adapted from Cornford and Fitzpatrick, 1985.)

growth of female "mesenchymal cells" was reported to be a consequence of the failure to mate (Severinghaus, 1928). In *S. haematobium* (Brumpt, 1936) and *S. mansoni* (Moore et al., 1954; Armstrong, 1965; Erasmus, 1973), the full growth and sexual maturation of female worms depend upon their physical contact with the male. It was also noted that females derived from unisexual infections were smaller and showed incomplete development of the reproductive system. In contrast, in *Schistosomatium douthitti* (Short, 1952) and *Heterobilharzia americana* (Armstrong, 1965), females will grow, but to a smaller size, in the absence of the male; but parthenogenetic production of eggs which produce viable miracidia has been reported. Vogel (1941) indicated that egg production and full somatic development of the human blood flukes required the stimulation of an enveloping male partner.

In a study where intact female worms were allowed to pair in vitro with male worm segments, Popiel and Basch (1984b) noted that vitelline gland differentiation in the females was restricted to the area of male contact and was

not propagated throughout the worm. They concluded that nutrients absorbed through the tegument were responsible for the survival of worms lacking a functioning gut; secondly, that worm-pairing male stimulation and female developmental response occurred in segments lacking cerebral ganglia; and thirdly, that males had no centralized location for the female-stimulating factor. It has also been demonstrated that when mature females were isolated from the male partners, a significant decrease in worm size was noted, and there was a concomitant regression in the reproductive system (Clough, 1981; Popiel et al., 1984). Autoradiographic studies of proline uptake in *S. mansoni* have demonstrated that the female appears to become more radioactive when an intimate contact with the male is established and that proline uptake in males is concentrated to the female contact zone in the gynecophoral canal (Strunk, 1967; Senft, 1968). Gender-specific responses of schistosomes to drugs have been described in several studies (Senft, 1969; Coles, 1973; Cioli and Knopf, 1980).

The influence of mating upon surface nutrient exchange in schistosomes can be studied by comparing the male and female tegumentary surfaces. Coles (1973) and Cornford (1985) have emphasized need for comparative study of surface phenomena in paired and unpaired male and female schistosomes. Minor differences in glucose uptake by male and female *Schistosomatium douthitti* have been reported (Cornford et al., 1981); in the human schistosomes, the rate of hexose uptake is greater in separated males than separated females, and paired worms always take up hexoses at a greater rate than unpaired schistosomes (Cornford, 1982; Cornford and Huot, 1981). Choline uptake is slightly higher in separated male than separated female *S. mansoni*, and lower in paired than unpaired schistosomes (Young and Podesta, 1984). In a study of nucleic acid precursors, Mattoccia et al. (1982) showed that paired *S. mansoni* take up approximately 13 times more uridine than seen in unpaired males, but this effect was not observed in studies of thymidine uptake. Paired worms reportedly took up two to three times the amount of leucine than did unpaired males. In comparisons of separated male and female schistosomes, these authors suggested the increased radioactive uridine and leucine seen in males was a function of worm size, but isolated females take up approximately four times as much thymidine as do isolated males. It was concluded that male-to-female differences in incorporation of nucleic acid precursors or amino acids should be considered in analyses of schistosome synthesis (Mattoccia et al., 1982). In another study of tyrosine uptake by female *S. mansoni* from unisexual infections, Popiel and Erasmus (1981) observed increased uptake in these females after stimulation by either male schistosomes, or lipid-soluble extracts isolated from male worms.

Other studies from this laboratory suggest that uptake of acidic amino acids in *S. mansoni* is quite different in paired and unpaired male and female schistosomes. We see little or no uptake of glutamate in paired male or female schistosomes. In contrast, the TUI is about 30% for glutamate in unpaired male or

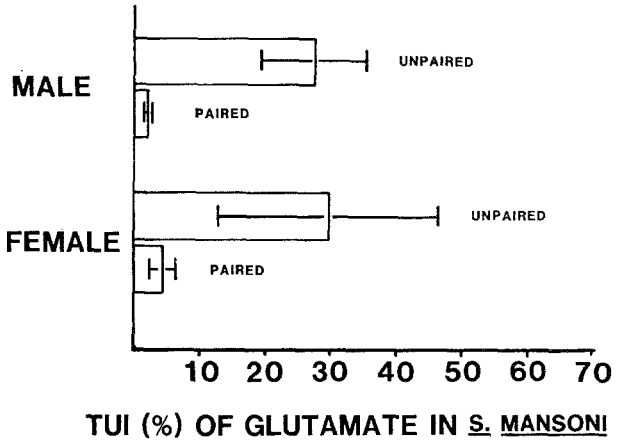

FIG. 7. Comparison of glutamate uptake in unpaired and paired male and female schistosome partners. Note that when the schistosomes are paired, TUIs measured in both males and females are approximately 3%. This approximates the measured TUIs of compounds such as sucrose and polyethylene glycol (Bocash et al., 1981), which are considered to be excluded by the schistosome tegument. (Vertical bars represent ± 1 SD.) In both males and females which were unpaired, however, a marked elevation of uptake of a tracer concentration of glutamate is observed.

unpaired female schistosomes (Figure 7). In paired schistosomes, the TUI is on the order of 3%; this approximates the measured TUIs of compounds such as sucrose and polyethylene glycol (Bocash et al., 1981), which are considered to be excluded by the schistosome tegument. However, a distinctly elevated uptake of glutamate is observed in unpaired males and females. Aspartic acid is the other dicarboxylic amino acid common in mammalian diets. Its uptake is also negligible in paired male and paired female schistosomes, but significant amounts of this amino acid are incorporated in unpaired males and unpaired females (Figure 8). Note also in this figure that the free diffusion of tritiated water in these schistosomes is not significantly different in paired and separated schistosomes; thus the reduced uptake of acidic amino acids is not an artifact of the water-reference technique. Furthermore, since significant amounts of tritiated water freely diffuse into the paired females, we are able to reject the possibility that a reduction in the available surface area of paired (female) schistosomes is responsible for the observed reduced uptake.

To ensure that this was not a time-dependent phenomenon, TUIs of acidic amino acids were examined over a 60-sec period, and similar results were observed. The amounts of aspartate and glutamate incorporated into paired schistosomes were equal to the amount of mannitol incorporated over a 60-sec period. Furthermore, in unpaired male and female schistosomes, the uptake of acidic amino acids was not increased in the presence of physiological concen-

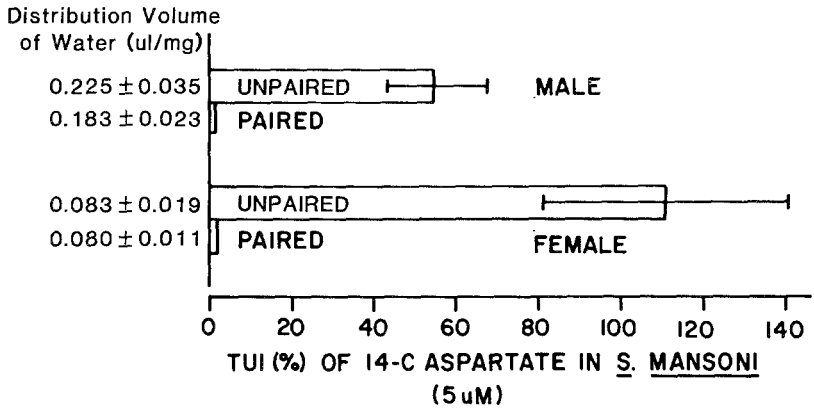

FIG. 8. Comparison of uptake of a trace concentration of aspartate in paired and unpaired male and female *S. mansoni*. Note that in paired males and females, the measured uptake of aspartate, like glutamate, is negligible. However, in separated male and female schistosomes, greatly elevated uptakes for this acidic amino acid are observed. Furthermore, note in the left column that the distribution volume of tritiated water is not significantly different in comparing paired and unpaired male schistosomes, nor is there a difference in the distribution volume of tritiated water in comparing unpaired and paired female schistosomes. Thus the reduced uptake of acidic amino acids, at least by female schistosomes, is not an artifact of the water reference technique, and since significant amounts of tritiated water are taken up by the paired females, the reduced uptake of aspartate, or glutamate (Figure 7), cannot be attributed to a reduction in the available surface area of the paired female schistosomes. (Each horizontal bar = ± 1 SD; $N > 5$ for each mean.)

trations of glucose, suggesting that tegumental incorporation of aspartate and glutamate was not mediated by an energy-dependent mechanism. In other studies, measurements of [^{14}C] glutamate uptake were performed over a wide range of specific activities, and clearly demonstrated the presence of a saturable, carrier-mediated transport mechanism in both unpaired male and unpaired female schistosomes (Cornford, 1985). It was also confirmed that this facilitated diffusion mechanism could not be identified in paired schistosomes. Similarly, when [^{14}C] aspartate uptake was measured over a wide range of substrate concentrations, a carrier-mediated transport mechanism for aspartate was apparent in both male and female schistosomes which were separated. In contrast, the tegument of paired schistosomes excluded aspartic acid over this same broad range of substrate concentrations (Cornford, 1985). These results observed with acidic amino acids contrasted sharply to studies of the uptake of the hexoses, where paired schistosomes were always observed to take up greater quantities of monosaccharides than seen in separated worms (Cornford and Huot, 1981).

Nonlinear regression analysis (Cornford et al., 1981) has subsequently been

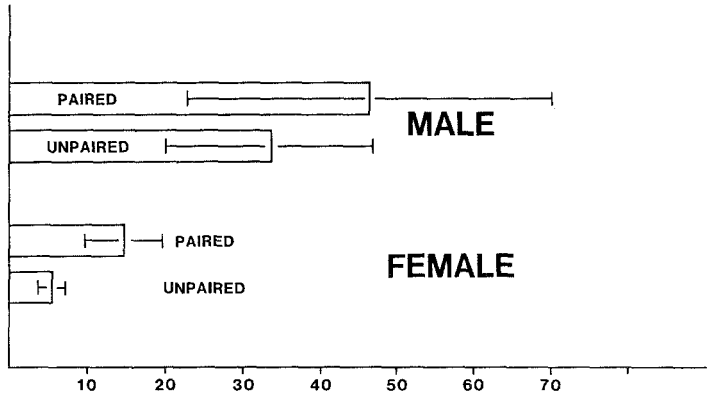

FIG. 9. Comparison of uptake of a trace concentration of [^{14}C]aspartate in paired and separated male and female *Schistosoma haematobium*. Note that in this species, both paired and unpaired male schistosomes take up significant quantities of aspartate. Mated females also incorporate significant quantities of this acidic amino acid, but in separated females, a minimal uptake of aspartate is apparent. Thus this species is unlike either *Schistosoma japonicum* (where no measurable uptake of acidic amino acids is seen in either paired or unpaired males and females; Cornford, 1985), or *S. mansoni*, where measurable uptake of acidic amino acids is seen only in separated males and females (Figures 7 and 8). (Each horizontal bar = ± 1 SD; $N > 5$ for each mean.)

employed to determine the half-saturation constants for uptake of these acidic amino acids. In separated female schistosomes, the half-saturation constant of glutamate was estimated to be 15 $\pm$ 4 μM, and in males, 14 $\pm$ 7 μM. The entry of any glutamate into schistosomes (which were unpaired in vivo) would be regulated by this high-affinity, low-capacity mechanism, since mammalian plasma contains 0.1–0.5 mM glutamate (Diem, 1962; Williamson and Brosnan, 1973). The half-saturation constant for aspartic acid uptake was significantly greater in separated male schistosomes (35 $\pm$ 8 μM) than in females (16 $\pm$ 4 μM), and comparable to plasma aspartate concentrations (about 30 μM; Diem, 1962; Williamson and Brosnan, 1973). The presence of a single acidic amino acid transport mechanism in unpaired *S. mansoni* males and females was suggested by the observation that 0.1 mM [^{14}C] aspartate uptake in male schistosomes could be reduced by a factor of 50% when 1 mM unlabeled glutamate was added to the incubation medium. The saturation of [^{14}C] glutamate uptake by the addition of 25, 100, and 1000 μM concentrations of unlabeled aspartate was observed in both unpaired male and unpaired female schistosomes. Nonlinear regression analyses indicates that the half-saturation constant for aspartate inhibition of glutamate uptake (K_i) was estimated to be 33 $\pm$ 38 μM in unpaired

females and 36 $\pm$ 54 μM in unpaired males. The fact that these K_i values are not significantly different from the estimated half-saturation constants of glutamate (15 and 14 μM in males and females, respectively) indicates that these two acidic amino acids do indeed share a single, facilitated-diffusion transport locus.

Studies utilizing the Japanese strain of *S. japonicum* indicated that no significant uptake of glutamate could be demonstrated over micromolar through millimolar concentrations in unpaired males or in paired males or paired females

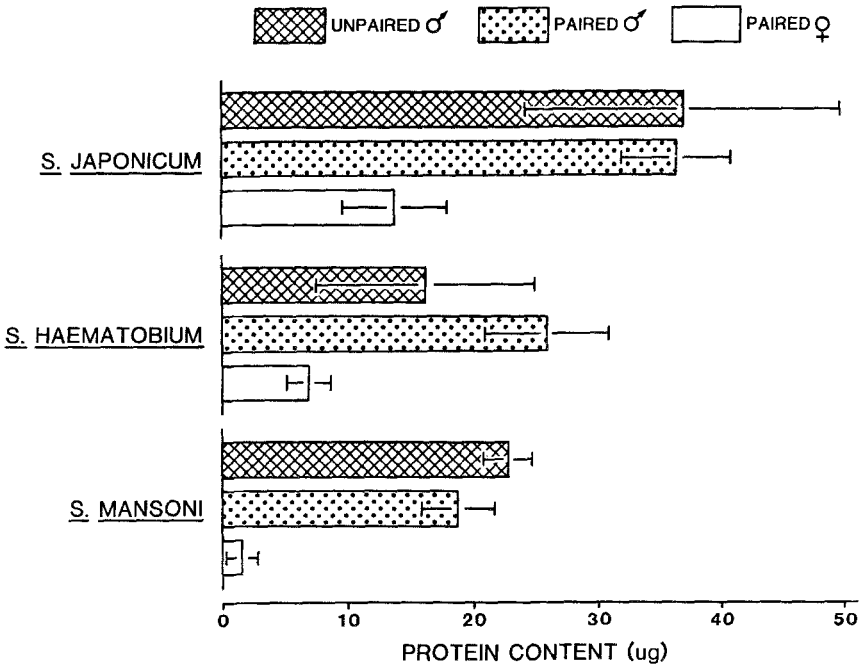

FIG. 10. A comparison of protein content (μg/worm) in unpaired male, paired male, and paired female human schistosomes. Note that in *S. japonicum* and *S. mansoni*, similar protein contents are observed in comparing unpaired and paired male schistosomes. However, in an Egyptian strain of *S. haematobium*, we have observed that males which were perfused unpaired from infected hamsters were significantly smaller than paired male. In this species, a stimulus provided by the paired female is apparently able to enhance the development of the male partner. As indicated in the text, it is typically the male partner which provides the stimulus responsible for full development of the female schistosome (Severinghaus, 1928; Moore et al., 1954; Brumpt, 1936; Armstrong, 1965; Erasmus, 1973; Short, 1952). *S. haematobium* is not only uniquely suited for the study of gender-specific differences (Aronstein and Strand, 1983), but would also represent an appropriate model system to define the nature of the stimulus which the female provides to the male partner.

(Cornford, 1985). However, in another human schistosome, *S. haematobium*, a situation exists which is unlike either *S. japonicum* or *S. mansoni*. Measurable quantities of radiolabeled aspartate are taken up by paired females, paired males, and separated male *S. haematobium*. Only in the separated female did we observe minimal uptake of aspartate (Figure 9). Thus the permeability of the teguments of human schistosome species to acidic amino acids may be modified in response to the copulative state in *S. mansoni* and *S. haematobium*, but these characteristics are not shared by *S. japonicum*.

CONCLUSIONS

The exchange of materials between paired male and female schistosome partners is not easily demonstrated. In the case of glucose, it is a rapid process, and it could be readily confirmed in three different human schistosome species. In these same schistosome species, a female situated in the gynecophoral canal has a negative effect upon glycogen reserves of the male partner; *Schaematobium* is unique in that the mate female affects an increase in tissue mass of the male partner (Figure 10). Most of the transferred glucose is converted to lactic acid by schistosomes, and the mechanism by which female schistosomes excrete lactate has not been defined. Does the paired female transfer lactate to the male partner, which is in turn externalized at the male dorsal surfaces? Alternatively, is lactate efflux accomplished by the osmoregulatory system of male and female schistosomes, and externalized at the excretory pores? Another topic worthy of further study would be to determine whether nutrients other than glucose may be transferred from male to female schistosomes. However, the present studies emphasize that caution should be exercised in analysis and interpretation of data obtained from in vitro investigations of separated male and female schistosomes. Furthermore, these conditions may not resemble in vivo, or paired, function. This caution should perhaps be applied in a wide range of disciplines (immunology, physiology, and pharmacology) involving study of surface phenomena where data are to be obtained in vitro from unpaired male and female schistosomes.

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PORTAL SERUM CONSTITUENTS
Possible Determinants for Anatomical Localization of *Schistosoma mansoni* During Maturation and Reproduction

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Abstract—Coupled adult pairs of *Schistosoma mansoni* were incubated in medium containing either peripheral or portal serum from rat, rabbit, hamster, or man, and egg production was measured daily. In all cases egg production was significantly increased for pairs in the presence of portal sera compared with that in the presence of peripheral sera. Fractionation of rabbit portal serum according to molecular weight demonstrated that the most active component(s) were in the range of 2000 to 50,000. Similarly, a rat portal serum fraction in the range of 2000 to 30,000 molecular weight was most stimulatory. These fractions were as effective in stimulating oviposition as whole portal serum. Conclusions: (1) portal serum factor(s) exist that stimulate *S. mansoni* oviposition in vitro; (2) they are present in susceptible and nonsusceptible hosts; and (3) the molecular weight range for the active components is larger than would be expected for simple carbohydrates, amino acids, or free fatty acids absorbed from the gastrointestinal tract.

Key Words—*Schistosoma mansoni*, portal serum, maturation, oviposition, reproduction.

INTRODUCTION

In susceptible hosts, the various species of schistosoma bear a remarkable predilection for particular anatomical sites (Cheever, 1965). Because of this phenomenon, the complications of schistosomiasis are generally limited to specific organ systems. In man, the preference of *Schistosoma hematobium* for the veins of the urinary system results in inflammation and ultimately fibrosis of the bladder and/or ureters (Warren, 1978a). Similarly, localization of *Schistosoma man-*

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soni and *Schistosoma japonicum* in the human portal–mesenteric venous system results in colonic and hepatic granulomatous disease with progressive hepatic fibrosis that becomes manifest as the morbid complications of portal hypertension (Warren, 1978b). From a teleological perspective, the localization phenomenon represents an advantageous evolutionary adaptation in that it simplifies the task of locating mates by bringing both sexes together in a relatively confined vascular region. In addition, deposition of eggs in these areas permits their elimination from the host for completion of the parasitic life cycle. The mechanism by which the parasite determines this site preference, however, is unknown.

In considering this problem, we noted the elegant series of studies by Miller and Wilson (1978, 1980), Wilson et al. (1978), and Knopf et al. (1983), who demonstrated that schistosomules of *S. mansoni* migrate through the venous and arterial vascular systems prior to their arrival in the portal–mesenteric system. In fact, it was determined that immature parasites make several passages through the pulmonary–systemic circulation before chance migration to the preferred site (Miller and Wilson, 1978). This occurs regardless of the site or mode of entry, e.g., percutaneous, subcutaneous, intrapleural, or intracardiac. When schistosomules were injected directly into the portal vein, the majority of the parasites remained at that site (Miller and Wilson, 1978). These data indicate that as a consequence of their migrations, the immature organisms are exposed to various regions of the circulatory system, and yet, they exhibit a definite preference for the portal–mesenteric venous system.

In addition, the work of Taylor et al. (1969), and Taylor (1971) showed that in single-sex infections, schistosomes also migrated to the portal–mesenteric venous system, indicating that each sex is capable of locating the preferred site independent of the other sex. Because blood draining to the portal vein is derived from the gastrointestinal tract, and therefore, different from peripheral blood in many respects (Ishikawa, 1975), we wondered whether the site preference of *S. mansoni* could be dependent on a constituent of portal blood that is not present in the periphery. This might take the form of a substance that the parasite recognizes or requires to develop or maintain a mature state.

We chose to test our hypothesis by measuring *S. mansoni* oviposition since reproductive capacity has been found to reflect the status of maturation in vitro and, furthermore, this feature is highly sensitive to the composition of the incubation media (Clegg, 1965; Newsome, 1962; Robinson, 1960; Senft and Senft, 1982; Michaels and Prata, 1968, Newport and Weller, 1982a,b).

METHODS AND MATERIALS

Animal Portal and Peripheral Serum. In our previous studies, we began by measuring oviposition of coupled pairs of *S. mansoni* incubated in medium

containing serum from different anatomical locations and from different hosts. Adult Sprague-Dawley male rats, adult golden hamsters, male and female, fed ad libitum, were anesthetized with ether. Male New Zealand White rabbits, also fed ad libitum, were anesthetized with a fentanyl and droperidol mixture. Portal blood was obtained by withdrawal into a syringe via a 20-gauge needle by direct puncture facing the mesenteric venous bed. Peripheral blood was obtained from the inferior vena cava. Whole blood was allowed to clot at 25°C for 10 min and then at 4°C for 1 hr. Serum was obtained by centrifugation at 3000 rpm at 4°C for 15 min.

Human Portal and Peripheral Serum. In collaboration with Drs. Michael Dunn and Refaat Kamel, human serum was obtained from an adult Egyptian male suffering from hepatosplenic schistosomiasis. Peripheral blood was drawn from the antecubital fossa and, after preoperative fasting for approximately 12 hr, the patient underwent a procedure for decompression of the portal vein during which portal pressures were measured by direct puncture and portal blood withdrawn through the catheter. The serum obtained was either used immediately or within 2 weeks of collection with storage at −70°C.

Assay for in Vitro Oviposition. Adult *S. mansoni* couples, Egyptian strain, were obtained by sterile perfusion (Duvall and DeWitt, 1967) from golden hamsters after seven weeks of infection with 100 cercariae each. Twelve coupled pairs were incubated, one pair per dish containing 2 ml of medium alone [RPMI 1640 with penicillin 100 units/ml and streptomycin 0.1 mg/ml plus 10% fetal calf serum (FCS)], medium plus 10% portal serum, or medium plus 10% peripheral serum. All media preparations were sterilized by filtration through a 0.20-μm membrane (Millipore Corporation, Bedford, Massachusetts). Incubations were carried out in 35-mm-diameter (35 × 10 mm) polystyrene dishes (Falcon Labware, Oxnard, California) at 37°C under 5% CO_2 in the dark. No microbial contamination occurred in any of the dishes.

Medium was changed and egg production was measured each day for five days by manual counting under a microscope. Repetitive experiments with duplicate dishes were counted for each preparation, and the results are expressed as means ±1 SD with statistical significance determined by two-tailed Student's *t* test. Microscopic examination demonstrated that all worms remained alive and coupled. By five days postdeposition, approximately 50% of the eggs contained motile embryos evaluated microscopically as evidence of viability during the experimental period.

Fractionation of Rabbit Portal Serum. Using a pressure dialysis chamber and filtration membranes (Amicon Corp., Danvers, Massachusetts), rabbit portal serum was fractionated according to molecular weight. Each fraction was washed with distilled water until UV absorption of the effluent as measured at 230 nm and 280 nm was identical to that of distilled water. Each fraction was concentrated by lyophilization and brought back to the original volume and isotonicity by addition of normal saline where necessary. To determine which

fraction contained the active component(s) of the portal serum, medium was prepared supplemented with each fraction to make a 10% solution. Prior to use, all preparations were sterile filtered as described above. Egg production was again measured microscopically with duplicate dishes for each medium preparation, and the results of repetitive experiments are expressed as the average ± 1 SD. Statistical significance was determined by Student's t test.

Recently, we have used a similar technique to separate fractions of rat portal serum and tested these fractions for oviposition stimulatory effect as described for the rabbit portal serum fractions.

RESULTS

Table 1 compares oviposition by coupled pairs of *S. mansoni* in media supplemented with sera from different sources. Regardless of the animal source, medium containing portal serum demonstrated a statistically significant ($P < 0.005$) enhancement of oviposition compared to medium prepared with peripheral serum. The ratio of total egg production of portal medium to peripheral medium was approximately 2.4 : 1 in the rat, 3 : 1 and 3.4 : 1 in man and rabbit respectively, and 11 : 1 for the hamster. Increases in the concentration of portal serum in the medium from 20% to 50% did not significantly increase egg production above that with 10% serum. Addition of rabbit peripheral serum to rabbit portal serum did not significantly change egg production (data not shown), indicating that the observed results are due to portal stimulation and not peripheral inhibition of oviposition.

Table 2 shows the effect of the different molecular weight fractions of rabbit portal serum on egg production: F1 <500; F2 500–2000; F3 2000–10,000; F4 10,000–50,000; F5 >50,000. Oviposition was maximally stimulated by fractions F3 and F4, indicating that the most active components of

TABLE 1. EFFECT OF PORTAL AND PERIPHERAL SERUM ON *S. Mansoni* OVIPOSITION IN VITRO[a]

Source	Portal	Peripheral	Significance[b]
Hamster	215 ± 25	19 ± 6	$P < 0.005$
Rabbit	357 ± 22	106 ± 16	$P < 0.005$
Rat	214 ± 32	90 ± 10	$P < 0.005$
Man	591 ± 37	195 ± 15	$P < 0.005$

Control (FCS 10%) 46 ± 5
Control (FCS 20%) 50 ± 6

[a] Egg production per coupled pair (for 12 pairs) mean ± SD, after five days of incubation.
[b] Statistical significance determined by two-tailed Student's t test.

TABLE 2. EFFECT OF RABBIT PORTAL SERUM FRACTIONS ON *S. mansoni* OVIPOSITION IN VITRO[a]

F1	F2	F3	F4	F5
152 ± 13	109 ± 10	266 ± 17	306 ± 22	21 ± 6

F1 <500
F2 500–2000
F3 2000–10,000
F4 10,000–50,000
F5 >50,000

[a] Egg production per pair (for 12 coupled pairs), mean ± SD after five days of incubation.

TABLE 3. EFFECT OF RAT PORTAL SERUM FRACTIONS ON *S. mansoni* OVIPOSITION IN VITRO[a]

F1	F2	F3	Control (FCS 20%)
77 ± 7	232 ± 13	64 ± 4	55 ± 10

F1 <2000
F2 2000–30,000
F3 >30,000

[a] Egg production per pair (for 10 coupled pairs), mean ± SD after five days of incubation.

rabbit portal serum are of molecular weight greater than 2000 and less than 50,000.

Table 3 shows the effect of different dialysis fractions of rat portal serum on egg production: F1 <2000; F2 2000–30,000; F3 >30,000. Fraction F2 showed the greatest stimulatory effect, three to four times that of the other fractions. This indicates that in the rat the stimulatory constituent(s) have a molecular weight greater than 2000 but less than 30,000.

DISCUSSION

The results of these experiments demonstrate that portal serum from various mammalian sources have components that stimulate *S. mansoni* oviposition in vitro. Significant enhancement of oviposition was seen with portal serum from man and hamster (highly susceptible hosts) (Cheever, 1965) as well as rat and rabbit (poorly susceptible hosts) (Stirewalt et al., 1957), indicating that the

active portal serum components provide the potential for preferential reproductive support regardless of the overall suitability of the animal as a host for *S. mansoni*.

Large differences are seen in egg production in medium containing peripheral serum supplements from various animal sources. In an early study Robinson (1960) also found large differences in egg production with peripheral sera from several animal species.

It has been clearly demonstrated that portal blood contains significantly greater quantities of nutrients (Ishikawa, 1975) compared to peripheral blood because of the drainage of blood from the gastrointestinal tract. Our data suggest that the active principles of portal serum are likely not to be amino acids, carbohydrates, or other simple metabolic substrates based on the effects of the fractions of rabbit portal serum. Our most recent data on rat portal serum fractions confirm the previous rabbit results and suggest that the substances are of similar size. It is possible that the same or similar substance is present in the rat as well as the rabbit. While an intermediate-sized molecule appears to be the most probable, the possibility of a low-molecular-weight substance that exists tightly bound to a macromolecule, as observed by Newport and Weller (1982a), must be considered in the isolation and identification of stimulatory substances.

Our results have not directly demonstrated an effect of portal serum component(s) on *S. mansoni* maturation or localization. We are aware that the observed enhancement of oviposition could be due to effects related to general metabolism and need not necessarily indicate that portal blood has a specific and direct effect on oviposition or on maturation. Nevertheless, we have demonstrated, for the first time, a difference in *S. mansoni* reproductive response in the presence of portal serum versus peripheral serum.

Although these studies are still quite preliminary, we are currently in the process of isolating and identifying the stimulatory substance(s) first from rat portal serum and ultimately from human portal serum. We plan to test the effects of portal serum components to determine if there are specific effects on the growth and maturation of immature *S. mansoni* schistosomula in vitro. In addition, we plan to examine sera from other important anatomical locations for effects on other schistosome species in order to determine if the phenomenon is parasite-species specific.

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LIMITATIONS TO SCHISTOSOME GROWTH AND MATURATION IN NONPERMISSIVE HOSTS

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Abstract—The life cycle of schistosomes is reviewed in its various steps, both in permissive hosts (in which the cycle is completed) and in nonpermissive hosts (which excrete no viable eggs as a result of the infection). A large worm loss occurs at (or after) the lung stage in both types of hosts ("normal attrition") and some nonpermissive hosts (like the rat) have an additional elimination of worms from the portal circulation. Worm growth and reproductive maturation are also impaired in several nonpermissive hosts and the possible host–parasite interactions leading to such limitations are discussed, with special reference to hormonal influences. Attention is also given to peculiar phenomena occurring in some hosts, like the late portal worm elimination in rhesus monkeys, the migration from mesenteric veins to lungs in rats, and the block to egg excretion in guinea pigs. The steps of the schistosome life cycle which appear vulnerable in several hosts are contrasted with the steps which are carried out successfully in the majority of hosts studied.

Key Words—Schistosomes, *Schistosoma mansoni*, permissive and nonpermissive hosts, reproductive maturation, hormones.

INTRODUCTION

The schistosomes which are responsible for human infections can also be infective for a variety of different mammalian hosts. The evidence is derived both from the occasional finding of naturally infected wild mammals and from the observation of schistosome development after controlled laboratory exposures to cercariae. Most of the available information on *Schistosoma mansoni* was

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obtained in the 1950s and early 1960s through an impressive series of studies designed to explore the suitability of a wide range of mammals as laboratory models for human infections (Moore et al., 1949; Stirewalt et al., 1951; Kuntz and Malakatis, 1955; Bruce et al., 1960; von Lichtenberg et al., 1962). The general picture emerging from these studies is that of a continuum of host behaviors, ranging from refractoriness to susceptibility, with a corresponding spectrum of schistosome stages at which parasite development and reproduction can be blocked. An operational distinction has been suggested for *S. mansoni* between "permissive" hosts, i.e., species in which the parasite can complete its life cycle, and "nonpermissive" hosts, i.e., species where development of worms is incomplete and/or no viable eggs can be isolated from the feces (Cioli et al., 1977; Knopf, 1982). Table 1 presents a list of the major permissive and nonpermissive species for *S. mansoni*. Such a dichotomy can be useful for broad descriptive purposes, but there are clearly borderline species, like the rhesus monkey, where the "self-cure" phenomenon observed under certain conditions may be suggestive of nonpermissiveness (Damian, 1984), whereas the presence of viable eggs in the feces seems to warrant at least the status of "temporarily permissive" to this host.

It would certainly be naive to think of directly applying to the human situation the striking limitations which nonpermissive hosts impose upon schistosome development, reproduction, and pathological effects. It may be fruitful, however, to look at each host–parasite combination as an *experimentum naturae* which can help dissect the schistosome biology by showing the multiple steps at which progression of the infection could be blocked. The subject has been recently reviewed by Knopf (1982), and Figure 1 is largely based on a scheme

TABLE 1. PERMISSIVE AND NONPERMISSIVE MAMMALIAN DEFINITIVE HOSTS FOR *S. mansoni*

Permissive	Nonpermissive
Man (*Homo sapiens*)	Guinea pig (*Cavia porcellus*)
Baboon (*Papio* spp.)	Skunk (*Mephitis nigra*)
Chimpanzee (*Pan* spp.)	Rabbit (*Oryctolagus cuniculus*)
Hamster (*Mesocricetus auratus*)	Norway rat (*Rattus norvegicus*)
Mouse (*Mus musculus*)	Raccoon (*Procyon lotor*)
Nile rat (*Arvicanthis niloticus*)	Cat (*Felis catus*)
Multimammate mouse (*Mastomys* spp.)	Muskrat (*Ondatra zibethica*)
Gerbil (*Gerbillus pyramidum*)	Nutria (*Myocaster coypus*)
Sheep (*Ovis* spp.)	Dog (*Canis familiaris*)
Goat (*Capra* spp.)	Fox (*Vulpes fulva*)
Calf (*Bos* spp.)	
Black rat (*Rattus rattus*)	
Rhesus monkey (*Macaca mulatta*)	

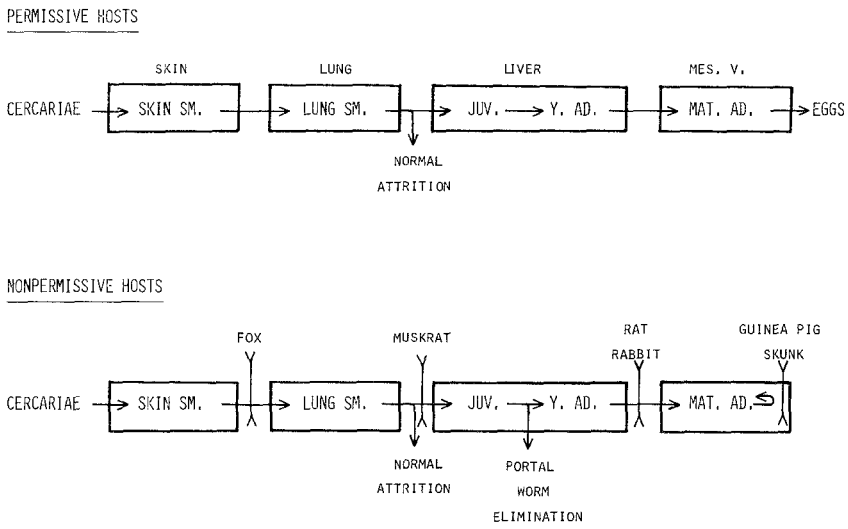

FIG. 1. Stages of *S. mansoni* life cycle which are blocked in nonpermissive hosts and in which worms are lost in permissive and/or nonpermissive hosts. Abbreviations: schistosomula (SM.); juvenile worms (JUV.); young adult worms (Y. AD.); mature adult worms (MAT. AD.); mesenteric veins (MES. V.).

proposed in this review. Although reference will be made to a variety of nonpermissive hosts of *S. mansoni*, most of the available information is derived from studies performed in the laboratory rat (*Rattus norvegicus*).

It may be appropriate, at this point, to mention that some instances of successful natural infection of *R. norvegicus* by *S. mansoni* have appeared in the literature. Thus, Martins et al. (1955) found that 46.8% of *R. norvegicus* captured in Belo Horizonte harbored *S. mansoni* and 13.3% of these rats passed viable eggs in the feces. Combes et al. (1975) reported that, out of a total of eight rats (four *R. rattus* and four *R. norvegicus*) captured in Guadeloupe, seven showed the presence of *S. mansoni* adults, of eggs in the intestine, and of eggs in the feces. Günther (1979) reported that, out of 38 *R. norvegicus* trapped in Egypt, six showed the presence of adult *S. mansoni*. He could also document that mature eggs were present in the liver, intestine, and feces; and was able in all six cases to obtain miracidia which were infective to snails. Our attempts to follow up on these observations with the original authors have not been successful, thus far. It would be quite interesting, however, if one could study a combination *R. norvegicus–S. mansoni* where the rat behaves as a permissive host. This might yield either a schistosome strain to compare with the commonly used ones, or a rat strain to cross with laboratory rats and ask whether permissiveness is a dominant or recessive trait (indicating that the presence or the absence of some factor, respectively, is responsible for permissiveness).

QUANTITATIVE ASPECTS OF PREADULT STAGES OF INFECTION

Skin and Lung Stages. The first step of the infection in mammalian hosts, i.e., skin penetration seems to show little selectivity in different species. Even in nonpermissive hosts, a large fraction of cercariae disappear from the infecting suspension (Warren and Peters, 1967), and there is histological evidence for the presence of schistosomula in epidermal and dermal layers of the skin (von Lichtenberg et al., 1962). In the fox, schistosomula have been documented in close proximity to skin blood vessels, but no evidence has been found of their penetration into these structures and no parasites have been found in the lungs (von Lichtenberg et al., 1962). The fox may thus represent an example of hosts in which the infection is effectively blocked at the skin level.

Experiments based on the recovery of schistosomula from minced skin have led to the conclusion that a significant fraction (10–30%) of penetrated cercariae die in the skin of permissive hosts such as the hamster and the mouse, whereas a larger fraction (50%) die in the skin of a nonpermissive host like the rat (Clegg and Smithers, 1968). The use of more recent techniques for the tracking of radioactively labeled schistosomula through the host tissues (Georgi, 1982), on the other hand, has convincingly demonstrated that, in the mouse, virtually all penetrated schistosomula leave the skin and can be quantitatively accounted for in the lungs (Mangold and Dean, 1983). Similar experiments performed in the rat have also shown that virtually no schistosomula remain in the skin of this host and that the number of lung radioactive foci is almost identical with the number of penetrated cercariae (Knopf, Cioli, Mangold, and Dean, manuscript in preparation). These results suggest that the nonpermissive characteristics of the rat are not displayed in the skin but only at later stages of the migration. It cannot be excluded, however, that schistosomula may actually suffer some early damage in the skin, but maintain their capacity to migrate and show only delayed effects when already in different tissues.

Lung-to-Liver Migration. The migration from lung to liver appears to be a critical stage in the *S. mansoni* life cycle. The muskrat may represent an example of nonpermissiveness where the block is exerted mainly at this stage (Figure 1), since von Lichtenberg et al. (1962) could find histological evidence for the presence of schistosomula in the lungs of this host, but not in the liver or in the mesenteric veins. In any event, it is interesting that, with respect to the migration from lung to liver, not even the mouse can be considered 100% permissive. Dean et al. (1984) have shown that, while essentially all penetrated schistosomula appear as radioactive foci in the lungs of mice, only about half of them eventually appear in the liver or in the mesenteric veins. The fate of the missing schistosomes is, as yet, totally unknown, but it would appear that they either disintegrate quite rapidly and/or are lost from the mouse tissue. In the rat, as in the mouse, about half of the parasites which appear in the lungs fail to reach the liver (Knopf et al., manuscript in preparation). Thus, it appears

that the "normal attrition" observed in permissive hosts after the lung stage is present in the rat with substantially similar characteristics (Figure 1).

Portal Worm Elimination ("Self-Cure"). After reaching the liver, the number of parasites will remain essentially constant throughout the rest of the infection in permissive hosts. In the rat, on the contrary, a further worm elimination will occur at the liver stage, beginning on the fourth week after infection ("self-cure"). A dramatic reduction in the number of worms which can be recovered by portal perfusion occurs between the fourth and the sixth week, followed by a slower rate of elimination. From the tenth week onward a very small number of schistosomes—generally corresponding to less than 2% of the infecting cercariae—is left in the rat. These surviving schistosomes are stunted, do not migrate to mesenteric veins, and produce limited numbers of infertile eggs. Their survival, however, can be very long-lasting: a mean of 5.6 worms per rat has been reported one year after infection with 1000 cercariae per rat (Phillips et al., 1978).

One of the possible interpretations of the portal worm elimination in rats is to assume that it is due to an acquired host immune response which becomes effective at the fourth week after infection (Smithers and Terry, 1965). Indeed, thoroughly immunosuppressed rats showed higher numbers of worms than intact controls at all times tested (Cioli and Dennert, 1976). However, portal worm elimination eventually occurred in immunosuppressed rats as well, and worms recovered from these animals were as stunted as those from intact rats, females did not achieve maturation, egg deposition remained abortive, and worm pairs failed to migrate from liver to mesenteric veins. Thus, it is possible that immunological phenomena may be restricted to the final phases of killing and disposing of worms which have been previously affected by some other non-immune mechanism.

Additional evidence against the hypothesis that portal worm elimination is primarily an immune phenomenon comes from a series of experiments designed to determine whether the timing of spontaneous rejection (four weeks) is due to the fact that the rat needs just about four weeks to mount an efficient immune response, or whether this timing is dictated by factors inherent to worm development. Schistosomes obtained from rats which had been infected two, three, or four weeks previously were transferred into the mesenteric veins of normal rats and the timing of worm elimination was determined in the recipients. Two-week-old worms were rejected beginning at two weeks after transfer, 3-week-old worms at one week after transfer, and 4-week-old worms immediately after transfer. It was concluded that the onset of *S. mansoni* rejection in laboratory rats is dependent on the total age of the parasites and independent of the length of contact with the host in which rejection occurs (Cioli et al., 1978). It looks as though around the fourth week schistosomes either have some special requirement which is not fulfilled in the rat, or become sensitive to some inhibitory factor which was not effective at previous stages.

As previously mentioned, a very small number of schistosomes do survive for a long time in the rat, although they never develop into mature adults. The question was raised as to whether the few survivors were genetically different from the rest of the population. Upon transfer of the survivors into permissive hosts, it was possible to obtain progeny of those worms and use them to infect new rats. Although this cycle was repeated eight times, there was no indication that these "selected" schistosomes had a better survival or a better development in the rat (Cioli, to be published). This result makes the genetic hypothesis a very unlikely one and leaves us with two unsolved questions: why are the majority of schistosomes eliminated from the rat, and why are just a few of them capable of long-term survival?

According to a classical hypothesis, schistosomes can survive for long periods of time in permissive hosts because they are disguised under a coat of "host antigens" (Smithers et al., 1969). When rat schistosomes were analyzed with respect to the host antigens, they were found to possess only a very small amount of these antigens, in comparison with the amount present in mouse worms (Cioli, 1976a). However, it is difficult to decide whether the scarcity of host antigens is a primary cause of stunting and death or whether it is a consequence of some other damage to the surface membrane resulting in poor fixation of host antigens. It is indeed shown that the tegument of rat schistosomes undergoes a delayed and incomplete development (Senft et al., 1978).

QUALITATIVE ASPECTS OF PREADULT STAGES OF INFECTION

Up to the fourth week after infection, schistosomes recovered from the rat are indistinguishable from those recovered from mice or hamsters with respect to size distribution, surface morphology, and gross internal appearance (Mackenzie and Clegg, cited by Smithers, 1972; Knopf, 1982). After this point, however, several differences become apparent (Cioli et al., 1977). The majority of rat schistosomes remain at the juvenile stage, fail to grow in size, and are largely eliminated by the mechanism of portal worm elimination. The survivors progress very slowly to the young adult stage and, although the pairing process occurs, worm length lags far behind the values observed in permissive hosts and vitelline gland development in the female is only rudimentary. Paired young adult worms fail to migrate to mesenteric veins and deposit only a moderate number of eggs which are confined to the liver. Only rarely do miracidia emerge from these eggs, but snail infections cannot be obtained from them.

It has been shown that the arrest in development observed in the rat is not an irreversible phenomenon, since complete progression to mature, fertile-egg laying adults can be obtained if rat worms are transplanted—at any stage—into a permissive host (Cioli, 1976b; Cioli et al., 1977). However, the limitation imposed by the rat does not consist only of an arrest in the progression of the

schistosome development from immature to mature stages. The rat will also prevent the maintenance of the adult stage, as shown by the rapid regression of fully developed mouse worms after transfer into rats (Cioli et al., 1977).

Schistosome Reproductive Maturation in Nonpermissive Hosts. It is commonly stated that an essential prerequisite for the reproductive development of female schistosomes consists of their pairing with male worms. The rat situation shows that pairing is essential but not sufficient, since—as previously mentioned—there is no evidence for a deficit in the pairing process in the rat (nor in other nonpermissive hosts), yet paired females possess a largely undeveloped reproductive system. We shall consider three possibilities for the failure of female worms to develop in the rat: (1) The male schistosome is defective in this host, thus failing to supply the stimulus necessary for reproductive female maturation. (2) Migration to mesenteric veins is required for female development. (3) The lack of essential chemical factors or the presence of inhibitory factors make the rat environment unsuitable for female maturation.

Experiments aimed at distinguishing between these possibilities are under way in our Rome laboratory. Our approach consists in exploiting the fact that the rabbit is a permissive host for *S. japonicum*, whereas it is nonpermissive for *S. mansoni* (more or less like the rat, Figure 1). We have performed preliminary experiments showing that in the Nile rat (which is permissive for both *S. mansoni* and *S. japonicum*) immature females of either species are brought to full reproductive development and oviposition by the heterologous male. By performing the hybrid crosses in the rabbit, we should be able to determine whether the *S. mansoni* female will be able to undergo growth, reproductive development, migration, and oviposition in a nonpermissive environment, i.e., we should be able to see whether an efficient male stimulation, possibly coupled with migration to mesenteric veins, is capable of overcoming whatever humoral deficiencies or inhibitions may exist in the rabbit for the *S. mansoni* female.

INFLUENCE OF HOST HORMONES

In the search for host factors which may be responsible for the nonpermissive status of the laboratory rat, a series of experiments on the effect of host endocrine gland removal have been performed (Knopf and Soliman, 1980; Knopf and Linden, 1985). The rationale for this approach was based upon the observation that worm development in rats was significantly retarded at the time when sexual maturation of the parasites occurs (26–28 days postinfection). The potential for an interaction between host hormones and parasites which might either stimulate worm sexual maturation in permissive hosts or interfere with this process in nonpermissive hosts was examined by infecting hypophysectomized (Hypox) mice and rats. Worm survival, development, and oviposition were not significantly altered in Hypox mice. In contrast, Hypox rats showed a two-

week delay in portal worm elimination, worm length was increased by 30%, vitelline gland staining was markedly improved in females, and oviposition was augmented. Further experiments showed that a similar increase in permissiveness could be obtained in thyroidectomized (Thyrox) rats: in these hosts, portal worm elimination was delayed by four weeks, worm length was increased by 80%, and some miracidia could be hatched from the liver eggs (Knopf and Soliman, 1980). These "rat-derived" miracidia from Thyrox rats successfully infect snails and generate cercariae which are capable of completing the parasite life cycle in mice (Knopf and Linden, 1985). Confirmation of the involvement of thyroid hormones in these phenomena has been recently obtained by demonstrating that administration of either T3 or T4 to Thyrox rats restores the portal worm elimination reaction (Knopf and Linden, 1985). Unexpectedly, the low number of worms which survived in hormone-restored Thyrox rats continued to sexually mature and produce increased numbers of eggs. These eggs failed to yield miracidia, however.

Since cell-mediated immune responses are impaired by thyroidectomy (Knopf, 1982), it is possible that the delay in onset of portal worm elimination in Thyrox rats may be due to a reduced activity of the immune mechanisms which, at least in part, contribute to worm elimination in these nonpermissive hosts. A direct inhibitory effect of thyroid hormones T3 and T4 on the maturation of schistosomes seems less likely. T3, incidentally, is included among the constituents of the medium formulated by Basch (1981) to optimize growth of *S. mansoni* in in vitro cultures. Other activities of the thyroid gland must be considered as potential candidates for inhibiting parasite maturation.

MIGRATION FROM LIVER TO MESENTERIC VEINS

This phase of migration is absent in the rat and the rabbit, but is present in other nonpermissive hosts like the skunk and the guinea pig (Figure 1). While most of the previous migration of schistosomes can be imagined to be largely passive (the worms being carried by the bloodstream), this phase represents a displacement in which the schistosome must engage in active movements to negotiate its way against the blood flow down the portal vein and the progressively smaller and smaller branches of mesenteric veins. The migration of paired worms from the liver is even more intriguing in the case of *S. hematobium*, and an interesting way of restating the problem could be to ask: what are the differences between the two schistosome species that guide them to such different final locations? It is in this final portion of the migration, therefore, that it becomes almost compelling to postulate the existence of some cue leading the schistosome to its final location. A concentration gradient of some chemical is usually hypothesized at this point: nutrients from intestinal absorption, oxygen, insulin, serotonin are all factors in which portal blood is particularly rich, but no evidence exists for a special role in worm migration of any of the above. A

fecal extract has been shown to exert some attraction on schistosomes in vitro (Awwad and Bell, 1978), but the possible relevance of this finding to the in vitro situation remains to be determined.

A prerequisite for migration to mesenteric veins seems to be represented by pair formation. In single-sex infections of the mouse, both the single males and the single females are confined almost exclusively to intrahepatic vessels and the main branch of the portal vein (Zanotti et al., 1982). In the rat, however, pairing occurs, but pairs fail to migrate. Sexual maturation, pairing, and onset of oviposition are all important events which roughly coincide with migration to mesenteric veins. Again, the cause–effect relationships (if any) between these phenomena are totally obscure. Thus, it might be that rat worms do not mature because they fail to reach the mesenteric veins, or it might be that they do not migrate because they are not mature (but mature mouse worms do not seem to migrate back to mesenteric veins after surgical transfer into the portal circulation of the rat). In any event, one would like to know which features make the rat different from permissive mammals with respect to the final schistosome migration: lack of chemical cues, presence of inhibitory factors, cumulated defects in worm development?

It may be worth stressing here that the migration from liver to mesenteric veins in permissive hosts is not done once and for all when the newly mature schistosomes first pair around the fourth week of infection. Quite convincing evidence has been derived from oogram studies that each schistosome pair may go up and down the portal system several times a day, depositing eggs in rather distant branches of intestinal vessels (Pellegrino and Coehlo, 1978; Cheever and Duvall, 1982). The same conclusion can be reached by transferring a single mature worm pair into the mesenteric veins of Nile rats (Cioli, unpublished). It is tempting to speculate that the stimulus to continuous migration might come just from the eggs deposited in a given site. Secretions from the eggs (or from the host tissues damaged by eggs) might stimulate schistosomes to move away from that site, a concept which is the reverse of the possible attraction of schistosomes towards a given site. The initial stimulus could come from the first eggs deposited in the liver at the onset of oviposition; if the abnormal, infertile eggs produced in the rat fail to give off the proper stimulus for migration, this might explain the failure of schistosomes to move away from the liver. Whatever the cause for the permanence of worms in the rat liver, this is certainly a dangerous location for a schistosome, since it has been shown that many parasites can be found dead or "trapped" by eosinophil-rich reactions within the blood vessels (Knopf, 1979).

LATE EVENTS IN MESENTERIC VEINS

As outlined in Table 1, while *Rattus norvegicus* (i.e., the laboratory rat) is a nonpermissive host, *Rattus rattus* (black rat or roof rat) is permissive for

S. mansoni. This latter host has been shown to present a peculiar further migration of schistosomes from the mesenteric veins to the lungs (Jourdane, 1978). This migration occurs after the seventh to eighth week of infection, and it has been proposed that adult worms may pass from the portal blood to the caval circulation by directly negotiating their way through the liver sinusoids (Imbert-Establet, 1980). A similar localization of adult *S. japonicum* in the lung of *Rattus rattus mindanensis* has been reported by Oshima et al. (1978), although in this case it has been suggested that this may not represent migration from mesenteric veins but rather a permanent settling in the lungs during the initial period of infection.

Another peculiar phenomenon occurs late in the infection of rhesus monkeys with *S. mansoni*. When this host is infected with a high dose of cercariae, a drastic reduction in the number of worms is apparent around the 16th week (late portal worm elimination), whereas the worm burden seems to remain indefinitely constant after low-dose infections (Cheever and Powers, 1972). It is not known whether the spontaneous worm loss occurring around the 16th week in the rhesus monkey is somehow related to the portal worm elimination occurring at four weeks in the rat, and equally unknown are the mechanisms of this most interesting phenomenon.

Mature adult schistosomes in mesenteric veins are actively engaged in egg production. In permissive hosts, a proportion of the eggs are capable, upon maturation, of passing into the feces. In some nonpermissive hosts (like the guinea pig and the skunk, Figure 1) this last step is blocked. Eggs are deposited in the intestinal tissues of the guinea pig and about half of them are viable, but they are not voided in the feces (Pearce and McLaren, 1983). However, passage of eggs into the feces has been documented in the guinea pig after transfer into this host of hamster-derived 8-day-old worms, an intriguing finding which seems to indicate that the nonpermissive effects of the guinea pig are exerted on worm development rather than against the egg itself (Michaels, 1970).

Apart from the many individual steps of the schistosome life cycle which may receive clarification from the study of single nonpermissive hosts, a very broad generalization may emerge from an overall view of the data available so far. It looks as though the survival mechanisms evolved by the schistosomes have enabled them to overcome certain of the obstacles encountered in their life cycle with remarkably good success in a wide variety of mammalian hosts. Thus, for example, skin penetration and male–female pairing appear to be performed very efficiently in all the hosts studied. Certain other steps of the life cycle, on the contrary, seem to encounter serious problems in several host species. Thus, migration from lung to liver is effected with great losses even in permissive hosts, migration from liver to mesenteric veins often fails, full growth and maturation are achieved only in certain cases, and some hosts are even capable of getting rid of established fertile schistosomes. It is totally up to the ingenuity of the applied biologists whether they prefer to exploit those steps

which appear most vulnerable in natural hosts, or whether they prefer to discover new weak links in the elaborate mechanism of schistosome survival.

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IDENTIFICATION OF SEX-LINKED ANTIGENS OF *Schistosoma mansoni* BY IMMUNOELECTROPHORESIS AND IMMUNOBLOTTING

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Abstract—Immunoelectrophoresis and immunoblotting techniques, using sera from hyperimmune rabbits and infected mice, were used to analyze antigens of *Schistosoma mansoni*. A comparison was made between the antigenic composition of adult male and female worms, isolated from monosexually and bisexually infected hamsters. A large number of female-specific and a few male-specific antigens were detected. The antigenic composition of females isolated from monosexually infected hamsters was shown to be very different from that of paired females. The presence of a major female-specific antigen with an apparent molecular weight of 32 kd in paired females only, suggests that synthesis of this antigen by female worms is initiated by the male partner. Analysis of the humoral immune response of mice receiving bisexual or monosexual infections showed that both mono- and bisexually infected mice develop a major humoral immune response to a 36-kd polypeptide antigen. A response to a major polypeptide antigen with an apparent molecular weight of 60 kd was observed in bisexually infected mice only. No antibodies reactive with this 60-kd polypeptide are formed during monosexual infections with either male or female worms. The 60-kd polypeptide was, however, present in monosexually reared females. This presence, and the presence in bisexually reared males, suggests a transfer of this antigen from females to males.

Key Words—*Schistosoma mansoni*, trematode, sex-linked antigens, monosexual and bisexual infections, immunoelectrophoresis, immunoblotting, rabbits, mice.

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INTRODUCTION

Granuloma formation around eggs is the major factor in the pathogenesis of schistosomiasis. The granuloma formation is succeeded by fibrosis, which causes obstruction to blood or urine flow. The occurrence of disease following schistosomal infection is related to the number of schistosome eggs in the tissues and the degree of the host's inflammatory response to them (Warren, 1975). The pathology would be minimized if the rate of oviposition could be substantially reduced. This might be accomplished by preventing the females from mating with the males.

There have been numerous investigations on the influence of mating on the sexual maturity of the females (Vogel, 1941; Armstrong, 1965; Erasmus, 1973; Shaw et al., 1977; Popiel and Basch, 1984). In monosexual infections of *Schistosoma mansoni*, female worms fail to develop normally. Growth is inhibited, and the development of the reproductive system is incomplete. Separation of sexually mature females from their male partners leads to a reversible degeneration of the female reproductive tract, and the production of viable eggs is terminated (Clough, 1981).

The mechanism by which the male matures the female is unknown. It has been postulated that female maturation is accomplished nutritionally, hormonally, tactually, and by sperm or components excreted with the sperm (Vogel, 1941; Armstrong, 1965; Moore et al., 1954). However, none of these postulations has been proven. Shaw et al. (1977) have shown that acetone or ether extracts of male worms can stimulate vitelline gland development. These experiments suggest that transfer of male-specific components during residence of the female in the gynecophoral canal of the male is an important factor in the development of the female reproductive system.

If sexual maturation and oviposition are dependent on transtegumental transfer of one or more male components to the female, it might be possible to prevent this transfer by the action of antibodies specific for the transfer component. Sex-linked components might also be part of postulated receptors, which are used to determine mating (Michaels, 1969). Antibodies to these receptor molecules might prevent mating and, as a result, stop egg production.

Comparative analysis of male and female *S. mansoni* proteins has been made mainly by electrophoretic techniques (Ruppel and Cioli, 1977; Cordeiro and Gazzinelli, 1979; Snary et al., 1980). To exclude contaminating host proteins, schistosome proteins have been metabolically labeled in vitro with radioactive amino acids (Atkinson and Atkinson, 1982; Aronstein and Strand, 1983). Atkinson and Atkinson (1982) used isoelectric focusing/SDS-polyacrylamide 2-dimensional gel electrophoresis to separate radiolabeled male and female proteins. Fluorographic analysis of separated peptides revealed the synthesis of 74 major polypeptides, among which were three male- and four female-specific

polypeptides. Atkinson and Atkinson (1980) also showed that a polypeptide with a molecular weight of 66 kd, synthesized by males, is transferred to female worms. However, other investigators were unable to detect transfer of a 66-kd polypeptide (Popiel and Basch, 1984). Aronstein and Strand (1983) showed that females cultured in vitro excrete a 66-kd polypeptide and a number of low-molecular-weight polypeptide antigens.

Only a few studies have been made on the antigenic properties of male- and female-specific components. Capron and Vernes (1968) studied the formation of specific antibodies in mono- and bisexually infected hamsters by immunoelectrophoresis. Precipitation patterns, obtained with male and female antigen preparations, showed no distinct differences. Rotmans et al. (1981) have reported the excretion of a low-molecular-weight female-specific antigen during cultivation of adult female *S. mansoni* in vitro. The presence of this antigen in the culture medium was demonstrated by immunoelectrophoresis using a rabbit antiserum directed against excretory and secretory antigens.

In this paper we report further studies on the analysis of sex-linked *S. mansoni* antigens by immunoelectrophoresis and the use of the immunoblotting technique to select potential target antigens for antibodies interfering with mating or the production of eggs.

METHODS AND MATERIALS

Collection of Parasites. Adult *S. mansoni* were collected from golden hamsters by means of perfusion with a sterile Dulbecco's balanced salt solution (DBSS), 48 days after exposure of the hamsters to 1000–1500 cercariae of mixed sex. Collected worms were washed twice with DBSS. For the most part, worms were used for antigen extraction. A smaller part of the worm pairs were allowed to separate by keeping the worms for 30 min at room temperature in the perfusion medium. Male and female worms were collected separately by manual transfer of individual worms to Petri dishes, using a Pasteur pipet. This procedure allowed the isolation of about 1500 worms of each sex in a 1-hr period. For the isolation of worms of single sex from monosexually infected hamsters, snails (*Biomphalaria glabrata*) were infected with a single miracidium. Each hamster was exposed to cercariae released by one infected snail and, 48 days postexposure, worms were collected by means of perfusion. The sex of the worms obtained from each hamster was determined, and male and female worms were collected separately in perfusion medium.

Antigen Extraction. About 1500 male, female, or mixed worms were homogenized in 15 ml phosphate-buffered saline (PBS) and centrifuged at 30,000*g* for 20 min. Centrifugation at this low *g* value, resulted in a preparation consisting of both water-soluble and membrane-bound antigens. The supernatant was dialyzed for 16 hr against deionized water at 4°C, lyophilized, and stored

at 4°C. For the purpose of immunizing rabbits with an antigen preparation as complete as possible, we extracted membrane-bound antigens from the pellet with a detergent. The pellet was suspended in a 1% solution of octyl-β-D-glucopyranoside (Calbiochem) in 35 mM PBS, pH 7.8. Homogenization was in a glass homogenizer with a Teflon pestle. After centrifugation of the extract at 30,000*g* for 20 min, the supernatant was dialyzed against deionized water and lyophilized.

For immunoblotting experiments, intact whole worms were also extracted in the electrophoresis sample buffer. Extraction was by homogenization in a glass homogenizer with a Teflon pestle. The homogenate was centrifuged (10,000*g*, 1 min) and the supernatant was either immediately subjected to SDS gel electrophoresis or stored at −20°C.

Protein Determinations. Protein was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

Sera. Rabbit antisera directed against male, female, and total worm antigen preparations were prepared as follows: One milliliter of a solution containing 1.0 mg antigen preparation was mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously. After three weeks, booster injections of 1.0 mg antigen preparation in 1.0 ml PBS were given weekly. Serum was obtained five weeks after the first injection and from then on every other week.

Infected mouse sera were obtained from pooled bleeding of mono- or bisexually infected Swiss mice 16 weeks after exposure to 100 cercariae.

Immunoelectrophoresis. Glass slides (7.5 × 2.5 cm) covered with 3 ml of a 0.95% (w/v) agarose gel (Koch-Light) in 0.1 M Veronal buffer, pH 8.2, were used for immunoelectrophoresis. Wells contained 1 mg antigen preparation dissolved in 10 μl Veronal buffer. Electrophoresis was carried out for 3 hr at 55 V and 3.5 mA per slide. After application of 100 μl of a threefold concentrated antiserum, the slides were placed in a moist chamber for 48 hr. At the end of this period, the slides were washed for 48 hr in PBS, and twice for 1 hr with deionized water. The slides were then dried and stained with Amido black 10B.

Polyacrylamide Gel Electrophoresis. One-dimensional gel electrophoresis was conducted essentially as described by Laemmli (1970). Homogeneous slab gels of 12% polyacrylamide or gradient slab gels of 8–15% polyacrylamide, containing 0.1% SDS, were overlaid with a 4.5% polyacrylamide stacking gel containing 0.1% SDS, inset with sample wells. Equal amounts of protein were dissolved in sample buffer at a protein concentration of 2.5 mg/ml. The samples were heated for 2 min at 100°C. The sample buffer contained 63 mM Tris HCl, pH 6.8, 3.3% SDS, 10% glycerol, and 2% 2-mercaptoethanol. Before applying the samples to the wells in the stacking gel, bromophenol blue was added to a final concentration of 0.05%. Electrophoresis was performed in a Pharmacia GE-2/4 LS electrophoresis apparatus at 15–20 mA per slab gel for approximately 15 hr, or until the tracking dye reached the bottom of the gel. Molecular

weight standards were phosphorylase b (M_r 94 kd), Albumin (M_r 67 kd), ovalbumin (M_r 45 kd), carbonic anhydrase (M_r 30 kd), and trypsin inhibitor (M_r 20.1 kd).

Immunoblotting. Separated polypeptides were blotted onto a nitrocellulose membrane (Schleicher und Schuell) essentially according to the procedure described by Towbin et al. (1979). A 25 mM Tris, 192 mM glycine buffer, pH 8.8, containing 20% (v/v) methanol, was used in a commercial blotting apparatus (Bio-Rad). Transfer was at 30 V for 15 hr.

The immunochemical detection of blotted polypeptide antigens was performed as described by Tsang et al. (1983). The blots were first soaked in PBS, containing 0.3% Tween-20 for 1 hr at room temperature. The blots were then washed twice for 30 min with PBS containing 0.05% Tween-20 and incubated with the appropriate antiserum. A mixture of rabbit antisera directed against water-soluble and membrane-bound male and female antigens was used to analyze sex-linked antigens, and mouse antisera from mono- and bisexually infected mice were used to study the immune response in mice. Control blots were incubated with negative rabbit and mouse serum. The rabbit sera were diluted 1:800 and the mouse sera 1:25. Incubation was for 90 min at room temperature. The blots were then washed four times with PBS containing 0.05% Tween-20 and incubated with a peroxidase-conjugated goat anti-rabbit IgG (Nordic Immunological Laboratories) or a peroxidase-conjugated sheep anti-mouse IgG (Institut Pasteur). Conjugates were diluted 1:1000 and incubated for 1 hr at room temperature. Once again the blots were washed with PBS, containing 0.05% Tween-20, and finally rinsed in PBS without Tween 20. Staining was in PBS, containing 1.3 mM 3,3′-diaminobenzidine (Fluka), 1.4 mM 4-chloronaphthol (Merck), and 0.012% hydrogen peroxide (Merck). Color development was stopped by washing with deionized water. The stained blots were dried protected from light.

RESULTS

Analysis of male and female antigen preparations was performed by immunoelectrophoresis. Figure 1 shows the results obtained with an antiserum prepared by immunization of a rabbit with male antigen preparations. The antigen preparations used in the middle electropherograph were prepared from male and female worms isolated from bisexually infected hamsters. Several male-specific or predominantly male antigens gave precipitation lines. These lines are indicated by figures (number 1–5). The major male-specific precipitation line is shown as number 2. Line number 3 represents an antigen, which seems to be more abundant in males than in females, and lines number 1, 4, and 5 are exclusively obtained with the male antigen preparation. The female antigen preparation gave only one female specific precipitation line, which is

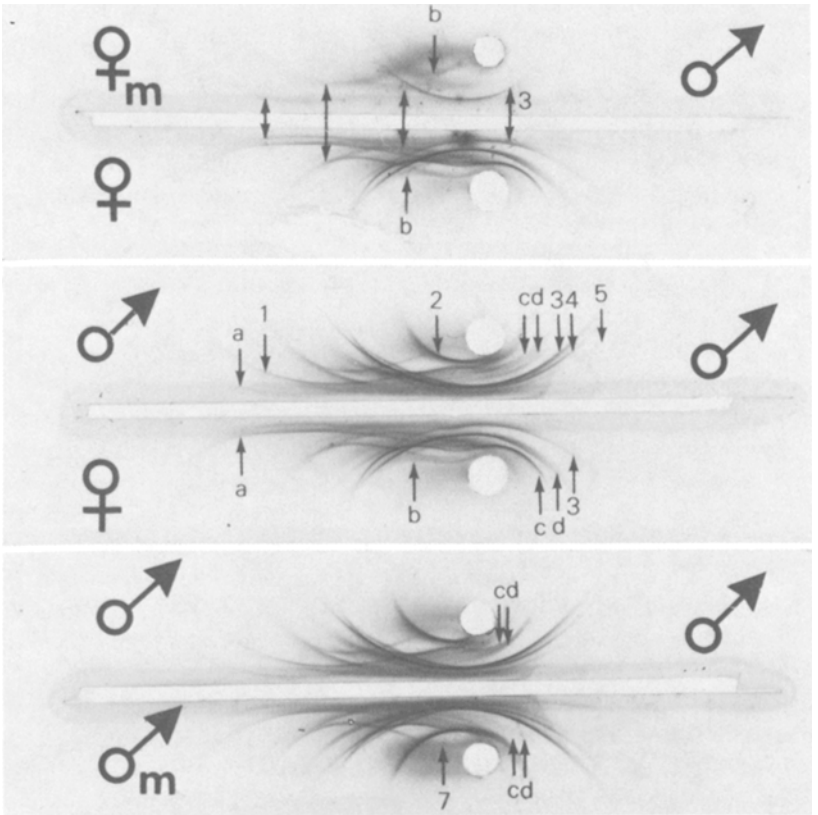

FIG. 1. Immunoelectrophoresis of antigens from bisexually reared males (♂), bisexually reared females (♀), monosexually reared males ($♂_m$), and monosexually reared females ($♀_m$) against serum from a rabbit immunized with antigens from bisexually reared male worms. Male-specific precipitation lines are indicated with numbers, female-specific lines with letters.

indicated by the letter b. Lines indicated by letters a, c, and d represent antigens which are more abundant in females than in males.

We also used antigen preparations obtained from monosexually reared male and female worms (upper and lower immunoelectropherographs of Figure 1). The antigen preparation from monosexually reared males gave a precipitation pattern very similar to that obtained with the preparation from bisexually reared males. A faint additional precipitation line, indicated by number 7, was obtained. The predominantly female antigens c and d, which could be observed in the preparation from bisexually reared males, are nearly absent in the preparation from monosexually reared males. A drastic reduction in the number of

precipitation lines was observed when a preparation from monosexually reared females was used. The presence of precipitation line number 3, which contains antigens predominantly present in the males, was very remarkable.

In a parallel immunoelectrophoresis experiment, we used an antiserum prepared by immunization of a rabbit with the preparation from bisexually reared females. Again, antigens extracted from monosexually and bisexually reared worms were analyzed. Surprisingly, another male-specific antigen was found (Figure 2, line 6). This antigen was present in bisexually reared males only, and not in males from monosexually infected hamsters. Two of the antigens, which with the "anti-male" serum appeared to be male specific, were found

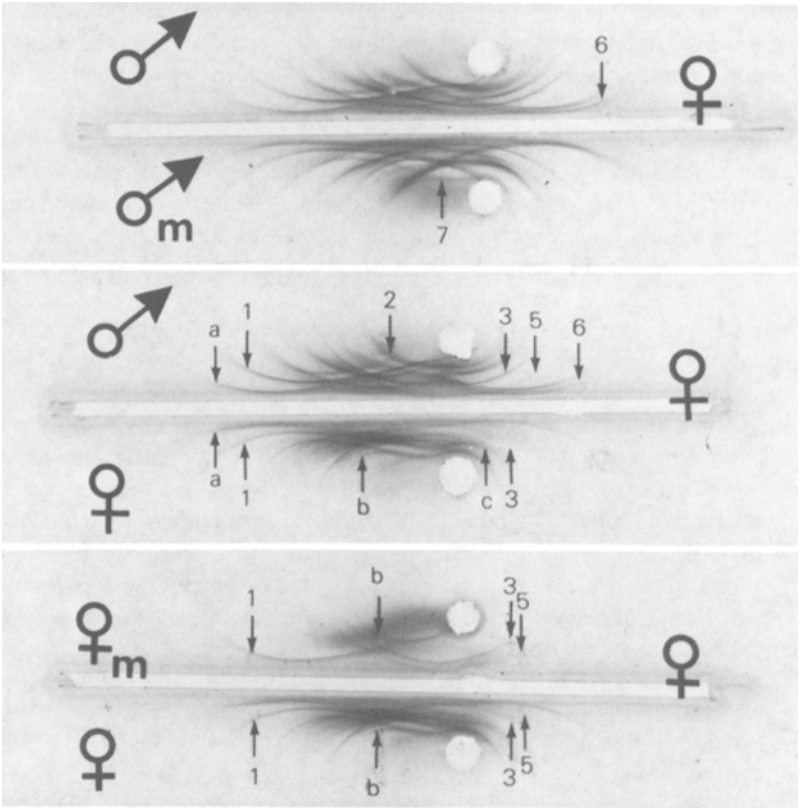

FIG. 2. Immunoelectrophoresis of antigens from bisexually reared males (♂), bisexually reared females (♀), monosexually reared males ($♂_m$), and monosexually reared females ($♀_m$) against serum from a rabbit immunized with antigens from bisexually reared female worms. Male-specific precipitation lines are indicated with numbers, female-specific lines with letters.

both in male and female antigen preparations (numbers 1 and 5). Similarly, an antigen previously found to be female-specific was also found now in both antigen preparations.

We also immunized rabbits with preparations from male and female worms reared in monosexually infected hamsters. The specificities of the antisera obtained after immunization of rabbits with the antigen preparations from the monosexually and bisexually reared males were very similar. However, immunization with a preparation from monosexually reared females gave very different results. None of the rabbits hyperimmunized with this "mono-female" preparation produced antibodies which gave precipitation lines with any of the male or female antigen preparations.

Antigen preparations extracted from males and females from mono- and bisexually infected hamsters were also analyzed by the enzyme-linked immunoelectrotransfer blot technique. Rabbits, used to prepare antisera, gave different humoral immune responses when immunized with male or female antigen preparations. However, considerable differences in response were also observed when different rabbits were immunized with the same antigen preparation. Identification of sex-linked antigens by immunoblotting is obviously only feasible when male and female preparations are compared using a single rabbit antiserum. To obtain a large diversity in reactivity towards male and female antigenic polypeptides in a single antiserum preparation, we mixed several rabbit antisera. These antisera were directed against water-soluble and detergent-extracted male and female antigens. A selection was made of those antisera containing antibodies reactive on blots with the largest number of antigenic polypeptides. Antigen preparations analyzed in the immunoblotting experiment were extracted from separated bisexually reared males and females as well as from monosexually reared males and females. Moreover, whole male and female worms were extracted with sample buffer and directly applied to the polyacrylamide slab gel. In order to subject the same amount of antigen preparation to each sample well, protein determinations were performed on all samples.

Figure 3 shows that a large number of antigenic polypeptides were detected in each of the six preparations. A considerable similarity in patterns was observed. Major antigens with an apparent molecular weight of 20, 24, 40, 45, 51, 92, and 150 kd were observed in all preparations. Many more female- than male-specific polypeptides were observed. Major female-specific polypeptide antigens with an apparent molecular weight of 32, 37, 60, and 66 kd were found. Control blotting experiments with egg antigens showed that the female-specific antigens did not originate from eggs. Only one male-specific polypeptide was detected. This polypeptide (M_r 33 kd) is present in the SDS extract of whole male worms. Two high-molecular-weight polypeptides (M_r 120 and 130 kd) were predominantly present in the male preparations.

The preparation from monosexually reared female worms contained several antigenic polypeptides not present in any of the other preparations (M_r 30,

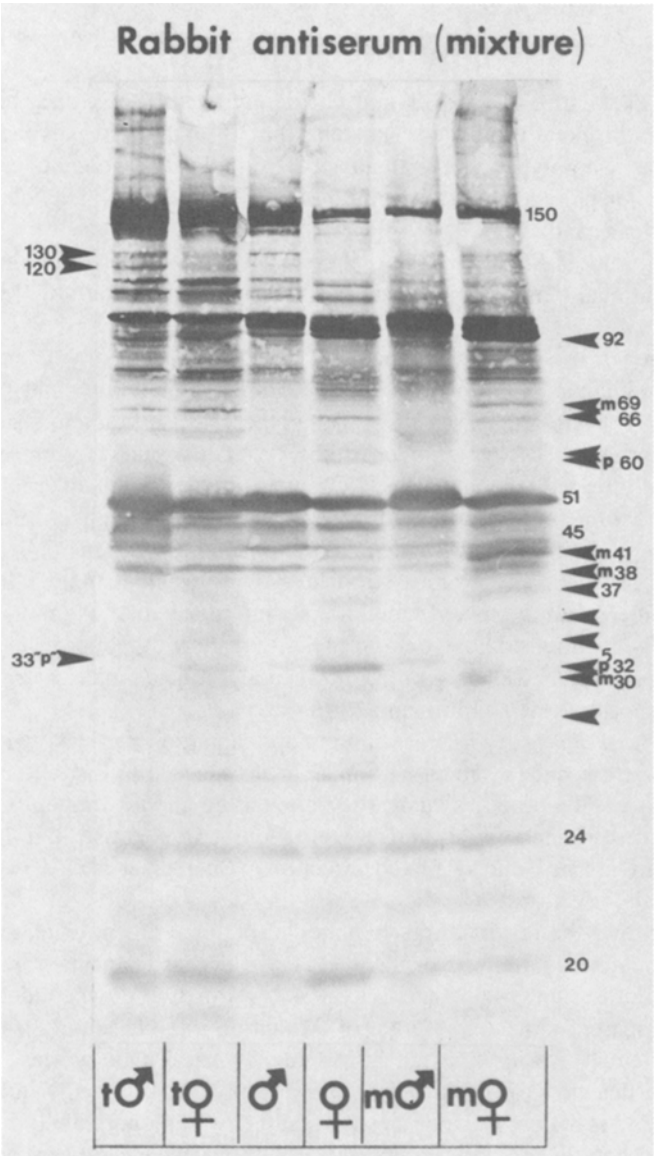

FIG. 3. Immunoblotting of antigens from bisexually reared males (♂), bisexually reared females (♀), monosexually reared males (m♂), and monosexually reared females (m♀). Male and female antigen preparations were also obtained by extraction of whole male and female worms with SDS-sample buffer (t♂ and t♀). A mixture of rabbit antisera against male and female water-soluble and membrane-bound antigens was used for the detection of blotted polypeptide antigens. Arrows on the left indicate male-specific antigens, arrows on the right, female-specific antigens. Antigens, specific for monosexually reared female worms, are indicated by the letter (m), and pair-specific antigens by the letter (p). The apparent molecular weight of major antigens and antigens of special interest, estimated from the position of molecular weight standards, is given in kilodaltons.

38, 41, and 69 kd). Especially the 30- and 41-kd polypeptides gave clear bands. In females from bisexually infected hamsters, the 30- and 41-kd polypeptides were absent, but two polypeptides with an apparent molecular weight of 32 and 60 kd, specific for paired females, were present. The 32-kd polypeptide gave an especially clear band.

Immunoblotting of SDS extracts of whole male and female worms showed only a few additional bands compared to the "standard" preparations (M_r 33, 50 and 67 kd).

The difference in antigenic composition of females from mono- and bisexually infected hamsters is very clear from Figure 4. Smaller amounts of antigen (20 μg/well) were applied to a 8–15% polyacrylamide gradient slab gel. The immunoblot shows a large number of polypeptides exclusively present in the monosexually reared female worms. Moreover, it is shown that the relative concentration of three polypeptides with apparent molecular weights of 92, 94, and 96 kd, is very different in the male and the female preparations.

Sera from infected mice were used to investigate whether the sex-linked antigens, identified with the mixed rabbit antiserum, also evoke a humoral immune response in an infected host. Sera from Swiss mice with monosexual male and female infections, as well as sera from bisexually infected mice, were used for immunoelectrophoresis and immunoblotting.

Figure 5 shows that very different immunoprecipitation patterns were obtained with sera from mice infected with male or female worms only. Remarkable is the absence of antibodies against the circulating anodic antigen (CAA) in serum from mice infected with female worms only. In contrast, high levels of antibodies are formed during mixed infections (Deelder et al., 1976) and monosexual male infections.

Figure 6 shows the results of an immunoblotting experiment using mouse antisera. The obtained pattern of bands was very different from that obtained with the rabbit antiserum. Major reactivity was towards two polypeptide antigens with an apparent molecular weight of 32 and 36 kd. These polypeptides are present in female worms, but not in bisexually reared male worms. Both antigens can be detected with sera from monosexually and bisexually infected mice. Remarkable is the presence of these two antigens in monosexually reared male worms. In conformity with this presence is the occurrence of antibodies against the two antigens in serum from mice infected with male worms only.

A remarkable difference between sera from monosexually and bisexually infected mice was the reactivity of the latter serum towards a 60-kd polypeptide. The presence of the polypeptide antigen in the SDS extract of mixed worms and in monosexually reared female worms was demonstrated.

Control immunoblotting experiments were performed to determine whether the female-specific antigens originated from eggs. However, no cross-reactivity of the female-specific antigens and egg antigens was observed.

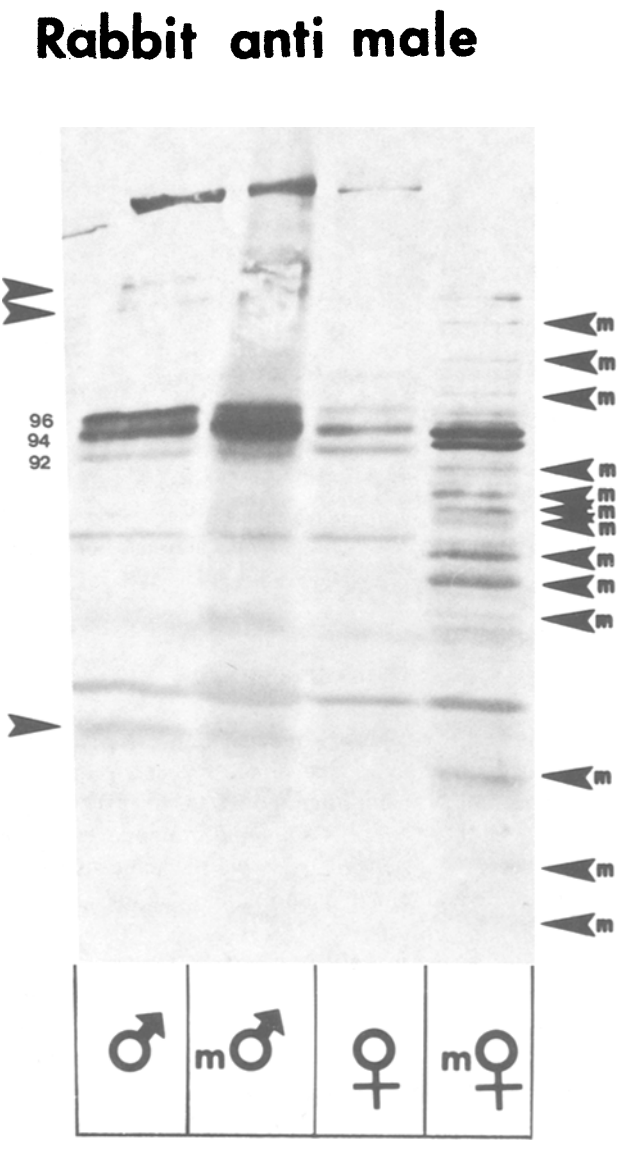

FIG. 4. Immunoblotting on a 8–15% gradient SDS-polyacrylamide slab gel of male and female antigen preparations, isolated from mono- and bisexually infected hamsters. Detection of blotted antigens was by reaction with an antiserum from a rabbit immunized with bisexually reared male worms.

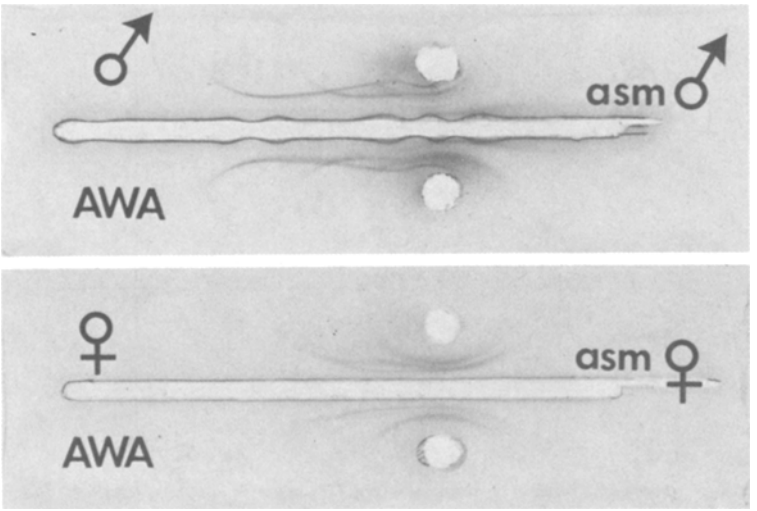

FIG. 5. Immunoelectrophoresis of male and female antigen preparations isolated from bisexually reared worms against antiserum from mice infected monosexually with male or female worms.

DISCUSSION

The aims of our studies on the sex-linked antigens of *Schistosoma mansoni* are to determine whether defined chemical substances are involved in the initiation of mating and female maturation and, if so, to investigate whether antibodies directed against these possibly antigenic components can interfere with the earlier mentioned processes. Moreover, we are interested in the direct or indirect effects of mating and female maturation on the immune response of the host.

Preceding research on the first objective, preliminary investigations on sex-linked antigens were performed. For comparative analysis of male and female antigen preparations, we used a single polyvalent antiserum reactive with a wide range of antigenic determinants. The obvious method for research on the second objective was the analysis of the humoral immune response of experimentally infected hosts. A comparison was made between the humoral immune response of monosexually and bisexually infected mice. Only mice receiving a bisexual infection develop a high degree of resistance to reinfection (Dean et al., 1978). This suggests that egg antigens or antigens which are produced exclusively by worm pairs play an important role in the development of resistance. In this study an antigenic polypeptide of 60 kd was identified which is presented to the immune system of the host by paired worms only.

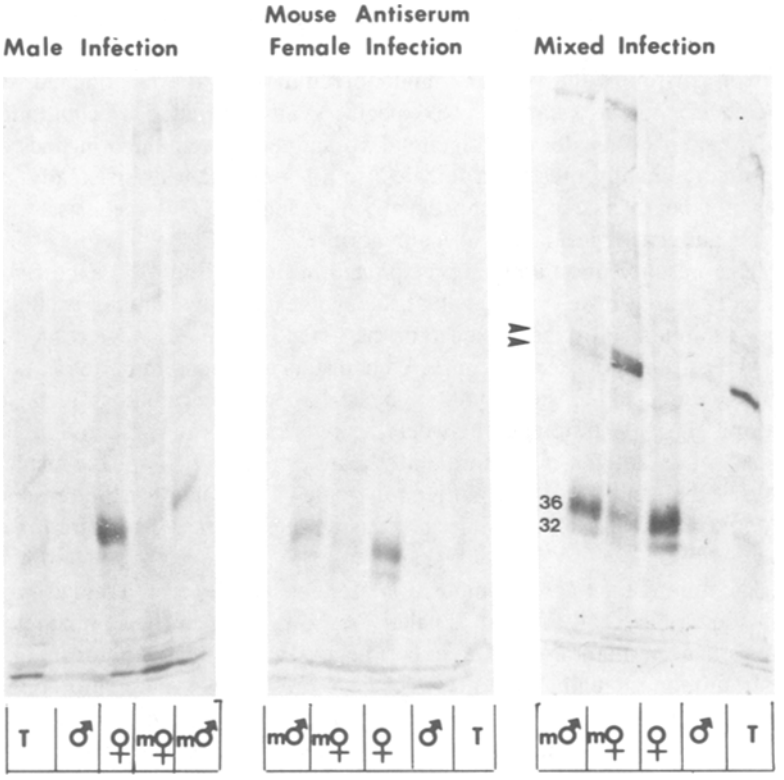

FIG. 6. Immunoblotting of male and female antigen preparations, isolated from mono- and bisexually infected hamsters. Freshly isolated worm pairs were also directly extracted with SDS-sample buffer (T). Electrophoresis was on a 8–15% gradient polyacrylamide gel. Blotted polypeptide antigens were detected with serum from mice monosexually infected with male or female worms or with serum from bisexually infected mice.

In contrast with earlier results obtained by Capron et al. (1965), analysis of sex-linked antigens by immunoelectrophoresis showed the presence of a number of male and female specific antigens. Even with sera raised in rabbits by immunization with a female antigen preparation, male-specific antigens were precipitated. Similarly, sera from rabbits immunized with antigen preparations from male worms can detect female-specific antigens. The same kind of observations were made when sera from monosexually infected mice were used for immunoblotting experiments. Aronstein and Strand (1984) reported a similar phenomenon in their studies on precipitation of antigenic glycoproteins with anti-male and anti-female mouse sera. Possibly, the female- and male-specific antigens are present in minute amounts in the worms of the other sex. This

minute amount might be sufficient or even optimal to evoke an immune response.

Comparison of the various immunoprecipitation patterns obtained by immunoelectrophoresis suggests a sex-specific synthesis, induced or inhibited by the partner, or a transfer of antigens. Two antigens precipitated in lines c and d are predominantly present in females, to a lesser extent in bisexually reared males, and nearly absent in monosexually reared males. This suggests a transfer of these antigens from female to male worms.

The presence of an antigen precipitated in line 6 (Figure 2) exclusively in bisexually reared males suggests that the synthesis of this antigen by the male worm is stimulated by the presence of the female worm.

Males from monosexually infected hamsters are very similar to males from bisexually infected hamsters, both in physical appearance and in antigenic composition. The only difference between the two male worm preparations is the presence of an antigen in monosexually reared males (line 7). This result suggests that the synthesis of this antigen by males is inhibited by the presence of female worms. It is also possible that this antigen is transferred from male to female worms.

The different antigenic composition of monosexually reared female worms, compared to bisexually reared females, is shown both in the immunoelectrophoresis and the immunoblotting experiments. Immunoelectrophoresis of antigens from monosexually reared females gave only a few precipitation lines, all of which were present also in bisexually reared females. In contrast to these results, results from immunoblotting experiments showed the presence of several antigens unique to monosexually infected females. The immunogenicity of the preparation from monosexually reared females is very low. Although several attempts were made, we failed to obtain any humoral immune response in rabbits immunized with this antigen preparation. Capron and Vernes (1968) reported a less marked precipitin formation during monosexual female infections in hamsters, in comparison to bisexual infections.

Immunoblotting experiments showed the presence of several female-specific antigens. The presence of a number of these antigens was limited to either mono- or bisexually reared female worms. Contact with male worms induced a switch in antigen synthesis, an additional processing by the females, or a transfer to and processing by male worms. Of particular interest are the disappearance upon mating of two major female-specific polypeptide antigens with an apparent molecular weight of 30 and 41 kd and the appearance of a major female specific polypeptide with an apparent molecular weight of 32 kd. Preliminary experiments have shown that the latter antigen is identical to one of the major microsomal antigens, as described by Tsang et al. (1984). The antigen, which is found exclusively in the microsomal fraction of a female worm homogenate, could be a surface antigen identical to the major 32-kd surface antigen described by Payares et al. (1985).

Although the use of rabbit antisera in the immunoblotting experiment showed clear differences between male and female preparations, these differences were much better expressed with sera from infected mice. The resemblance of the immunoblotting patterns obtained with sera from mice monosexually infected with male or female worms is striking. Both sera reacted strongly with a female-specific polypeptide antigen with an apparent molecular weight of 36 kd. This antigen was not present in eggs. Compared to the rabbit serum, the mouse serum reacted less strongly with the 32-kd polypeptide antigen. Of particular interest was the reactivity of the serum from bisexually infected mice with a 60-kd polypeptide. The absence of antibodies reactive with this antigen in sera from monosexually infected mice suggests that presentation of the 60-kd antigen by worm pairs is essential for evoking a humoral immune response in mice. The presence in monosexually reared females suggests a transfer of the antigen from females to males when worms are paired.

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BIOLOGY OF TEGUMENT ASSOCIATED IgG-Fc AND C3 RECEPTORS IN *Schistosoma mansoni*

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Abstract—Mechanisms by which schistosomes escape host immune responses are reviewed, with particular emphasis regarding the possible contributions of host or host-like Fc and C3 receptors on adult parasites.

Key Words—*Schistosoma mansoni*, Fc receptors, C3 receptors, developmental stage antigen expression, host immunoglobulins, antigenic modulation, immune escape.

INTRODUCTION

Parasites which inhabit the bodies of their hosts are subjected to an awesome panoply of defense mechanisms, some of which are highly specific while others are more general in nature. Successful parasites have evolved mechanisms for accommodating themselves to these defenses and are capable of surviving their onslaught, at least until they can reproduce. One of the most successful parasitic adaptations to murine and primate hosts is the genus *Schistosoma*. This parasite is capable of penetrating the primary barrier to infection, the skin, migrating via the circulatory system to the heart, lungs, and ultimately, the liver, and establishing reproducing adult worms which survive for decades in the definitive host. The adult parasite avoids immune destruction while using the host's immune response to exercise population control by eliciting effector mechanisms which destroy newly invading larvae. Furthermore, the schistosome uses the host's immune response to protect itself from the immune response by a series of biologically sophisticated molecular interactions at the host–parasite interface.

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The primary research interest of our laboratory at Texas A&M has been, is, and will, at least in part, continue to be the elucidation of those mechanisms by which parasites escape host immune responses. We have been actively involved in pursuing two of the most accepted theoretical mechanisms, namely host antigen mimicry (Damian, 1964) and host antigen adsorption (Smithers et al., 1969). This presentation will focus on one aspect of our work regarding the presence of host or hostlike Fc and C3 receptors on adult parasites, since these molecules may well form a convenient bridge between those two theories.

Research on these receptors began indirectly in the early 1970s with the demonstration by Campbell (1973) that repeatedly frozen and thawed adult worms from mice released host IgG, as detected by immunodiffusion. Direct evidence for the presence of host immunoglobulin associated with the surface of adult parasites was presented for murine worms by Sogandares-Bernal (1976) by immunofluorescence and for baboon worms by Kemp et al. (1976) by immunoperoxidase procedures. The prevailing theory of immune escape at that time was immunological blockade or enhancement, a concept borrowed from tumor immunology which maintained that non-complement-fixing host antibodies of low affinity bound to tumor-specific antigens on tumor cells, thereby denying access to those antigens of more effective host antibodies or effector cells. This hypothesis was made particularly attractive by the observation of Phillips et al. (1975) that passively transferred immunoglobulins in rats from a certain stage of larval parasite migration enhanced the ability of the parasites to survive. Since enhancing or blocking antibody in the tumor systems had been shown to be tumor-antigen-specific IgG_{2b} in the murine model, the next logical step in establishing the schistosome immune escape mechanism to the immunological blockade was to demonstrate that schistosome-specific IgG_{2b}, and only schistosome-specific IgG_{2b}, was bound to the adult worm's surface. As it turned out, however, experiments designed to document these two critical points supported neither.

An immunocytochemical and immunodiffusion survey of tegument associated immunoglobulins on and from mouse worms established that not only was IgG_{2b} present, but also IgG_1, IgG_{2a}, IgG_3, IgA, and IgM (Kemp et al., 1978). The absence of IgD and IgE suggested a certain specificity of uptake by the parasite. It was further demonstrated that worms taken from mice which had been immunized against known antigens such as human red blood cells, bovine serum albumin, or horseradish peroxidase specifically bound those antigens to their surfaces through an antibody-mediated event (Kemp et al., 1977). These data not only established that a considerable amount of the tegument-associated immunoglobulin was specific for antigens other than the parasite but that the antibodies were oriented in such a manner as to be able to present their Fab regions for binding. This suggested an Fc region–parasite tegument linkage.

These observations led our laboratory to consider the possibility that the adult worm was escaping the immune response by masquerading as a host im-

mune effector cell such as a macrophage or a neutrophil. Were this consideration valid, one would predict certain host or hostlike components to be found on the parasite's surface, namely histocompatibility antigens, Fc receptors (FcR), and C3 receptors (C3R). It is of interest to note that histocompatibility antigens have been shown to be bound to both larval (Sher et al., 1978) and adult worms (Gitter and Damian, 1982), although the adult associated H_2 antigens proved incapable of stimulating a mixed lymphocyte reaction (Gitter et al., 1982).

The first direct evidence for FcRs and CRs associated with schistosomes was reported by Torpier et al. (1979) and Santoro et al. (1979). These workers identified receptors for IgG, C1q, and C3 on cercariae and new schistosomula, but not on lung stage schistosomula or adult worms. The negative results regarding adult worms was due to the tegument modulation mechanism (Kemp et al., 1980) which allows adult worms to selectively shed immunologically compromised antigens from their surfaces in less than 20 min at room temperature. The characterization of the modulation process was being conducted in our laboratory at the time of the receptor localization experiments and was not available to the French scientists.

Direct localization of IgG FcRs and C3Rs on adult *S. mansoni* was accomplished by the incubation of adult parasites in intact or modified antigen–antibody or antigen–antibody–complement complexes or in C3-coated bacteria in the presence of tegument modulation inhibitors (Tarleton and Kemp, 1981; McGuinness and Kemp, 1981). It was demonstrated that the receptors were generally distributed on the dorsal tegumental surfaces of adult male worms and that the receptors were probably different since the C3R, but not the FcR, proved to be formalin sensitive.

STUDIES OF RECEPTOR FUNCTION

Recent studies in our laboratory have focused on attempting to ascertain the origin and biological functions of these receptors in the host–parasite relationship.

FcR Studies. The only FcR exhaustively documented as to specificity has shown an affinity for IgG. Our data indicating the presence of other classes and subclasses of immunoglobulins prompted us to ask if there was one receptor which had a broad-spectrum binding range (a general FcR) or if each class and subclass of immunoglobulin had its own specific FcR. We reasoned that if one general receptor were present, preincubation of a worm in one class of immunoglobulin would saturate the available receptors and block the binding of any other class or subclass to the parasite. In performing these experiments, we found that incubation of the parasite in purified IgG prior to incubation in purified IgA did not block the uptake of IgA. Likewise, the binding of IgA did not

onstrated that the binding of either had no effect upon the uptake of the other. These data indicate that there are specific FcRs for IgG_1, IgG_{2b}, and IgA. The other classes and subclasses are being studied at this time to establish their binding specificities. These data also suggest that the receptors are of host rather than parasite origin, since it is difficult to envision a parasite evolving a genome for all murine or primate FcRs, while selectively leaving out those for IgD and IgE (these latter being the only isotypes not previously shown to be associated in any manner with the adult parasite's tegumental surface).

In related studies (Rasmussen and Kemp, 1985) we attempted to ascertain the possible contribution of the FcR to immune escape by defining under what conditions host immunoglobulin specifically binds to the parasite and what conditions or factors are required by the parasite to obviate that binding. Neither normal mouse serum, normal mouse IgG, nor immune mouse serum bound specifically to fresh worms or to worms that had their adsorbed host components removed from their tegumental surfaces by incubation in RPMI-1640 for 2.5–4 hr at 37°C (elution). Immune IgG does not bind to fresh worms, but does bind rather dramatically to eluted parasites. This specific binding was blocked by preincubation of the eluted worms in normal mouse serum or normal mouse serum minus IgG and was partially blocked by preincubation in immune mouse serum or normal mouse IgG. The specificity of this blocking phenomenon is illustrated by the failure of preincubation in mouse albumin, bovine albumin, or fetal bovine serum to contribute to immune IgG binding inhibition. These data suggest that, at least in vitro, adsorbed host components do contribute to the inhibition of specific antibody binding, therefore immune escape, and that the host components involved have yet to be completely identified. It is further apparent that the parasite is selective in what host serum components it adsorbs, host immunoglobulins being only a portion of what the parasite needs to protect itself.

C3R Studies. Although the presence of complement receptors has been firmly established to be associated with the tegumental surfaces of larval and adult parasites, the presence of complement components naturally bound to those surfaces has only recently been unequivocally demonstrated. Kabil (1976) reported the localization of C3 on the surfaces of female, but not male adult worms, while Sogandares-Bernal (1976) found no complement whatsoever on schistosome surfaces. Studies in our laboratory using anti-mouse C3 and fluoresceinated *Staphylococcus aureus* localization procedures (Kemp et al., 1980) confirmed the observations of Sogandares-Bernal (1976) that there was no detectable C3 on the free tegumental surfaces of either sex of adult parasite. However, employing peroxidase labeled anti-mouse C3 at the transmission electron microscope level, we found copious amounts of C3 bound to the membranes of the tegumental infoldings of male worms (Rasmussen and Kemp, in preparation). No C3 was localized on the free tegumental surface, which explains our negative data with the FB assay.

Further studies on the possible functions of C3Rs in the host–parasite relationship led us to observe that adult worms incubated for short periods of time (20–30 min) in normal mouse serum evinced pronounced tegumental damage, while worms incubated for up to 1.5 hr in immune mouse serum displayed no such damage. We also noted that the damage inflicted by normal mouse serum seemed confined to the apical areas of the male doral tubercles. Such observations were reminiscent of the fate of cercariae in normal vs. immune sera (Stirewalt and Evans, 1955; Tavares et al., 1978) and stimulated us to look further into the sources of tegumental damage. Normal mouse serum damage was abrogated by heat inactivation and by EDTA or zymosan treatment. EGTA treatment had no effect, indicating that the alternate rather that the classical complement pathway was responsible. Immune mouse serum which had had the immune IgG removed by protein A affinity chromatography proved as damaging as normal mouse serum, and the addition of immune IgG to the incubation media prior to either normal mouse serum or immune mouse serum minus IgG protected the tegumental surface from damage. It is obvious from these experiments that specific antigen–antibody interaction at the parasite's surface protects the worm in some manner from the destructive effects of alternative complement pathway activation. How these data relate to our previous work suggesting limited specific antibody interaction at the parasite's surface has yet to be determined and appears contradictory (and is always a sure sign that the system being studied is more complex than currently envisioned).

As an extension of these observations, we designed a series of experiments to test if the classical complement pathway could damage a schistosome's surface under conditions which would damage another membrane system. We immunized an infected mouse with rabbit RBCs so that we could obtain an antiserum which contained both anti-schistosome and anti-RBC antibodies. We fractionated this serum to obtain IgG by protein A affinity chromatography, then incubated it with a mixture of adult worms and rabbit RBCs. After 10 min at 30°C, we added normal mouse or guinea pig serum and observed the reactions with phase-contrast microscopy. In certain experiments, we added a solution of acridine orange and ethidium bromide to the media to document membrane disruptions. Intact cells and worms absorb the acridine orange and appear green with fluorescence microscopy, while dead or damaged cells or worms absorb the ethidium bromide and appear orange. The rabbit RBCs either swelled and burst or acquired an orange color, while the parasites appeared totally unaffected by the incubations.

We feel these experiments demonstrate that: (1) factors present in immune serum (perhaps anti-schistosome antibodies) protect the parasite from potential alternative pathway activation by mechanisms as yet not understood. It is possible that the antibodies bind to and disarm those surface molecules which would activate the alternative pathway, thereby sparing the worm one of the host's most effective nonspecific responses: (2) the classical complement pathway,

although active and uninhibited apart from the parasite's surface, is ineffective in dealing with the parasite. These conclusions support the previous observations of Santoro et al. (1979) and Levy-Schaffer et al. (1982).

Studies of Developmental Stage Expression of FcRs and C3Rs. Preliminary observations performed in our laboratory, intended to establish the developmental stage expression and specificity of the FcRs and C3Rs, have confirmed the observations of Torpier et al. (1979) as to the absence of receptors on lung stage worms. We have observed that at day 16 postinfection there is a mixed population of parasites in the liver of the mouse host, some of which are distinctly schistosomular and some of which are adult-like. Those parasites retaining their schistosomular characteristics have no detectable tegument-associated immunoglobulins, no FcRs, no C3Rs, and are unable to modulate their surfaces. The adult-like worms do possess immunoglobulins, FcRs, C3Rs, and can modulate their surfaces. If these observations can be confirmed, they would indicate a dramatic, and potentially critical, developmental shift between days 14 and 20 postinfection and might further suggest the point in the developmental sequence when the schistosome metamorphoses from being at the mercy of the immune response into being its master.

SPECULATIONS

The following comments are speculative in nature, but are based upon our experience in working with the receptors.

1. We feel that the parasite is probably borrowing both FcRs and C3Rs from the host. If this is true, it should also be recognized that the parasite is displaying an impressive degree of control over its interface with the host. The fact that receptor-bound molecules are modulated when immunologically compromised indicates an intimate molecular association between the receptor and the cytoskeleton of the tegumental cytoplasm. If our working hypothesis is correct and the parasite is borrowing the receptors from host cells, the candidate donor host cell or cells should prove to possess histocompatibility antigens, receptors for some fragment of C3, and Fc receptors for all classes and subclasses of host immunoglobulins except IgD and IgE. The elucidation of the mechanism(s) of host membrane antigen acquisition by the parasite is of profound importance in understanding the host–parasite relationship at the molecular level. The future directions of research in this area may already have been suggested by the work of Caulfield et al. (1980a, b) where the apparent transfer of membrane from neutrophils to schistosomula was observed.

2. The adsorption and flagrant display of host immunoglobulins on the parasite's surface may well serve other purposes than simple steric blockade. The removal of significant quantities of antibody from the circulation by the parasite, if only for limited periods of time, may influence the idiotype regu-

lation network resulting in altered immune responses. This idea is particularly attractive when one considers that the FcR-bound immunoglobulins are oriented so that their idiotypes are prominently displayed. The possible function of these antibodies in immunoregulation is not a new idea, given the work of Auriault et al. (1980, 1981), which suggests the proteolytic cleavage of schistosomular FcR-bound antibodies are involved in inhibition of immune responses.

3. The actual role of C3Rs in the host–parasite relationship remains unclear. It is apparent that the parasite is capable of using facets of the immune response to negate the effects of other manifestations of immunity. The specific binding of antibody to the adult parasite's surface is probably an unusual event, but even in experimental situations where antibody binds and complement is activated by the classical pathway, no one has ever reported tegumental damage. Activation of the alternative pathway is extremely damaging to the worm under experimental conditions in normal serum, but we have no evidence that such activation occurs in vivo, where the worm is constantly exposed to immune serum. The presence of C3 in the tegumental infoldings, but not on the free surface, is intriguing and suggests a membrane difference or a differential distribution of membrane characteristics. It is possible that activated C3, from either pathway, is complexed to and inactivated by the C3R, which then moves from the free surface to the infoldings in order to facilitate degradation of that potentially hazardous molecule.

4. The C3R may have nothing whatsoever to do with complement inactivation. Given the well-established biochemical associations between the complement and clotting cascades, perhaps the C3R is involved in some way in aiding the parasite to avoid clot formation. Taking into account the established presence on the parasite of an inhibitor of activated Hageman factor, dubbed bilharzin by its discoverers (Tsang and Damian, 1977), such a suggestion is reasonable.

The purpose of this presentation was to briefly describe the background of the research on immunological receptors associated with *S. mansoni* and to superficially communicate the present status of our work in that area. These studies are consistently suggesting that we have only scratched the surface of an extremely complex series of biological interactions between a well-adapted parasite and its host. We expect to be quite busy sorting out these interactions for some time to come.

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STRATEGIES TO DETERMINE THE MOLECULAR BASIS OF CHEMICAL COMMUNICATION BY TREMATODES[1]

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Abstract—The identification of pheromones and chemicals that may inhibit or stimulate growth and reproduction necessarily leads to the consideration of biochemical methods to isolate and characterize these molecules. Analysis of *Schistosoma mansoni* surface antigens by direct radioiodination, metabolic labeling with tritiated and ^{14}C-tagged hexose precursors, affinity chromatography, isoelectric focusing, hydrophobic chromatography, and competitive inhibition is presented to illustrate methods of immunochemical analysis and antigen purification. Technical problems that may arise when investigating parasite molecular biology are described. Evidence of diminished fecundity of female worms in acutely infected mice supports the theory of a "crowding effect" in murine schistosomiasis and suggests the possibility that worm secretions or metabolites may function as chemical messages to inhibit oviposition. There is also evidence that the immune response of mice to an isolated surface antigen from adult worms results in the attenuation of hepatic granulomata of challenge infections. Several hypotheses are proposed to elucidate the molecular basis for chemical communication between trematodes and analytical approaches to test these hypotheses are outlined.

Key Words—Trematoda, *Schistosoma mansoni*, surface antigens, radiolabeling, affinity chromatography, hydrophobic chromatography, lectins, receptors, pheromones, proteins, glycoproteins, crowding effect.

[1] The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences. The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Animal Resources, National Research Council, DHEW Pub. No. (NIH) 78-23.

INTRODUCTION

There is increasing evidence for communication between parasites with regard to host location (Kearn, 1986), sexual attraction and pairing (Fried, 1986; Childs et al., 1986; Shirazian et al., 1986), and vitellogenesis (Popiel, 1986); and in many instances, the existence of a chemical message is implied. Elucidation of the molecular basis for chemical communication would not only increase our understanding of parasite biology, but would also suggest practical methods to control parasite maturation, egg deposition, and transmission of disease. Chemical communication implies that messages are both sent and received. The transmission of chemical messages may include the active secretion of specific functional molecules, the routine turnover of surface membrane proteins and glycoproteins, or some feedback mechanism involving the release of metabolic by-products. If chemical receptors are present on trematodes, they most likely occur on the tegumental surface. For this reason, an understanding of the molecular composition of the tegument—that active interface between the parasite and its microenvironment (terminology of Dogiel, 1958)—becomes essential.

The identification of pheromones or receptor sites necessarily leads to the consideration of methods to isolate and characterize these molecules. However, the biochemical characterization of trematode secretions or membrane-bound molecules is not a simple task. For several years, our laboratory has been actively engaged in the immunochemical examination of surface antigens on *Schistosoma mansoni* adult worms. Although this work has primarily dealt with large molecular weight, polar antigens rather than small nonpolar or lipophilic pheromones, the basic conceptual approach to molecular analysis is similar for both. Our attempts to isolate and characterize surface antigens are not only particularly relevant to the identification of specific tegumental receptor sites, but also help to illustrate many of the technical problems associated with any investigation of the cell biology of complex metazoan parasites.

PITFALLS IN ANTIGEN ISOLATION AND CHARACTERIZATION

Direct Radioiodination of Surface Proteins. Initial attempts to characterize the surface antigens of *Schistosoma mansoni* sought to apply the well-defined methods of protein radiolabeling that had proven successful in the study of lymphocytes and tumor histocompatibility antigens (Kusel et al., 1975; Ruppel, 1978). It was learned, however, that lactoperoxidase-catalyzed radioiodination of *S. mansoni* adult worms yielded highly inconsistent results (Hayunga and Murrell, 1982). A representative SDS-PAGE profile of detergent-extracted tegumental proteins following lactoperoxidase labeling in phosphate-buffered saline (PBS) is shown in Figure 1; several protein peaks are clearly discernible. In contrast, when worms were labeled under identical conditions in Earle's balanced salt solution (EBSS), no protein peaks were present (Figure 1). It had

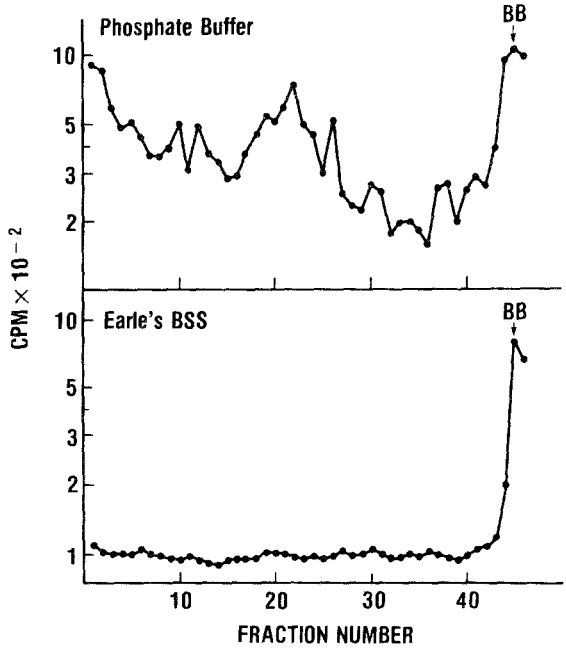

FIG. 1. Comparison of detergent-extracted tegumental proteins from *S. mansoni* adult worms following lactoperoxidase-catalyzed radioiodination in phosphate-buffered saline (upper graph) and Earle's balanced salt solution (lower graph), 10% gels, 2-mm slices.

been found previously by Ruppel (1978) that when worms were incubated in PBS, internal proteins leaked into the media where they became radiolabeled and subsequently adsorbed by the tegument, giving erroneous results. In a balanced salt solution, neither leakage nor labeling took place. Thus, the choice of buffer in radiolabeling experiments was found to be critical.

Metabolic Radiolabeling of Tegumental Glycoproteins. Metabolic labeling has yielded considerable information about membrane turnover by adult schistosomes. We have examined the incorporation of several labeled hexose precursors, as shown in Table 1, and found *S. mansoni* tegumental glycoproteins to have an average biological half-life ranging from 15 min to 2 hr. This is in general agreement with the turnover rates of approximately 2 hr or less determined by Wilson and Barnes (1977), Nash (1979), and Podesta (personal communication), but differs from those reported by Kusel and MacKenzie (1975) and Samuelson and Caulfield (1982), who calculated a longer turnover time. Figure 2A shows the amount of labeled tegumental extract containing tritiated glucosamine that precipitates in 20% trichloroacetic acid (TCA) after varying "chase" incubations in media without the labeled precursor. The points on this graph would be in general agreement with an exponential decay curve, $y = ae^{bx}$, where $a = 10$ and $b = -0.45$. Loss of label is virtually complete by 12

TABLE 1. ESTIMATE OF TURNOVER RATES FOR TEGUMENTAL GLYCOCONJUGATES BASED UPON PULSE-CHASE LABELING WITH HEXOSE AND HEXOSAMINE PRECURSORS

Labeled precursor	Turnover rate (expressed as half-life)
[^{3}H]Galactose	30 min
[^{3}H]Glucose	15 min
[^{3}H]Glucosamine	$1\frac{1}{2}$ hr
[^{3}H]Fucose	1 hr
[^{14}C]Glucose	50 min
[^{14}C]*N*-Acetylglucosamine	45 min
[^{14}C]Mannose	40 min
[^{14}C]Galactosamine	2 hr
[^{14}C]*N*-Acetylgalactosamine	75 min

hr, and by extrapolation, the average half-life of tegumental glycoproteins labeled with this precursor can be estimated to be approximately $1\frac{1}{2}$ hr.

If we assume that all the label lost from the tegument is shed into the media, then we can calculate an "expected" curve for accretion of labeled TCA-ppt material in the media, $y = a(1 - e^{bx})$, as shown in Figure 2B. Although the total cpm recovered from the media corresponded to the total label lost by the tegument, the amount of TCA-ppt material (presumptive glycoprotein) was less than expected. This discrepancy is probably due to breakdown of large molecules incubating in the culture media for several hours, although recycling (ingestion) of labeled glycoprotein by the worms may also occur. The observed values in Figure 2B would be in general agreement with an exponential decay curve superimposed on the "expected" curve, $y - a(1 - e^{bx}) + a'x^{b'}$, where $a = 10$, $b = -0.45$, $a' = -0.01115$, and $b' = 2$.

Ordinarily, a metabolic labeling experiment would require a "pulse" label with a small amount of radioactive precursor followed by a "chase" incubation in media containing an excess (40 mM, for example) of unlabeled precursor. Such was the initial design of our experiments. However, we soon learned that our protocol needed revision because concentrations of glucosamine as low as 1.8 mM (0.4 mg/ml) were shown to be toxic for *S. mansoni* (Bueding et al., 1954). Glucosamine appears to be a normal component of some tegumental glycoproteins (Figure 3). However, it is thought that with high concentrations of this molecule, a build-up of metabolic by-products, such as a phosphate ester of glucosamine, may interfere with chemical reactions essential for the survival of the worms (Bueding et al., 1954). Regardless of the mechanism involved, a "chase" incubation using 40 mM glucosamine would probably have made a serious impact on the normal physiology of the parasite, and thus compromised

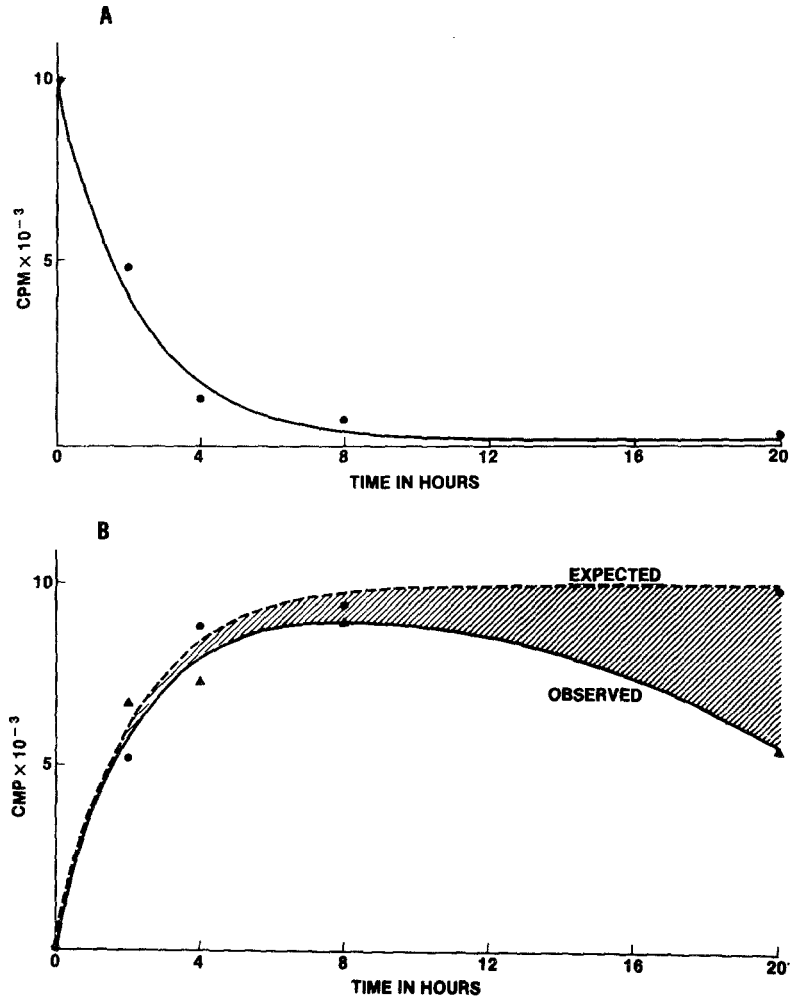

FIG. 2. Amount of TCA-precipitable, detergent-extracted tegumental material (expressed as counts per minute per 100 worms) recovered after a 2-hour incubation with tritiated D-glucosamine. Time refers to the number of hours of incubation in media without label subsequent to radiolabeling. (A) Labeled glycoprotein present in the tegumental extract; (B) labeled glycoprotein present in the culture media; "expected" values are calculated from the previous graph.

the experiment. For this reason, our protocol to examine turnover of glucosamine-containing tegumental glycoconjugates utilized "chase" incubations in unaltered NCTC-135 media which contains only 18 μM glucosamine (Evans et al., 1964). A complication such as this illustrates the importance of a sound knowledge of the experimental animal when designing in vitro studies.

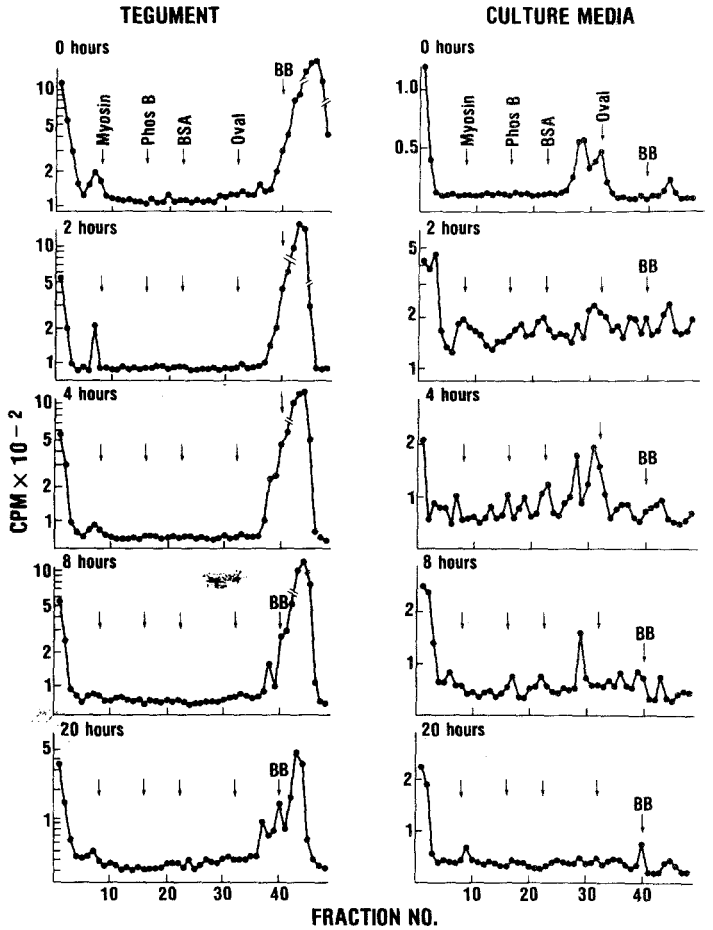

FIG. 3. SDS-PAGE profile of [^{3}H]glucosamine-labeled *S. mansoni* detergent-extracted tegument and culture media following various lengths of "chase" incubation, 7½% gels, 2-mm slices.

Comparison of Tegumental Antigens and Culture Antigens. A qualitative analysis of tegumental glycoproteins and "culture secretory antigens" was performed by running SDS gels on samples taken at varying intervals after a "pulse" incubation in tritiated glucosamine. As shown in Figure 3, two glycoprotein peaks with apparent mol. wt. ≥ 300,000 and 210,000 are distinctly visible immediately after labeling ($t = 0$ hr); however, over 90% of the label migrated ahead of the dye front, which suggests incorporation into glycolipid or low molecular weight glycopeptides. Increasing incubations in unlabeled media result in decreased radioactivity of the tegumental extract, until by 8 hr, the 210,000 mol. wt. peak is barely discernible. At 8 and 20 hr, an additional peak of 30,000 mol. wt. is seen. We believe that this peak was present initially, but

was obscured by the large peak migrating ahead of the dye front; at 4 hr, the 30,000 mol. wt. peak appears as a distinct shoulder on this larger peak.

In contrast, the SDS-PAGE profiles of the culture media are more complex. The profile at $t = 0$ hr represents culture media taken immediately following the "pulse" incubation and contains three distinct glycoprotein peaks with apparent mol. wt. $\geq$ 300,000, 50,000, and 43,000. The 50,000 and 43,000 mol. wt. peaks were not present in the tegumental extracts and may represent nontegumental (gut?) secretions, enzymatic degradation of larger tegumental glycoproteins previously released into the media, or metabolic by-products excreted by the worms. The large ($\geq$300,000 mol. wt.) peak may represent early turnover of a tegumental component, or it may represent a totally different (secreted?) glycoprotein, as resolution of apparent molecular weight is poor in this region of the gel. Turnover of the 210,000 mol. wt. tegumental glycoprotein is not yet detected at $t = 0$ hr.

The profile of the culture media becomes more complex at $t = 2$ hr (i.e., "pulse" labeling followed by a 2-hr "chase" incubation in media without label) and later. The 50,000 and 43,000 mol. wt. peaks found at this time doubtless correspond to similar peaks at $t = 0$ hr, while the $\geq$300,000 and 210,000 mol. wt. peaks are probably tegumental glycoproteins shed into the media. The other peaks may represent additional excretions or secretions, or breakdown of the larger molecules. By 20 hr, few of these peaks can be identified, a finding that is consistent with extensive proteolysis, as very small labeled components would have been lost during the dialysis step that precedes SDS-PAGE.

The presence of the large ($\geq$300,000 mol. wt.) peak in the culture media prior to the appearance of the 210,000 mol. wt. peak suggests a faster rate of turnover for the former glycoprotein. Likewise, pulse-chase experiments using tritiated galactose suggest that a 260,000 mol. wt. component is shed more rapidly than other tegumental glycoproteins (data not shown). Since histochemical localization was not done, we do not know whether such apparent differences in turnover rate reflect gender differences between parasites or regional variation in glycoprotein composition and tegumental turnover as might be expected when comparing dorsal and ventral surfaces of male worms. Results of metabolic labeling with hexose precursors and lectin affinity chromatography (described below) reveal the complexity of the surface membrane glycoproteins and allow for a preliminary characterization of these molecules (Table 2).

Clearly, the surface membrane glycoproteins of *S. mansoni* do appear in time in the media and may constitute a significant portion of the so-called "culture secretory antigens." However, as depicted in Figure 3, the culture media also contains a variety of other worm-derived molecules and breakdown products which only compound the complexity of the preparation. For this reason, the harvesting of secretions or surface antigens from culture media should be approached with caution.

Lectin Affinity Chromatography. Receptor sites for concanavalin A and a variety of other lectins have been demonstrated on the surface of adult worms

TABLE 2. PARTIAL CHARACTERIZATION OF RADIOLABELED GLYCOPROTEINS FROM *Schistosoma* PRECURSORS AND

Containing labeled precursors							
gal	glc	man	fuc	galNH	galNAc	glcNH	glcNAc
≥300,000	≥300,000[b]	≥300,000	≥300,000	≥300,000	≥300,000	≥300,000	≥300,000
260,000	250,000	—	—	—	260,000	—	—
210,000[b]	—	—	—	220,000	—	210,000	—
165,000[b]	163,000	(170,000)[e]	—	170,000	165,000	(170,000)[e]	—
—	—	—	—	—	—	—	155,000
—	—	—	—	—	(143,000)	—	—
—	—	—	—	—	—	—	135,000
—	—	—	—	—	—	—	—
—	(108,000)	—	—	—	—	—	—
100,000[b]	—	—	—	—	—	—	—
85,000[b] (79,000)[e]	—	—	—	—	—	—	—
—	(76,000)	—	—	71,000	(74,000)	—	—
67,000[b]	—	—	—	—	65,000	—	64,000
59,000[b]	—	(61,000)[e]	—	59,000	—	(56,500)[e]	56,000
53,000[b]	—	—	—	—	52,000	—	—
46,000[b]	45,000	46,000	—	44,500	—	—	44,000
(43,000)[b] 41,000	—	43,000	—	41,000	42,000	—	38,000
—	—	36,000	—	—	—	(34,000)[e]	—
32,500[b]	—	—	—	—	(31,000)	30,000	—
—	—	—	—	—	—	—	—

[a] Apparent molecular weights are calculated from SDS-PAGE profiles. Appearance of molecular weight data appearing in parentheses () were obtained from only one experiment and were not reproduced.
[b] Antigenicity demonstrated by immunoprecipitation.
[c] Detected in [^{3}H]galactose-labeled samples only.
[d] Detected in [^{3}H]galactose- and [^{3}H]mannose-labeled samples.
[e] Detected only after reaction with concanavalin A.
[f] Apparent shift in molecular weight in NP40.
[g] Apparent shift in molecular weight in borate eluate.
[h] Detected only in borate eluate; not significantly greater than control.
[i] Detected only in NP40 sample.

and schistosomula (Bennett and Seed, 1977; Murrell et al., 1978; Simpson and Smithers, 1980), and lectin affinity chromatography has been proposed as a method to isolate surface membrane antigens from *S. mansoni* (Bennett and Seed, 1977; Brink et al., 1980; Strand et al., 1982). However, efforts to isolate antigens in quantity were hindered by the heterogeneity of crude antigen preparations, by low yields of the final product, and by the apparent irreversible

mansoni ADULT WORMS BASED UPON INCORPORATION OF HEXOSE LECTIN BINDING[a]

Binding to lectins						
Con A	Lentil	WGA	SBA	RCA120	RCA60	Helix
(≥300,000)[c]	(≥300,000)[d]	—	—	—	—	—
—	—	—	—	—	—	—
(215,000)[c]	—	—	—	—	—	—
168,000	—	—	—	—	—	—
—	—	—	152,000	—	—	(155,000)[i]
—	—	—	—	—	—	—
134,000	—	—	—	—	—	—
—	122,000	—	122,000	120,000	125,000	—
—	(112,000)	(115,000)[h]	—	108,000	—	—
—	—	—	—	—	—	—
83,000 (79,000)[c]	83,000	83,500	88,000	(80,000)	—	—
—	—	(74,000)	—	—	—	—
—	—	—	—	—	—	—
58,000[b]	57,500	(60,000)[c] 56,500	59,000	56,000	58,000	—
—	—	53,000	—	—	—	—
—	(46,000)[c]	—	45,000	—	47,000	—
41,500 (36,000)[f]	—	38,000 (35,000)[f]	(44,000)[g] 42,500	40,000	41,500 (38,500)[f]	(42,000)[h]
34,000	34,000	—	—	—	—	—
30,500 (27,000)[f]	30,500	31,000 (27,000)[f]	31,500 (28,000)[g]	33,000	31,000	(30,000)[h]
—	—	—	(25,000)[g] 23,500	(22,000)[i]	—	—

on the same line, indicates our opinion that the glycoproteins detected are identical. Data

binding of material by Con A–Sepharose (Hayunga et al., 1983). When preparative columns were used a second time, the yield was markedly diminished. After three or more sample applications, the column took on a permanent yellow-brown color that was not removed by alternate washings to regenerate the column. It was also found that the temperature at which elution is done can have a major effect on antigen recovery (Hayunga, 1986). As shown in Figure

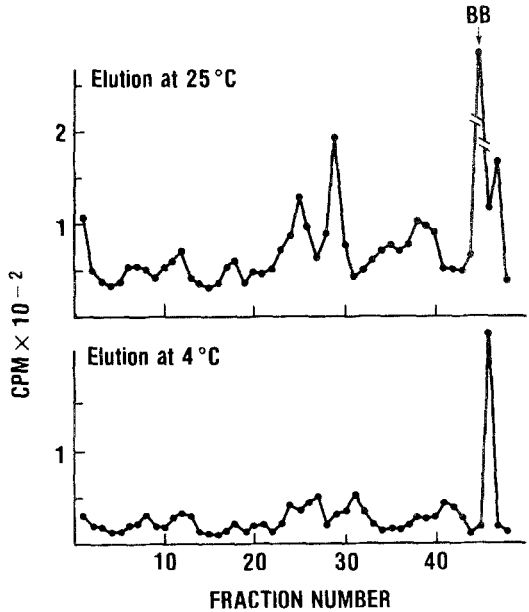

FIG. 4. SDS-PAGE profile of equal aliquots of Bolton-Hunter-labeled tegumental glycoproteins of *S. mansoni* adult worms eluted from identical concanavalin A–Sepharose 4B chromatography columns at different temperatures, $7\frac{1}{2}\%$ gels, 2-mm slices.

4, elution of a Con A–Sepharose column in a cold room results in far less recovery of adsorbed glycoprotein than elution of an identical sample and column at room temperature. Yet temperature can easily be overlooked when attempting to scale up production.

These problems were finally resolved by removing lipid from the crude preparation and by using a sequence of chromatography columns as shown in Figure 5. The purified glycoprotein migrated as a single peak in Ampholine isoelectric focusing. It reacted with sera from humans infected with *S. mansoni*, *S. haematobium*, or *S. japonicum*, and surface binding of fluorescein-labeled Con A by adult worms was inhibited by the purified antigen. Metabolic labeling experiments revealed significant incorporation of galactose into this molecule (Hayunga et al., 1983).

Analytical-scale affinity chromatography was also performed using immobilized *Lens culinaris* (lentil) lectin, wheat germ agglutinin, soybean agglutinin, and the agglutinins from *Ricinus communis* and *Helix pomatia* (Hayunga and Sumner, 1986). It was found that several glycoproteins reacted with more than one lectin. Therefore, to control for nonspecific adsorption of material by the lectin columns, we prepared control columns as follows: CNBr-activated Sepharose 4B or Sepharose 6MB was reacted with ethanolamine to block re-

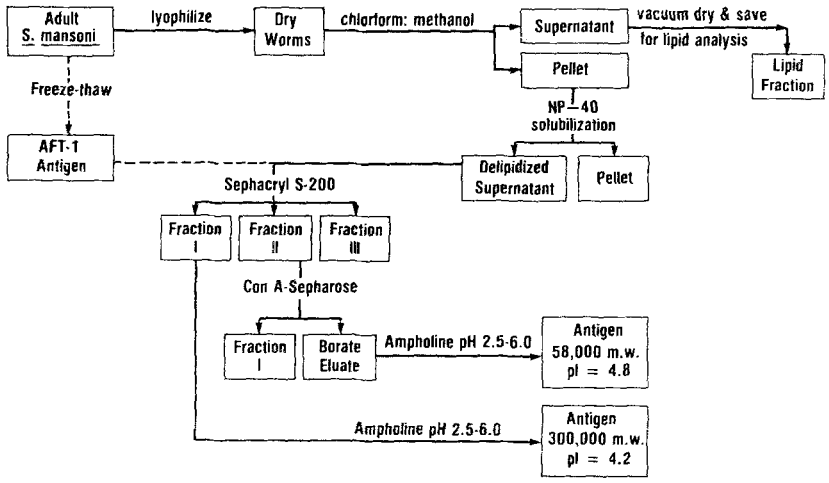

FIG. 5. Flow diagram depicting the fractionation protocol for purifying the Con A-binding surface antigen from *Schistosoma mansoni* adult worms. (Reprinted from Hayunga et al., 1983. Courtesy of Allen Press.)

active groups, followed by alternate washing in 0.1 M $NaHCO_3$, pH 8.3, and 0.1 M sodium acetate, pH 4.0, then final equilibration. In this way, the control columns were identical to the commercially produced lectin columns with the exception that they contained no immobilized ligand.

In Figure 6, it can be seen that the Sepharose 6MB control column yielded two peaks of 45,000 and 30,000 mol. wt. Although these peaks were small, they were identical, both qualitatively and quantitatively, to the sugar eluate from the *Helix pomatia*-Sepharose 6MB column. Thus, without such exhaustive controls, the interpretation of lectin affinity chromatography would have been flawed.

TESTING SOME HYPOTHESES ABOUT CHEMICAL COMMUNICATION

Although many observations of parasite behavior have implied chemical communication, the demonstration of a chemical message requires isolation of an active substance. However, the isolation and subsequent characterization of such substances is often confounded by the complexity of the parasite-host system, by limitations of in vitro models, and by difficulties in distinguishing between parasite and host components in vivo. By way of illustration, several hypotheses are proposed to determine the molecular basis for chemical communication by trematodes. Specific problem areas are addressed and possible solutions suggested. In each instance, the central question posed is, "How does one identify the molecule used as a chemical message?"

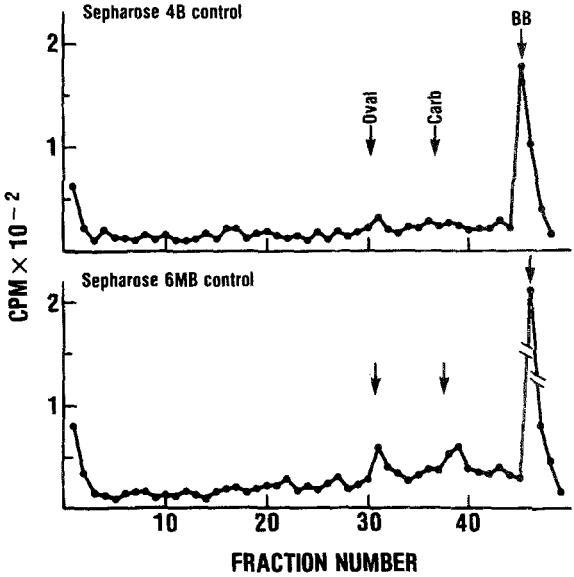

FIG. 6. Borate elutates of Bolton-Hunter radioiodinated *S. mansoni* adult freeze-thaw (AFT) antigen applied to control columns of Sepharose 4B and Sepharose 6MB without ligand, 7½% gels, 2-mm slices. (Reprinted from Hayunga and Sumner, 1986. Courtesy of Allen Press.)

Hypothesis I: Free-Swimming Stages of Trematodes Migrate Directionally in Response to Specific Chemical Signals Produced by the Prospective Host. In the first hypothesis, it is presumed that the behavior of the parasite in proximity to its host has been adequately observed and is well defined. The experimental approach to test such a hypothesis for free-swimming stages would be rather straightforward, involving the use of an environmental chamber (perhaps something as simple as a Petri dish), adding synthetic chemicals or defined fractions derived from the host, and making direct observations. In addition, the experimental design should compare the response between concentration gradients and uniform concentrations of the test material and allow the observer to distinguish between directional movement and nondirectional stimulation of activity.

Such an approach is seen in experiments to determine the behavioral response of *S. mansoni* miracidia to snail-conditioned water by Roberts et al. (1979). Although miracidia did not appear to orient themselves to a gradient of snail-conditioned water, their change in angular velocity while swimming enabled them to remain in regions of higher concentration. Identification of the active component in the water could be done by testing sequential fractions following the kind of protocol described by Gloer et al. (1986) to isolate sexual

chemoattractants. Alternatively, Plorin and Gilbertson (1985) used a more intuitive approach to select glucose as one of the possible stimulants present in snail-conditioned water.

Hypothesis II: Adult Trematodes Produce Secretions that Influence Mating Behavior and Sexual Maturation. The testing of this hypothesis may at first glance also seem rather straightforward. However, identification of chemical signals used by parasitic stages in vivo is inherently more complicated than similar assessments for free-swimming stages. In the first place, the definition of a secretion is problematic. In a preliminary analysis, we may simply designate everything left in the culture dish after incubation under defined conditions as the "culture secretions," but this is a very crude working definition. Eventually, this material will have to be characterized, and it has generally been found that "culture secretions" are very heterogeneous mixtures of proteins, carbohydrates, and lipids (Murrell et al., 1974). It is known that the tegumental surface membrane, and hence its associated proteins and glycoproteins, are continuously shed into the media, as depicted in Figures 2 and 3. How, then, is one to distinguish between true secretions, excretions, and membrane turnover? Furthermore, the heterogeneity of even the best defined preparations is overwhelming. One-dimensional and two-dimensional SDS-PAGE have revealed numerous components in parasite gene products (Ruppel and Cioli, 1977; Atkinson and Atkinson, 1982) and surface membrane extracts (Hayunga et al., 1979; Siddiqui and Podesta, 1985). Even when one selects for molecules with specific carbohydrate moieties by lectin affinity chromatography, the preparations are still rather heterogeneous (Aronstein and Strand, 1984; Hayunga and Sumner, 1986).

Although there is probably no easy solution to the problem of heterogeneity, chemical analyses can nevertheless proceed. In an accompanying paper, Gloer et al. (1986) followed a very rational scheme of chemical fractionation by extracting parasite material with a series of solvents of increasing polarity, and they found an active sexual chemoattractant to be a nonpolar component present in the *n*-pentane extract. Another approach to molecular fractionation might involve affinity chromatography. For example, it seems intuitively obvious that a sexual attractant secretion or its corresponding surface receptor should be unique to one sex. One might therefore consider raising antibodies against male worms and preparing an anti-male affinity column; extracts from female worms could be applied to this column and female gender-specific antigens collected in the "fall-through" fraction. Alternatively, monoclonal antibodies might be used to isolate sex-linked antigens. In this regard, the groundwork for identifying gender-specific antigens has already been laid (Ruppel and Cioli, 1977; Atkinson and Atkinson, 1982; Aronstein and Strand, 1984; Rotmans et al., 1986).

Finally, even when individual components are identified and isolated in relatively homogeneous preparations, yields are low and subsequent biochem-

ical analyses are necessarily limited. For this reason, investigators must rely on highly sensitive analytical probes, such as monoclonal antibodies, or the labeling of parasite molecules with isotopes that can be detected by radiation emission (Hayunga and Murrell, 1982) or by nuclear magnetic resonance (NMR) as described by Mansour et al. (1982) and Thompson and Lee (1985). Molecular structure may also be determined by the analysis of charge differences of glycoproteins following sequential enzymatic degradation by exoglycosidases (Kobata, 1979; Poretz and Pieczenik, 1981), or by competitive inhibition of antibody or lectin binding. Recently, Hayunga et al. (1983) described a "competition ELISA" to infer the structure of antibody binding sites of glycoproteins by competitive inhibition using various sugars and amino sugars; each test required only 1 μg of purified antigen.

Hypothesis III: Adult Female Trematodes Produce a Factor that Suppresses Egg Production in Other Females. The notion of a crowding effect for *S. mansoni* adult worms is somewhat controversial. An inverse relationship between worm length and intensity of infection has been reported in some instances (Radke et al., 1957; Grimaldo and Kershaw, 1961). Yet, in an equally convincing study, Lennox and Schiller (1972) were unable to demonstrate such a crowding effect. With regard to egg production, data from human autopsies (Cheever, 1968; Cheever et al., 1977; Anderson and May, 1982) and from experimental infections of the grivet monkey *Cercopithecus aethiops aethiops* (Cheever and Duvall, 1974) indicate a tendency towards decreased oviposition in heavier infections. However, interpretation of data from these studies is complicated by possible changes in the fecundity of older female worms and by the destruction of tissue eggs in chronic infections.

We have found evidence for reduced fecundity in relation to population density in acutely infected mice (Hayunga et al., 1985). As shown in Figure 7, as the number of female worms increases, the number of eggs produced per female decreases, which is indicative of a crowding effect. Such data also suggest that even when worm burden is reduced, the egg burden in the liver may still be unacceptably high—an important consideration in vaccine development. A similar finding was also reported by Harrison et al. (1982), who concluded that reduced fecundity was related to excess numbers of unpaired female worms.

Clearly, some kind of negative-feedback mechanism is at work. Although reduced egg production by *S. mansoni* suggests a "chemical message," such as the recently reported pheromone that inhibits reproduction in the tick *Argas arboreus* (Khalil, 1984), it is perhaps just as likely that worms may be responding to a build-up of metabolic wastes or to competition for limited resources. The presence of a factor in portal blood that stimulates oviposition (Wu and Wu, 1986), and the observation that tyrosine uptake is increased during vitellogenesis (Erasmus, 1986), both lend support to the notion that egg production by *S. mansoni* may be resource limited. However, there is little evidence to indicate a molecular basis for the crowding effect. With the exception of work

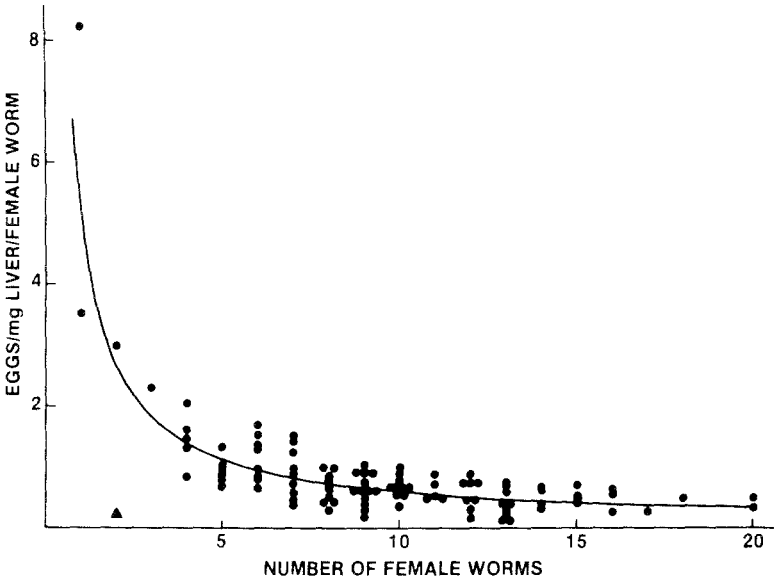

FIG. 7. Number of eggs recovered from the liver per female worm in mice experimentally infected with *Schistosoma mansoni*, eight weeks postinfection. (Reprinted from Hayunga et al., 1985. Courtesy of Allen Press.)

reported in the accompanying papers (Shirazian et al., 1986; Wu and Wu, 1986), in vitro experiments to examine egg production by *S. mansoni* have been sorely lacking and may not always be directly extrapolated to the in vivo situation. We do not know what effects host nutrition and metabolism may have on parasite fecundity, and the effect of infection serum on oviposition in vitro reported by Rotmans et al. (1986) suggests that the immune response of the host may be a major factor in the modulation of egg output. Finally, to further complicate matters, there is even evidence of variation in egg production between individual female worms cultured in uncrowded conditions (Shirazian et al., 1986).

Hypothesis IV: Adult Trematodes Produce a Factor that Stimulates Host Resistance to the Infective Stage of the Parasite. The last hypothesis attempts to establish a molecular basis for concomitant immunity, and implies chemical communication between the parasite and its host in order to limit the establishment of additional parasites. This is by far the most difficult of the hypotheses to test, involving not only the characterization of parasite molecules, with all its inherent problems, but also an assessment of the host's immune response to these molecules and to living parasites. Implicit in this hypothesis, too, is the assumption that concomitant immunity represents an immunological response to infection, and is not simply an ecological phenomenon, as suggested by Warren (1973) and Wilson et al. (1983).

The immunochemical characterization of schistosome antigens is, by itself, a formidable task. Yet testing of the hypothesis also requires manipulation of the host immune system. The complexity of the immune response can be illustrated by contradictory evidence regarding immunization with crude antigen extracts (Murrell et al., 1975) and by the impact that the route of antigen presentation may have on protective immunity (James, 1985). The presence of protective antigens is supported by the observation that specific antibodies bind to cercarial surface membranes and kill parasites in vitro (Harn et al., 1984) and by the reduction of worm burden in challenge infections of mice following inoculation with defined antigen preparations (Hillyer and Sagramoso de Ateca, 1979).

At present, several antigens have been isolated in relatively homogeneous form: the MSA_1 fraction from soluble egg antigen or SEA (Pelley et al., 1976); FhConA, a concanavalin A-binding glycoprotein isolated from the tegument of *Fasciola hepatica* (Hillyer and Sagramoso de Ateca, 1979); SmConA and SmFxI, two glycoproteins isolated from *S. mansoni* adult worms (Hayunga et al., 1983); and a purified protease from *S. mansoni* (Chappell and Dresden, 1984). Of those that have been tested for protection, the results have been equivocal. FhConA, which has been extensively studied, has produced reductions in worm burden as high as 70% (Hillyer and Sagramoso de Ateca, 1979). In our laboratory, FhConA was less effective in reducing worm burden but did appear to reduce the size of egg-induced hepatic granulomata, as did SmConA. SmFxI was ineffective in protecting mice against challenge infection despite high specific antibody titers following inoculation (Hayunga et al., 1985). In general, progress in antigen isolation has been slow, and the matter of establishing a molecular basis for concomitant immunity is far from resolved.

CONCLUSIONS

Attempts to characterize trematode surface antigens and secretions have been complicated by the heterogeneity of even well-defined preparations. Peculiarities of the parasites with regard to radiolabeling and of parasite extracts with regard to nonspecific binding to chromatography columns emphasize the need for meticulous experimental controls. When individual molecules are isolated in homogeneous form, subsequent analysis is often thwarted by low yields of the final product.

In the accompanying papers (Gloer et al., 1986; Eveland and Hajeeb, 1986), low-molecular-weight, nonpolar molecules have been implicated as sexual chemoattractants for *S.mansoni*. This suggests the possible application of hydrophobic chromatography using immobilized alkanes or aminoalkanes to isolate lipophilic molecules on the basis of affinity interactions of their exposed hydrophobic patches, as originally proposed by Shaltiel (1974). We have re-

cently applied this method to the analysis of tegumental extracts from *S. mansoni* adult worms and isolated a stage-specific hydrophobic protein of 40,000 mol. wt. (Hayunga and Sumner, 1983). Hydrophobic chromatography ought to be of value in isolating smaller nonpolar molecules as well.

Although considerable attention has been given to small, lipophilic molecules, the possible role of larger hydrophilic proteins in chemical communication by trematodes should not be overlooked. According to Wilson (1975, p. 235), "proteins, in fact, make up a large fraction of the known waterborne pheromones," while smaller more volatile molecules are usually associated with airborne message transmission. It has been shown that oviposition of *S. mansoni* is stimulated by a large molecular weight protein component in portal serum (Wu and Wu, 1986) and it should not be surprising if other water soluble proteins were used as chemical messages by blood flukes residing in an aqueous environment. Finally, the concept of chemical communication implies not only transmission but also the reception of messages. Yet, except for the descriptions of Fc and complement receptors (Kemp et al., 1986) or benzodiazepine binding sites on the tegument of *S. mansoni* (Bennett, 1980), little attention has been paid to identifying functional surface receptors on trematodes.

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FUNDAMENTAL ASPECTS AND POTENTIAL ROLES OF ECDYSTEROIDS IN SCHISTOSOMES
An Update Overview

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Abstract—The human digenic trematode *Schistosoma mansoni* produces ecdysteroid hormones. Both ecdysone (22*R*)-2β,3β,14,22,25-pentahydroxycholest-7-en-6-one and 20-hydroxyecdysone (22*R*)-2β,3β,14,20,22,25-hexahydroxycholest-7-en-6-one were detected during the critical development stages of this bisexual parasite: during the exponential growth phase to adult stage (day 11 postinfection) and during the sexual maturation of adult and egg laying (day 40 postinfection). The parasites released their ecdysteroid hormones in the biological fluids of the infected vertebrates as soon as six days postinfection. In addition, the time course of the ecdysteroid titer was correlated with the susceptibility or the innate resistance of the host to schistosome infection. Moreover, we demonstrated that after a schistomicide therapy, ecdysteroids from urine of infected children decreased markedly four days after drug administration. We also have demonstrated that immunization of rodents with an ecdysone-BSA complex led to the reduction of the worm burden. By in vitro studies, we have shown that the ecdysterone antibodies were able to kill the juvenile worms within 24 hr. In addition, we have demonstrated that the enzymes superoxide dismutase (SOD, EC 1.15.1.1) were present in schistosomes and that the total superoxide dismutase activities in both males and females could be correlated with the 20-hydroxyecdysone within parasites.

Key Words—*Schistosoma mansoni*, ecdysteroid, antiecdysteroid, serum/kinetic, urine/diagnosis, superoxide dismutase (EC 1.15.1.1), mass spectrum, chromatography.

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INTRODUCTION

The ecdysteroids were first demonstrated in arthropods (Butenandt and Karlson, 1954) where they were associated with their moulting process (for review, see Karlson, 1980). Because the growth of nematodes has involved a series of evident moults (Rogers, 1962), the detection of ecdysteroids in worms was done first in this parasitic species (Rogers, 1973; Horn et al., 1974). Our freeze fracture studies of *Schistosoma mansoni* membrane differentiation allowed the observation of a membrane exuviation during the period of 10–20 days after infection (Figure 1.) (Torpier et al., 1982). Such a phenomenon occurring at a precise period of the worm development is reminiscent of the moulting process of arthropods in which metamorphoses are induced by two specific hormones: namely ecdysone and 20-hydroxyecdysone. Since we demonstrated that the produced ecdysteroids (Nirdé, 1982; Torpier et al., 1982, Nirdé et al., 1983a) were released in the biological fluids of the infected hosts (Nirdé 1982, Nirdé et al., 1983b, 1984), particular interest has been devoted to this topic in platyhelminthic worms. (Whitehead, 1983; Koolmann et al., 1984, 1985; Rees and Merdis, 1984; Rees et al., 1985). The present paper is an update review on the ecdysteroids in the human trematode *Schistosoma mansoni* and on the potential roles of these hormones in schistosomiasis.

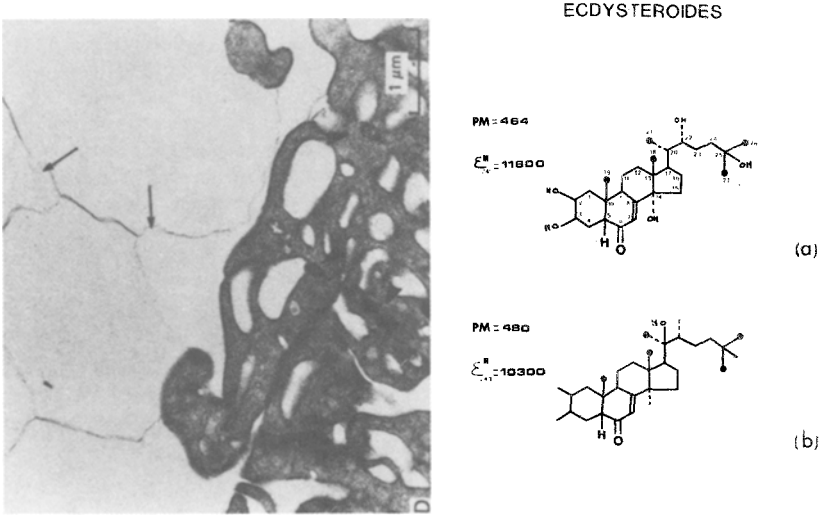

FIG. 1. Illustration of the membrane exuviation (arrow) observed during the 10- to 20-day period after infection (left) and structure of ecdysone (a) and 20-hydroxyecdysone (b). (Modified from Torpier, et al., 1982.)

METHODS AND MATERIALS

Infection of rodents was performed by exposure to cercariae of *Schistosoma mansoni* as initially described by Smithers and Terry (1965). Recovery of parasites of required age was performed by perfusion of the rodents killed by cervical dislocation. Eleven-day-old and 20-day-old worms were collected by perfusion of the liver with MEM. Forty-day-old schistosomes were recovered by total perfusion of the whole blood system with NaCl 0.9% or only from the mesenteric and portal system of hamsters. Eggs were recovered by a 54% Percoll gradient in MEM as described by Nirdé et al. (1983a).

Ecdysteroid extracts of worms were performed by a methanol–water–hexane (10:5:3 by v/v) homogenization as described by De Reggi et al. (1975). For the biological samples such as urine and sera, a methanol–water (3:1 v/v) extraction according to Nirdé et al. (1984) was performed. To 1 ml serum was added 3 ml methanol (Merck). The mixture was then vortexed prior to a 60°C incubation for 15 min and centrifuged (800*g* for 10 min at 4°C). The supernatant was then dried under nitrogen flow and resuspended in 0.1 M citrate buffer, pH 6.1, prior to the RIA assay (De Reggi et al., 1975).

The ^{125}I-labeled hormone was a succinyl ecdysterone coupled to [^{125}I]tyrosine-methyl-ester (Immunotech, Marseille). The binding assays were made in a Plexiglas apparatus containing 10 dialysis compartments, each divided into two 200-μl chambers by a cellulose membrane. One chamber was filled with 150 μl of ecdysteroid antibody (Immunotech) and the other one with 150 μl of a mixture of equal parts of the sample to be analyzed and the ^{125}I-labeled hormone. After 24 hr shaking at 4°C, equilibrium was achieved and 100 μl of each compartment was counted. The sum of the bound (B) and free (F) labeled analog was found on the antibody side, the free (F) alone on the other. The ratio of the binding was computed as $r = B/T$, where $T = B + F$. The standard curve was run with pure 20-OH-ecdysone (Sigma) from 10^{-7} M to 10^{-11} M. Bound fractions were then computed with a WANG 2200 S system, with a linear regression of the standard curve (Nirdé, 1982). The results were expressed as picomoles of 20-OH-ecdysone equivalent per 100 mg of protein or per milliliter of biological fluids (urine or sera).

Mass spectroscopy analysis was done with 600 ml urine of children infected with *Schistosoma haematobium*. A butanol extraction was performed on this material as previously described (Nirdé et al., 1984) The butanol extract was then analyzed by thin-layer chromatography (silicagel plates, 20 × 20 cm, F 254, Merck) with chloroform–methanol as solvent (80:20, v/v). The plates were scraped by 1-cm bands and the products eluted with three washes with 95% ethanol. Only the immunoreactive samples were analyzed by HPLC (Waters, reverse phase) with methanol–water as solvent (40:60 v/v, 1 ml min,

isocratic elution). Gas chromatography was performed on immunoreactive HPLC fractions after derivatization. Fractions to be analyzed were added to 100 μl of trimethylsilylimidazole (TSIM) and heated at 80°C under argon. Separation of derivatized ecdysteroids from reagent was performed through HPLC on silica column with ethanol–hexane as solvent. After evaporation of the solvent under argon, acetone was added to dried extract and 1–2 μl was injected into a gas chromatographic apparatus (Varian 1200, capillary glass column SE/30) at 290°C. A mass spectrometer (LKB 9000S) was coupled to the gas chromatograph. This spectrometer was equipped with an INCOSS computer system which allows the recording of one mass spectrum per second.

The complex ecdysone–BSA was used as immunogenic compound. Hamsters were inoculated by subcutaneous injections by the Vaitukaïtis technique (1971). Each hamster received a primary injection of 200 μg of protein with 1 vol of Freund's complete adjuvent (Bio-mérieux) in a total volume of 500 μl. Boosts of 100 μg were given on day 30 and day 60 after the primary injection. Hamsters were infected 10 days after the last injection with 100 cercariae of *S. mansoni*. Antibody titer was determined by the RIA method.

The in vitro cytotoxicity of sera from hamsters immunized with an ecdysone–BSA complex was performed on skin schistosomula. The skin schistosomula were collected in vitro after penetration of cercariae through isolated pieces of Swiss mice abdominal skin according to the method initially described by Stirewalt and Uy (1969). For the in vitro cytotoxicity assay about 120 schistosomula were incubated in each well of a flat-bottomed microtiter plate (Nunclon, Denmark) with 110 μl of 10% FCS in MEM, 50 μl of heated sample serum to be tested, and 40 μl of fresh guinea pig serum. For studies without complement, the guinea pig serum was heated for 30 min at 56°C. The percentage of mortality was measured by direct visual examination and by scanning microscopy.

For scanning microscopy studies, schistosomula were fixed in 0.1% glutaraldehyde in 0.05 M sodium cacodylate buffer. The samples were then post-fixed with 1% osmium tetroxide solution for 1 hr at 4°C and dehydrated in a ethanol series. A gold–palladium coating (60:40) was then performed before examination.

Superoxide dismutase was assayed by the inhibition of the cytochrome *c* reduction using a xanthine–xanthine oxidase system as originally described by McCord and Fridovich (1969). Batches of 4–10 adult worms or 20,000 schistosomula, or 20,000 day-11 parasites were used. Parasite material was suspended in 2 ml of 50 mM phosphate buffer (pH 7.8), sonicated on ice for 45 sec at 20 kHz (Sonotrode TC 4C, type 20-200 S), and centrifuged for 10 min at 800 *g* (Jouan E96 4°C). Triton X-100 was added to the supernatant to a final concentration of 0.2% (v/v) prior to 30 min incubation at 4°C. Centrifugation was then performed (45,000 *g* for 10 min at 4°C) and the supernatant used in

the SOD assay at pH 10 with or without 1 mM KCN. The final reaction mixture was 2.85 ml and the standard curve was set using bovine erythrocyte SOD (Sigma). Crude extract activities were then computed on a WANG 2200 MVP system by using a polynomial fitting of the standard curve as described by Nirdé (1985). Results were expressed as Sigma SOD units equivalent per milligram of protein.

RESULTS

Figure 2 shows the variation in ecdysone levels during *Schistosoma mansoni* development. Line A indicates that newly derived schistosomula contain 4.3 pmol ecdysone equivalents per 100 mg of protein. Line B corresponds to the day-11 schisotomes. The amount of ecdysteroids at this stage is 25 pmol. Line D represents both males and females collected on day 40 after infection

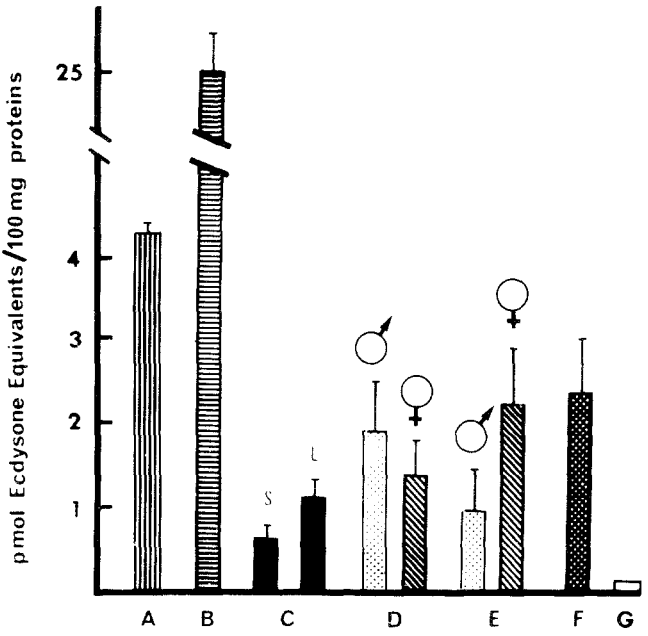

FIG. 2. Ecdysteroid levels during *Schistosoma mansoni* development using the RIA method. (A) Newly derived 3-hr schistosomula ($N = 2$); (B) 11-day-old schistosomes ($N = 4$); (C) 20-day-old schistosomes ($N = 4$); (D) 40-day-old schistosomes (adults) from the total perfusion of hamsters ($N = 6$); (E) 40-day-old schistosomes from mesenteric vessels ($N = 4$); (F) Eggs of *Schistosoma mansoni* ($N = 6$); (G) control performed on uninfected animal tissues ($N = 10$). Bars are standard deviation. (From Nirdé et al, 1983b.)

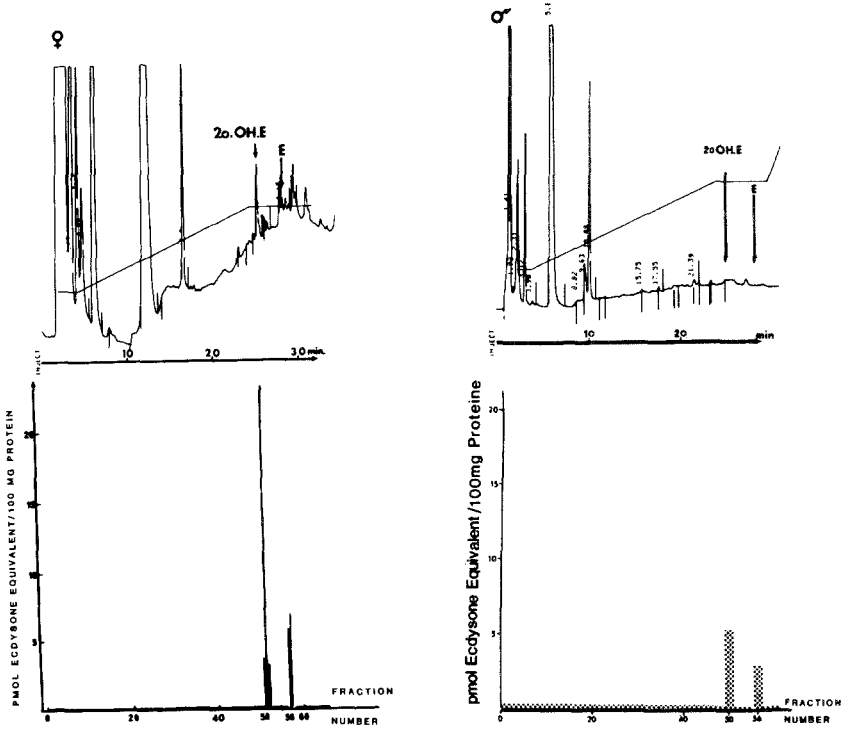

FIG. 3. High-pressure liquid chromatography of the extract of parasite from total perfusion and RIA on collected fractions (♀, females; ♂, males). Column: Whatmann, Partisil 10-25/ODS 3; detection: Waters UV–visible detector M 440, 245 nm; programmer: Waters M 650; flow: 1 ml/min; gradient: 30-min linear gradient of 30% acetonitrile in water was performed; arrows: standard elution of 20-hydroxyecdysone or ecdysone from Sigma run in the same conditions. (Modified from Nirdé et al., 1984a.)

by total perfusion of hamsters exhibiting an ecdysone level at about 2 pmol. No significant sexual differences can be observed by the radioimmunoassay method. In Figure 3, HPLC analyses demonstrate that both ecdysone and 20-hydroxyecdysone are present. The 20-OH-ecdysone is more abundant than ecdysone in both sexes. The ratio, r, of 20-hydroxyecdysone ecdysteroids is $r = 0.62$ for the males and $r = 0.68$ for the females. In contrast, within adults collected exclusively by local puncture of mesenteric vessels (line E), the normal location of these worms for mating and egg laying, sexual differences in ecdysteroid levels were observed: less than 1 pmol for the males and 2.5 pmol for the females.

In addition, HPLC analyses shown in Figure 4 demonstrate that parasites from mesenteric vessels exhibit an inverse ratio of 20-hydroxyecdysone to total

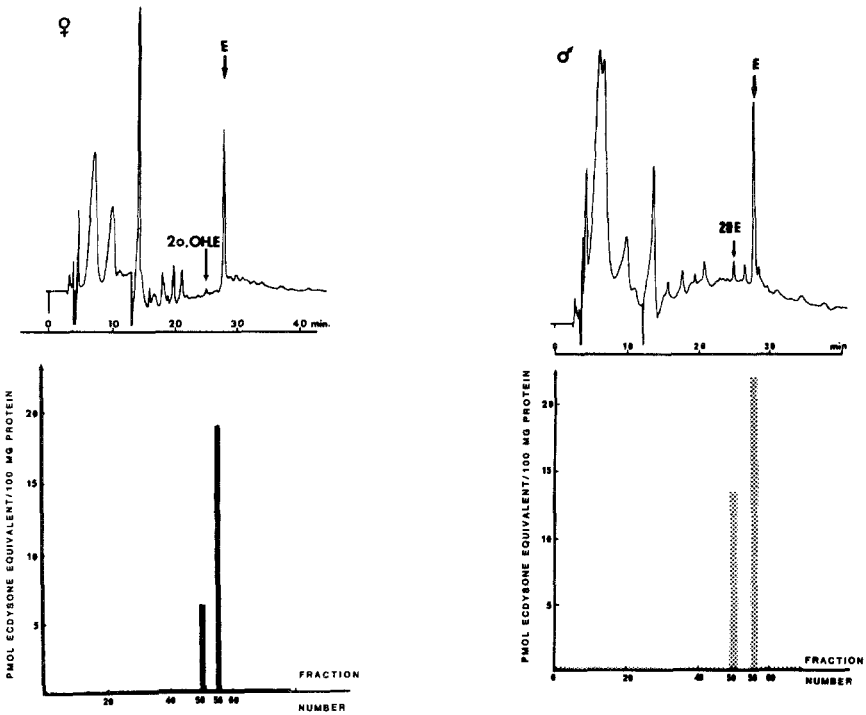

FIG. 4. High-pressure liquid chromatography of the extract of parasite collected exclusively by local puncture of mesenteric vessels and RIA on collected fractions. Column: Waters Microbondapack-phenyl; detection: Waters UV–visible detector M 441; 254 nm; calculator: Waters M 730 and system controler M 720; flow: 1 ml/min with Waters 6000 A pumps; gradient: After 2 min of isocratic conditions with water–acetonitrile (90:10) as solvent, a linear gradient of 30% of acetonitrile:water was performed in 30 min; arrows: standard elution of 20-hydroxyecdysone and ecdysone from Sigma run in the same conditions. (Modified from Nirdé et al., 1984a.)

ecysteroids compared with parasites collected by total perfusion: $r = 0.40$ for the males and 0.24 for the females. In mesenteric vessels, ecdysone is far more abundant than 20-hydroxyecdysone in both sexes. In the eggs, line F, the hormonal content and the ecdysteroid ratio are comparable to those of the females from mesenteric vessels ($r = 0.24$).

As shown in Figure 5, the trematode *Fasciola hepatica* also contains ecdysteroids. The ratio of 20-hydroxyecdysone to ecdysone plus 20-hydroxyecdysone was 0.44. HPLC analyses also indicate that a third ecdysteroid peak was detected. This peak could be specific for this species. Besides the physiological role of these hormones in the parasites, it would be interesting to follow their fate in the biological fluids of the infected hosts. Vertebrates do not pro-

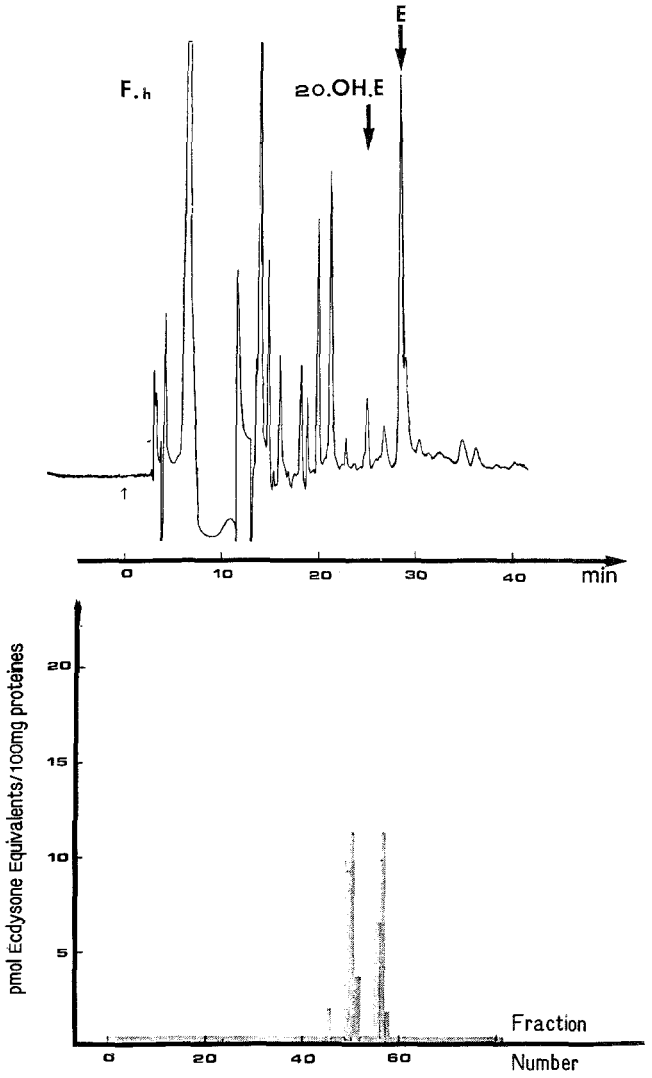

FIG. 5. High-pressure liquid chromatography and RIA analysis in extracts of the trematode parasite *Fasciola hepatica*.

duce insect moulting hormones nor specific enzyme systems to catalyze these hormones and, a priori, the ecdysteroids would not be metabolized by the hosts.

As shown in Figure 6, the serum of infected rodents contains ecdysteroids. As early as six days postinfection, ecdysteroids could be detected in sera of infected rats or hamsters, whereas no detection is possible in uninfected animals, or during the first five days of infection (Nirdé et al., 1984a,b). As shown

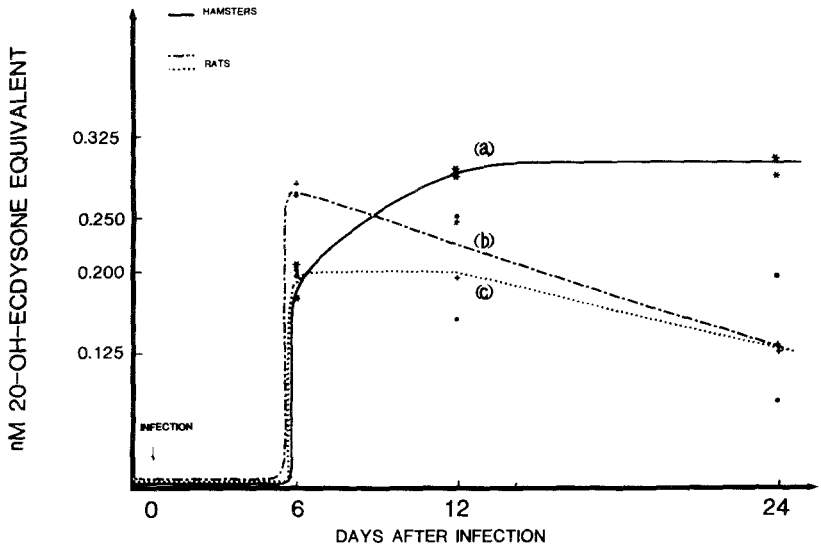

FIG. 6. Changes in ecdysteroid concentration in the serum of permissive and nonpermissive rodent hosts infected with *Schistosoma mansoni*. Hamsters (*M. auratus*, a) and rats (*R. rattus*, b) were infected with 2000 cercariae of *Schistosoma mansoni*, whereas other rats (c) were infected with only 500 cercariae. (From Nirdé et al., 1984a.)

in this figure, the changes in hormone level are different between permissive (curve a) and nonpermissive hosts (curves b and c). In permissive hosts such as hamsters, the RIA activity reaches 0.3 nM on day 12 postinfection, and this value remains unchanged on day 24. In contrast, in the nonpermissive hosts such as rats, the ecdysteroid level reaches 0.3 nM on day 6 postinfection, and, more important, it decreases slowly from day 10–12 onwards. This figure also shows that the rise in ecdysteroid concentration, which occurs on day 6, is significantly lower in rats that received a weaker challenge of 500 cercariae compared to rats challenged with two thousand cercariae. After day 12, the two groups of rats do not differ significantly with regard to the ecdysteroid titer.

Further analyses have shown that ecdysteroids were also present in the blood of infected primates, even in the absence of parasitological markers (Nirdé, 1982, Nirdé et al., 1984a). Based on these data, ecdysteroid titers were determined in the urine of humans infected with *Schistosoma haematobium*.

Figure 7 demonstrates that children have a very high concentration of insect-molting hormones in their urine (Nirdé et al. 1984). Urine of 60 infected children between 10 and 14 years of age were assayed. The average level in hormone before chemotherapy was 4.5 nM. However, in three children, the highest value found was more than 180 nM. As seen in this figure, four days after drug administration, the hormonal concentration decreases markedly to one third and then slowly reaches 6 nM some 100 days after treatment.

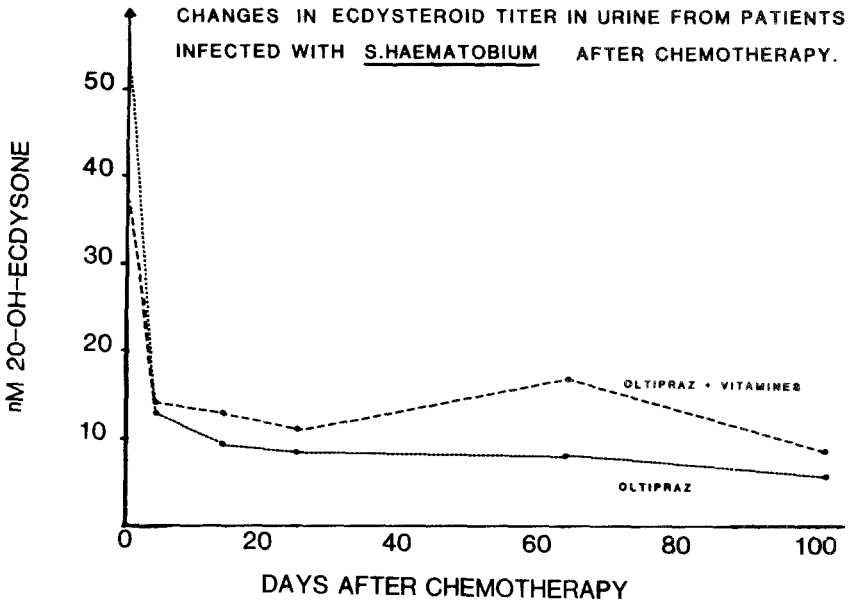

FIG. 7. Changes in ecdysteroid concentration in urine from patients infected with *Schistosoma haematobium* before and after chemotherapy. Day of chemotherapy: D_0, Single dose of Oltipraz (30 mg/kg, $N = 20$) or Oltipraz plus vitamins (30 mg/kg, $N = 20$).

Chemical characterization of ecdysteroids found in urine of infected humans has been achieved by mass spectroscopy as shown in Figure 8. Figure 8A, curve a, is the total ion recording. It indicates that several molecules are present in the urine sample. In curve b, the fragmentograph ion recording allows the detection of four ecdysteroid peaks. Arrow 1, the 20-hydroxyecdysone, and arrow 2, the 5 epi-20-hydroxyecdysone, show the two major peaks. One of the minor peaks is the 3-oxo-20-hydroxyecdysone. The last one has not been identified yet. Figure 8B(a) shows the mass spectrum of the compound yielding peak 1 in the fragmentogram. Spectrum b exhibits the mass spectrum of standard 20-OH-ecdysone run under the same conditions. The differences observed in the molecular weight of these ecdysones compared to crude molecules are caused by the derivatization of the ecdysteroids for the mass spectrum analysis. The fragment at *m/e* 561 corresponds to the nucleus of the ecdysone. The fragments with a larger *m/e* correspond to the fragmentation of the side chain of ecdysone after cleavage between C-20 and C-22 and to the loss of a silanol in the case of fully silylated derivatives.

In Figure 9, we notice that ecdysteroids are also detected in the sera of rodents infected with the nematode *Dipetalonema vitae*. Whereas low levels of ecdysteroids were measured on days 43 and 73 postinfection, high amounts of these hormones could be detected on day 63 postinfection.

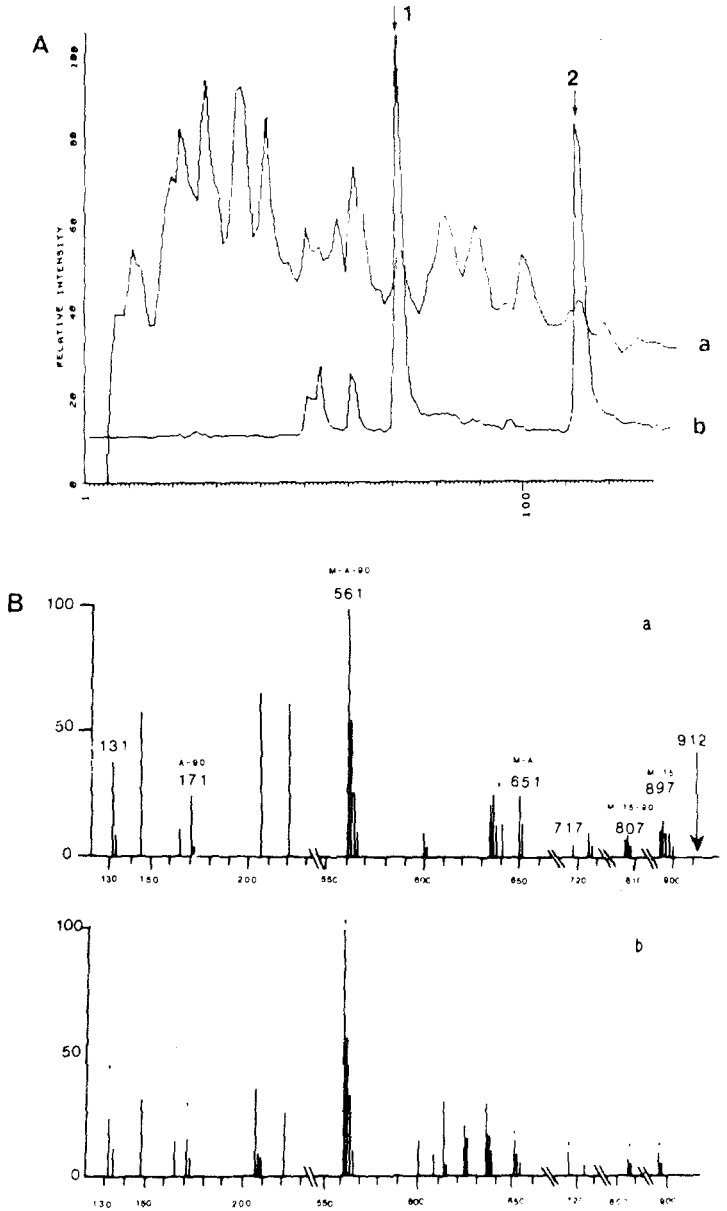

FIG. 8. Gas chromatography and mass spectrometry of hexamethylsilylated natural compounds from the infected patients. (A) Total ion gas chromatograph: (a) total ion recording, (b) recording of ion fragment at *m/e* 561. (B) Mass spectra: (a) mass spectrum of hexatrimethylsilylated reference 20-hydroxyecdysone (reference from SIMES, Milan); (b) mass spectrum of released ecdysteroid yielding peak 1 in A, line b. (From Nirdé et al., 1984a.)

STAGE	ECDYSTEROID AMOUNT *	N
D.43	0.18 +0.08	2
D.63	23.00 + 0.13	2
D.70	0.30 + 0.01	2

* PMOLE ECDYSONE EQUIVAL / ML SERUM

FIG. 9. Ecdysteroid levels in the sera of the nematode *Dipetalonema vitae*.

Figure 10 demonstrates that schistosomes contain the enzyme superoxide dismutase. Two isoenzymes have been detected during the development stages of the parasite: cytosolic Cu/Zn SOD and mitochondrial Mn SOD.

Both SOD activities are present in schistosomula and 11-day-old schistosomes. Adults collected on day 40 after infection by total perfusion of hamsters exhibit more SOD than adults recovered by local puncture of mesenteric vessels. Sexual differences in the ratio between mitochondrial and copper-zinc SOD can be observed. The females contain more copper-zinc SOD than the males.

In addition, as shown in Figure 11, total SOD activities in both males and females can be correlated with the ratio of 20-hydroxyecdysone to total ecdysteroids within parasites. When this ratio increases, the total SOD activities also increase. This is true for both sexes. The presence of ecdysteroid hormones around parasites could suggest that these hormones would be necessary for parasite development. As well as the biological role of these ecdysteroids at the parasite level, it seems interesting to investigate the biological activities of antiecdysteroid antibodies against schistosomes.

Figure 12 shows the effects of immunization of rodents on the parasite burden. In hamsters, without any boosts, the reduction of the parasite population is around 38%. However, the females are more sensitive to the ecdysteroid antibodies than the males. In this case the reduction was over 50%. In the same way, in mice the reduction is around 30%. Once again, the females are far more sensitive than the males: the reduction is 60%. For a long time, it was impossible to reduce the parasite burden when rodents were boosted. Thus, another immunogenic compound has been synthesized.

As shown in Figure 13, the immunization of hamsters, followed by two boosts, leads to a significant reduction of the worm population, compared to hamsters which have received no injection (control) or a nonprotective antigen (placebo).

In addition, as shown in Figure 14, the corresponding ecdysteroid anti-

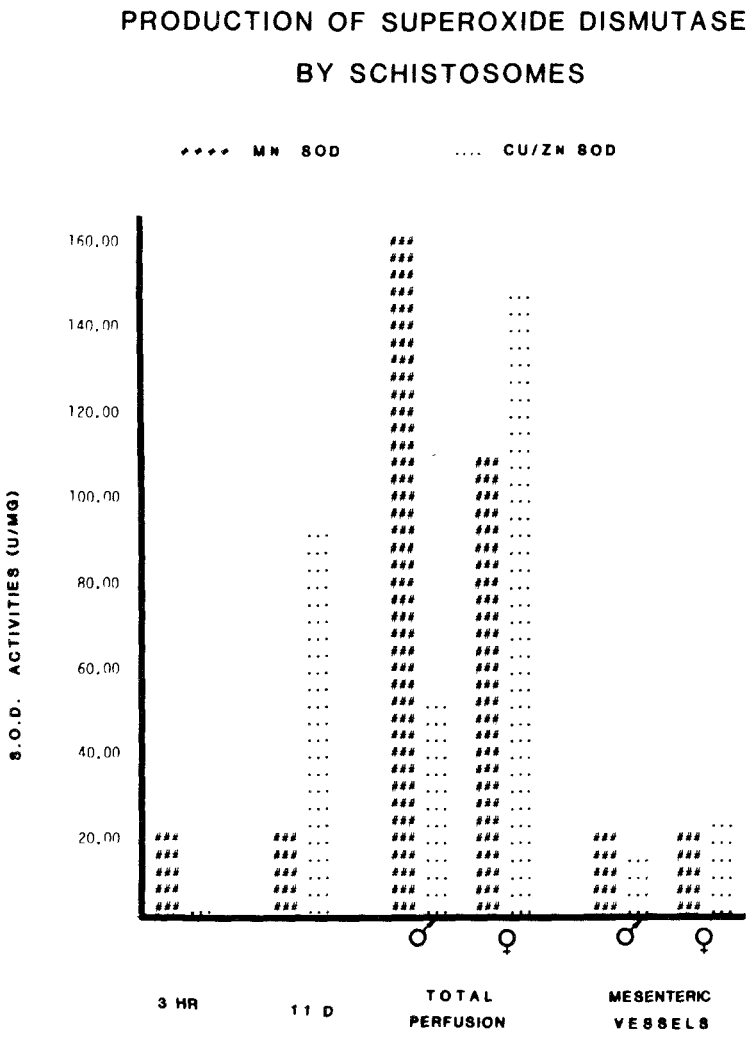

FIG. 10. Variation in superoxide dismutase activities during the development stages of *Schistosoma mansoni*.

bodies thus produced appear to be cytotoxic for schistosomula within 24 hr. For low dilution of the sera (1/4), this cytotoxicity reaches 100% within 24 hr of incubation; otherwise no cytotoxicity occurs. When samples were heated before incubation, the cytotoxicity remained at the same value. As shown in Figure 15, scanning microscopy on the in vitro cultured schistosomula has been investigated. This figure indicates that parasites incubated with the antibodies from immunized hamsters shed their membrane complex. This phenomenon

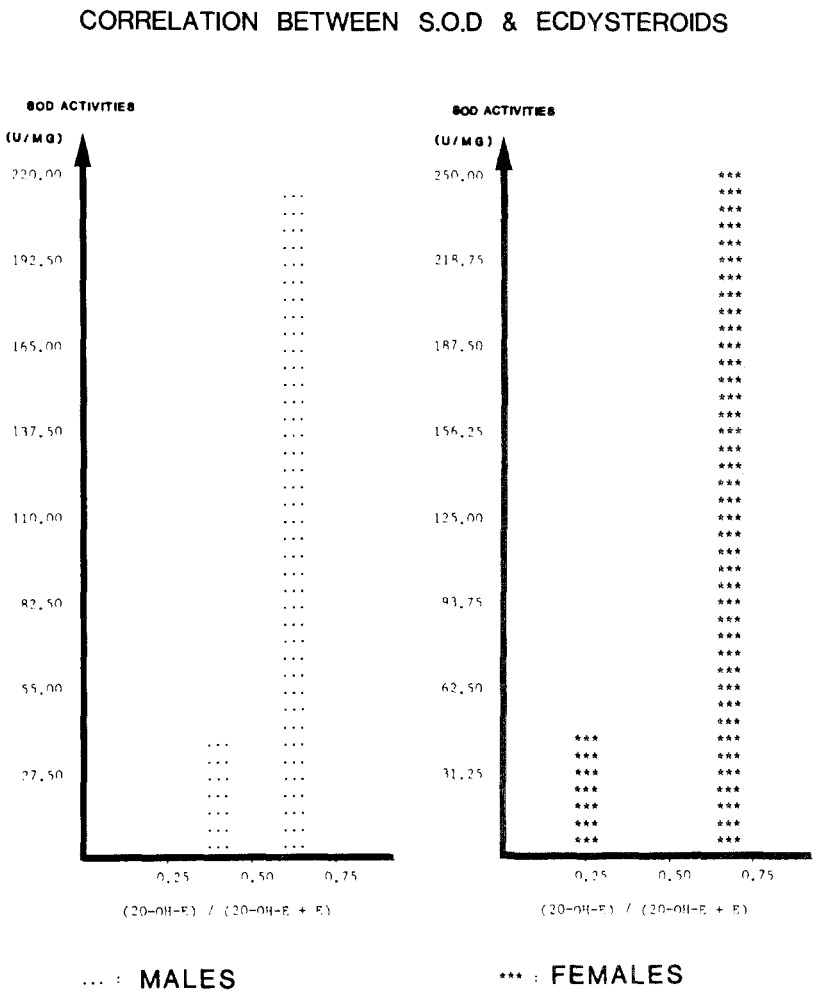

FIG. 11. Correlation between total superoxide dismutase and the ratio of 20-hydroxyecdysone to total ecdysteroids within adult *Schistosoma mansoni*.

leads to the death of the worms. The velvet-like appearance on the surface of the scoured parasites corresponds to the loss of the external tegument of *S. mansoni*.

DISCUSSION

We have demonstrated that the two hormonal activities, ecdysone and 20-hydroxyecdysone, were present at two crucial periods of the development of the bisexual trematode *Schistosoma mansoni*.

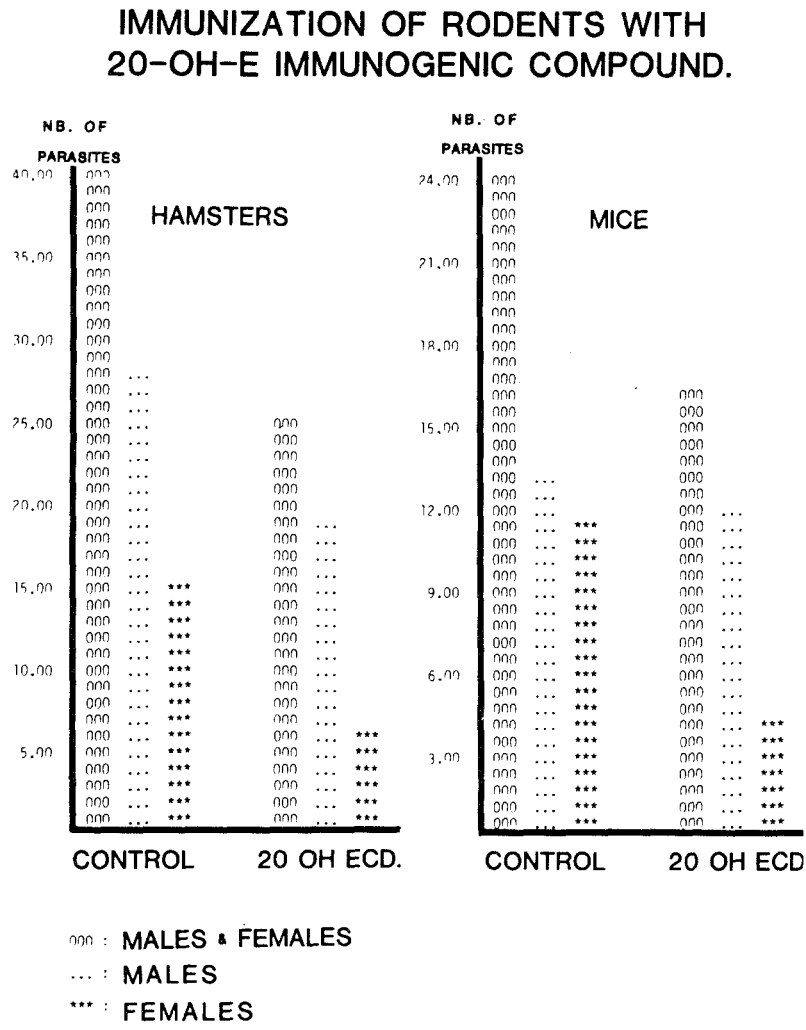

FIG. 12. Effects of a single immunization of an ecdsyone immunogenic compound on the parasite burden.

Firstly, the day 6 to day 11 period after infection is a transition period between the juvenile worm and its exponential growth phase to the bisexual stage. The high amount of ecdysteroids in 11-day-old worms, compared with others, indicates an endogenous synthesis by *S. mansoni*. Because the definitive host does not produce ecdysteroid hormones, passive incorporation of ecdysteroids from the host can be excluded. This rise in ecdysone levels can be correlated to three linked events: (1) The first event is migration and the development of juvenile worms which have left the lungs and just reached the liver.

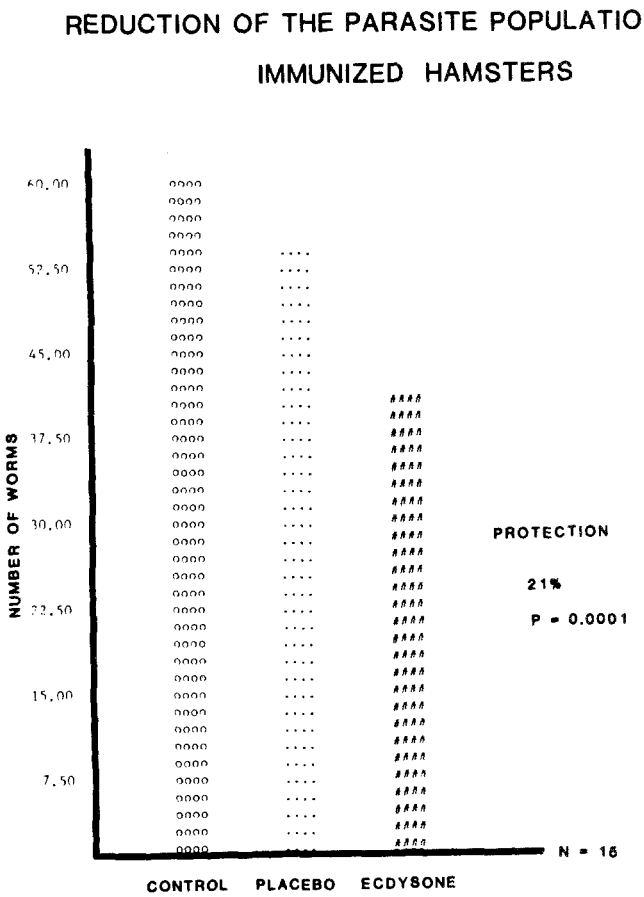

FIG. 13. Effects of immunization with two boosts of an ecdysone immunogenic compound on the parasite burden.

Wilson and coworkers (1978) have demonstrated that the process of transformation from the lung to liver form is characterized by a number of changes in the biological functions of schistosomes. They lose their ability to migrate, and their length and shape are modified. (2) During the same period, metabolic changes occur in the parasite, which leaves a semiquiescent metabolic state. As demonstrated by Lawson and Wilson (1980), an increase in density, nitrogen content, and growth initiation occur. (3) The rise in ecdysteroid is also correlated to membrane modifications of *S. mansoni*, as shown by Torpier et al. (1982).

The second crucial period in adult males and females is the priod of sexual maturation characterized by egg laying. The ratio between ecdysone and 20-hydroxyecdysone appears to be different according to the anatomical localization of the adult worms in mammalian hosts.

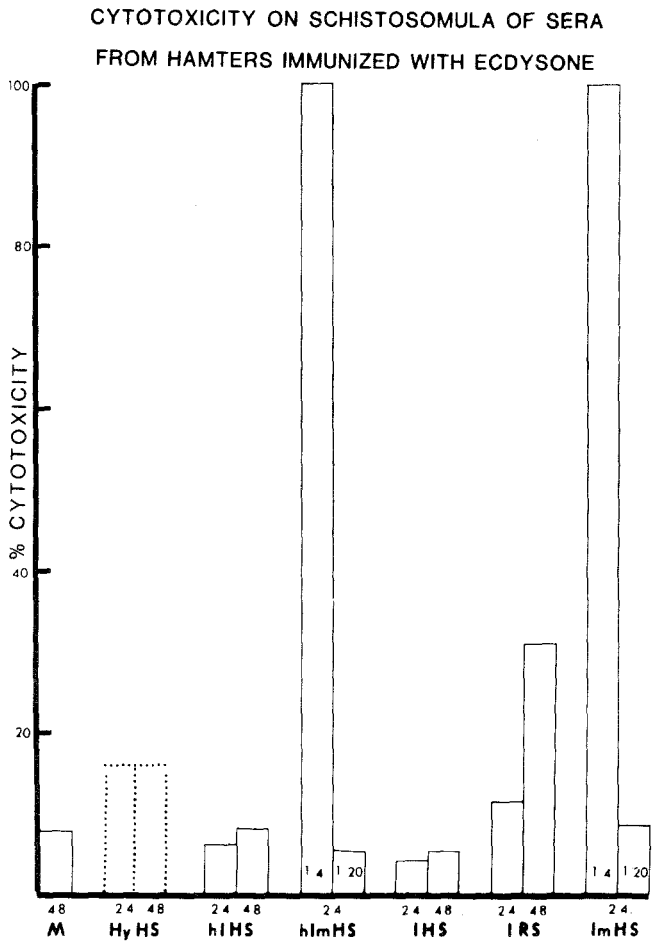

FIG. 14. In vitro cytotoxicity of natural ecdysone antibodies on schistosomules of *Schistosoma mansoni*.

These data indirectly suggest that ecdysteroids would have an important role in inducing the maturation of the female reproductive system. Our work has also demonstrated that the newly derived schistosomula contain ecdysteroids. As our experiments were conducted at 4°C and as the intermediate snail host produced ecdysteroid hormones (Nirdé and Herbert, unpublished), our hypothesis was that this large amount of ecdysteroid hormones found in 3-hr schistosomula might come from the snail host rather than from the parasite itself. In addition, the relatively high level of ecdysteroids during this first larval stage indicates that juvenile schistosomes leave the intermediate snail host with a reserve of ecdysteroids. If the *Schistosoma mansoni* model has allowed some interesting observations in the field of the production of ecdysteroids in para-

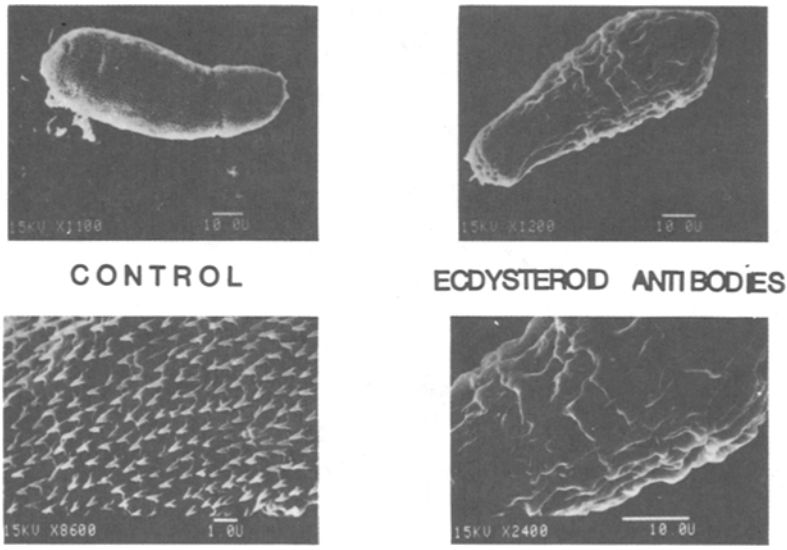

FIG. 15. Scanning microscopy on cultured schistosomula after a 24-hr period of incubation with the natural ecdysone antibodies.

sites, it is obvious that this model is not the most suitable for further investigation in the pathway of ecdysteroid hormones in worms. It must be emphasized that the metabolic pathway of ecdysterone in insects has been studied in ovaries where the hormonal level was more than 10^{-3} M. Therefore, the low hormonal level found in parasites, around 10^{-15} M, has not allowed the chemical characterization of these products by mass spectroscopy.

Our work has demonstrated that the serum of infected rodents contains ecdysteroids as early as six days postinfection. Moreover, the changes in hormone level are different between permissive and nonpermissive hosts. These important observations suggest that the concentration of ecdysteroids could be an early biological parameter of parasite infection and may be correlated with the permissiveness of the host. We also have shown that ecdysteroids were present in the serum of rodents infected with the nematode *Dipetalonema vitae*. In addition, we have demonstrated that ecdysteroids were present in the urine of infected children. It is the first demonstration of excreted ecdysteroids by organisms. These hormones have been characterized by GC and MS as the 20-hydroxyecdysone, the 5-epi-20-hydroxyecdysone and the 3-oxo-20-hydroxyecdysone. Our work also shows that the moulting hormones decrease markedly after an antischistosome chemotherapy. According to these data, we therefore confirmed that the ecdysteroids were correlated with the viability of the parasites. Moreover, the release of ecdysteroids by schistosomes might be a general

feature of parasite infection. This is true for trematodes and nematodes. Our work on this point has been confirmed by other teams (Koolmann et al., 1984). This important observation, that human beings may have insect-molting hormones in their biological fluids, could lead to an early diagnosis of parasite infection.

In arthropods, it is well known that ecdysteroids induce protein synthesis. Two of them appear interesting to study with regards to parasite physiology. The first one is the enzyme superoxide dismutase. This enzyme catalyzes the conversion of oxygen anion to hydrogen peroxide (McCord and Fridovich, 1969). Superoxide dismutase could be involved in escape mechanisms of worms against oxygen anions produced by the immune cells: macrophages and eosinophils.

Our work demonstrated that superoxide dismutase was produced by schistosomes. The two SODs were present during the critical development stages of the parasite. Once again, the ratio of Mn SOD to Cu/Zn SOD was different between males and females. The females contained more cytosolic SOD than the males in both the mesenteric and the total perfusion. However, parasites collected from total perfusion of hamsters exhibited more SOD activities than parasites collected exclusively from the mesenteric vessels. Moreover, the total SOD activities could be correlated with the ratio of 20-hydroxyecdysone to total ecdysteroids within parasites. As 20-hydroxyecdysone is more active than ecdysone, in the physiological sense, our hypothesis is that the 20-hydroxyecdysone, or its conjugates, induces this enzyme in *Schistosoma mansoni*.

The second interesting enzyme is the dopa decarboxylase. Gathering together the publications on ecdysone, serotonin, and dopa decarboxylase, the schema in Figure 16 indicates what the action of ecdysone on egg shell formation may be. Two pathways are possible for the synthesis of melanin. The first one involves the serotonin molecule, the enzymes dopa decarboxylase (EC 4.1.1.28), and hydroxyindole-*O*-methyltransferase. On one hand, the enzyme dopa decarboxylase is known to be under the control of ecdysone (Kraminsky et al., 1981), and, in schistosomes, decarboxylates 5-OH-tryptophan as well as L dopa (Catto, 1981). On the other hand, serotonin is abundant in mesenteric vessels, and *S. mansoni* contains high concentrations of this biogenic amine. In addition, the enzyme hydroxyindole-*O*-methyltransferase is inhibitied by estradiol around 1 mM whereas it is activated by low steroid levels (Cardinali et al, 1981). Thus the amount of circulating estradiol could modulate the production of melanin. This fact must be correlated to the experiments of Barrabes and coworkers (1980), who demonstrated that large amounts of estradiol reduce egg production by schistosomes.

The second pathway involves L-dopa and dopa decarboxylase. At present, no direct experiments have been done to verify this schema, but it must be kept in mind when the induction of the female reproductive system is investigated.

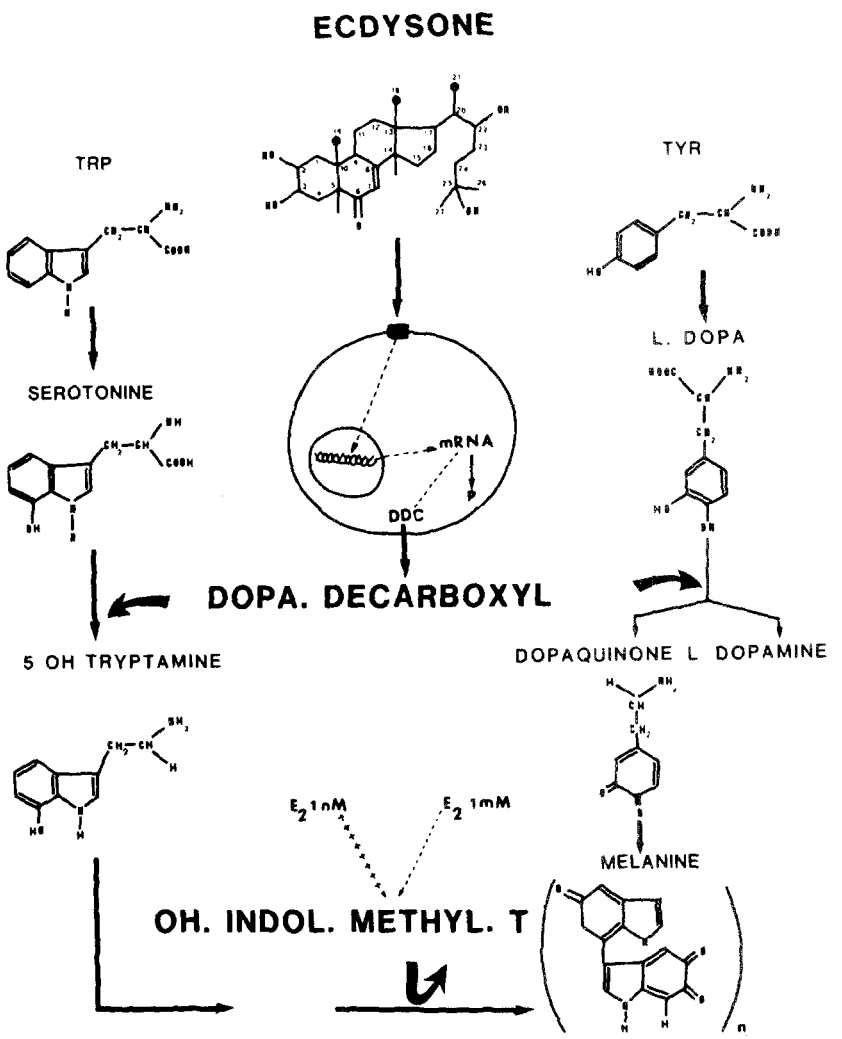

FIG. 16. Action of ecdysone on egg shell formation.

We certainly do not claim that we have achieved a high degree of protection. It is obvious that our experimental protocols have to be optimized. However, this partial result indicates that immunization with ecdysteroids might lead to a reduction of the worm burden. Although no direct evidence has been obtained on the physiological role of ecdysteroids within parasites, close correlations between the presence of significant amounts of hormones and some physiological phenomena are thought provoking. In this regard, the involvement of ecdysteroid hormones seems evident: (1) in parasite morphogenesis,

(2) in sexual maturation of the female reproductive system, and (3) in the induction of enzymes implicated in parasite escape mechanisms. Moreover, because humans do not produce ecdysone, the cytotoxic effect of ecdysteroid antibodies might be a novel strategy for immunoprophylaxis of parasite infections in man.

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DEVELOPMENTAL CHANGES IN ENERGY METABOLISM OF *Schistosoma mansoni* AND PHYSIOLOGICAL ROLE OF OXYGEN IN MAINTAINING PARASITE FUNCTION

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Abstract—The time course of the conversion from a cyanide-sensitive to a cyanide-insensitive energy metabolism in immature *Schistosoma mansoni* was followed by correlating transitions in CO_2 and lactate formation with physiological properties of the parasite. Volume conducted electrical potentials and measurement of CO_2 evolution indicate that 3-hr posttransformational schistosomula are highly sensitive to 1 mM cyanide. By 24 hr after transformation, evolution of CO_2 under control conditions is reduced by 77% from 3-hr levels, while lactate excretion rises by 84%. At the 24-hr stage, neither cyanide nor rotenone affects the frequency or magnitude of endogenous electrical transients, but cyanide does eliminate 83% of the already reduced levels of CO_2 evolved in 24-hr schistosomula. The adult parasite evolves a low level of CO_2 which is reduced by 88% in the presence of 1 mM cyanide. No significant Pasteur effect is detected, however, and endogenous electrical activity as well as mechanical responses of the adult musculature are unaffected by cyanide exposure. Furthermore, adult schistosomes were not adversely affected in terms of the physiological parameters measured by 24-hr incubations in oxygen-free medium. Adults were only marginally affected by 24-hr exposure to several respiratory inhibitors, but responded rapidly to some uncouplers of oxidative phosphorylation, including 2,4-DNP, carbonyl cyanide 3-chlorphenylhydrazone, and closantel. Our results indicate that schistosomula continue to rely on cyanide-sensitive respiratory components for at least 3 hr after transformation; by 24 hr, however, the parasites are metabolically similar to the adult stage, i.e, they depend on lactate fermentation for most of their energy requirements.

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Key Words—*Schistosoma mansoni*, glucose metabolism, electrophysiology, cyanide, antimony.

INTRODUCTION

Fermentation of glucose to lactate in the adult trematode, *Schistosoma mansoni* occurs both under aerobic and anaerobic conditions. Glycolysis is the principal source of energy, and no Pasteur effect is observed (Bueding, 1950; Schiller et al., 1975; Bueling and Fisher, 1982). During the cercarial stage of the parasite's life cycle, however, most energy is derived by oxidative metabolism (Oliver et al., 1953; Coles, 1972a). Cercariae are killed within 4 hr in the presence of 1 mM cyanide, and addition of glucose (1 mg/ml) does not prolong their lives (Coles, 1972b). However, Coles (1972b) reported that cercariae do exhibit a Pasteur effect under nitrogen and that lactate is excreted under anaerobic conditions only.

Between the aerobic cercariae and anaerobic adult stages of the parasite's life cycle, there is a period of transition during which the recently penetrated schistosomulum undergoes a number of important changes. Morphologically, the parasite loses its tail and develops a heptalaminate outer tegumental membrane (Hockley and McLaren, 1973). Immunologically, the worm undergoes a rapid, nearly linear decline in susceptibility to rejection by passively transferred immune serum (Sher, 1977), possibly due to surface changes which inhibit recognition and/or binding by host immunoglobulins (Dean, 1977). Most evidence suggests that these changes occur rapidly and are, for the most part, completed within 24 hr after the parasite enters the host.

The conversion from the aerobic metabolic state of cercariae to the predominantly anaerobic one of adult schistosomes is important because of its possible functional relationship with changes in immuno- and chemosensitivity. The time course of this metabolic conversion, however, is not well documented. Previous studies have failed to provide quantitative data correlating metabolic conditions with the physiological status of the parasite. Additionally, most of these studies fail to measure evolved CO_2, the major by-product of oxidative metabolism, and rely primarily on enzymatic assays which are less sensitive than readily available chromatographic techniques.

METHODS AND MATERIALS

Parasite Preparations and Incubation Media. A Puerto Rican strain of *S. mansoni* was maintained by passage through Swiss Webster mice and *Biomphalaria glabrata* snails. Cercariae were collected in dechlorinated water by exposing 100–150 infected snails to light for 1–2 hr. Cercarial suspensions were passed through a wire grid to remove snail feces and other debris. Cercariae

were counted under a microscope and maintained in dechlorinated water at 30°C prior to use. Bacterial contamination in all suspensions of organisms was reduced by using sterilized glassware and by adding 100 units/ml penicillin plus 100 μg/ml streptomycin to media prior to sterile filtration.

Schistosomula, the larval or skin stage parasites, were prepared from the same batch of cercariae by two methods. Mechanically transformed schistosomula were obtained by a modification of the method of Ramalho-Pinto et al. (1974). Cercariae were placed in 15 ml conical centrifuge tubes and cooled to 4°C for 10 min to reduce motility. After centrifugation at low speed for 1 min, the supernatant was decanted and replaced by a cold 1 : 1 mixture of filtered horse serum and RPMI-1640 (HS/RPMI; Gibco, Grand Island, New York) buffered to pH of 7.4 with 25 mM HEPES. To remove the parasites' tails and thereby initiate transformation, the packed cercariae were resuspended and stirred for 1 min at a moderate speed on a vortex mixer. After the suspension was centrifuged, the tail-rich supernatant was discarded and the body-rich pellet resuspended in additional HS/RPMI. This process was repeated six times, so that the final suspension of parasites contained fewer than 6% tails or nontransformed cercariae.

Skin-penetrated schistosomula were prepared by a modification of the method of Cousin et al. (1981). Approximately 1000 cercariae in dechlorinated water were pipetted onto the cleaned inner surface of the ears of uninfected, ether-anesthetized Swiss Webster mice and allowed to penetrate for 45 min. The mice were killed by cervical dislocation and nonpenetrating cercariae were removed by cotton swabs. The ears were removed and finely minced in a 20-ml Petri dish containing 5 ml HS/RPMI. After a 1-hr incubation at 37°C with continuous agitation, motile schistosomula were individually transferred by pipet into vials containing HS/RPMI and maintained at 37°C. Schistosomula were transformed from cercariae 3 hr or 24 hr prior to testing, and maintained in HS/RPMI. Transformation was verified by means of the methylene blue dye exclusion test (Clegg and Smithers, 1968).

To determine the effects of protein synthesis inhibition on the metabolic conversion of the schistosomulum, 200 μg/ml puromycin (in distilled water; Sigma Chemical Co., St. Louis, Missouri) was added to cercarial preparations 30 min prior to mechanical and skin transformation. These parasites were maintained in a similar concentration of puromycin during all subsequent incubations and electrophysiological recordings which were conducted 24 hr after transformation. Electrophysiological (but not biochemical) assays were also obtained from schistosomula transformed and maintained in 0.1 mM colchicine or 10 μM cytochalasin B to assess the importance of microtubule and microfilament-mediated processes on the conversion to a physiological cyanide-insensitive state.

Adult parasites were obtained according to the method of Fetterer et al. (1977) by extracting worm pairs from the portal veins of cervically dislocated

mice 55 days after infection. Worm pairs were separated, and the males were placed in vials containing HS/RPMI and maintained at 37°C prior to use.

Electrophysiological and Mechanical Recordings. Endogenous electrical activity was recorded by a modification of the method described by Fetterer et al. (1977). Glass suction electrodes were manufactured from 1.25 mm capillary tubing (W.P. Instruments, New Haven, Connecticut) pulled to a fine tip on a horizontal electrode puller (Narishige, Tokyo, Japan). The electrodes were cut with a diamond pencil to obtain a flat tip, with an outside diameter of 15–30 μm. The tip was then heated on a microforge (Aloe Corp., New York, New York) which melted the glass from the inside, until the flattened tip became smooth, and the aperture was reduced to approximately 2 μm for recording from schistosomula, or 15–20 μm for recording from adults. Electrodes were firmly attached to the parasite's surface during recordings by way of negative pressure created by a 10-ml syringe. Prior to attachment of the parasite, Hanks' balanced salt solution (HBSS) was drawn into the electrode until it made contact with a silver wire connected to an amplifier (model P-15, Grass Instruments, Quincy, Massachusetts). Electrical signals from the parasites were filtered with the low pass set to 0.3 Hz and the high pass to 1 kHz. Signals were displayed on an oscilloscope (model 5113, Tektronix Inc., Beaverton, Oregon) or a chart recorder (Narco Biosystems Inc., Houston, Texas), and passed onto an analog-to-digital converter prior to computer analysis (Alis I-10 Data Acquisition and Control System, Ecotech Inc., Lansing, Michigan).

All recordings of endogenous electrical potentials from parasites exposed to potassium cyanide (CN^-), rotenone (both from Sigma Chemical Co.), or potassium antimony tartrate (KSb tartrate; Fisher Scientific, Fair Lawn, New Jersey) were obtained either 1–2 hr or 24–28 hr after the parasites were preincubated in the presence of the drug. Parasites incubated in the presence of vehicle served as controls.

After electrode attachment, a 2–4 min equilibration period was allowed prior to electrical recording. Electrical activity was quantified by counting all negative field potentials in excess of 20 μV over six 10-sec intervals. Data from these intervals were then averaged. Potentials under 20 μV were not counted as they were within the noise range of the system. Electrical responses were measured in at least eight control parasites and eight parasites exposed to each experimental substance. Statistical analysis for significance of difference between means used Student's nonpaired t test.

The effects of oxygen-free medium, or exposure to respiratory inhibitors or an inhibitor of the TCA cycle on tegumental membrane ionic gradients, were measured as previously described (Thompson et al., 1982). In the present study, all recordings were obtained after 24-hr exposures. Under each condition, recordings were obtained from at least five parasites during each of at least three separate experiments.

Muscle tension recordings were obtained from adult schistosomes using the method described by Fetterer et al. (1977). The effects of inhibitors on the energy-dependent muscle tension changes induced by exposure to elevated potassium (60 mM), which increases muscle tension in schistosomes, or the acetylcholine analog carbachol (0.1 mM), which reduces muscle tension in schistosomes, were assessed by preincubating the parasites for 1–2 hr or 24–28 hr in 1 mM CN^- or 0.1 mM KSb tartrate-containing media prior to exposure to the tension-altering treatments. Mechanical responses were measured in at least five control parasites and five parasites preincubated in drug.

Incubation in [^{14}C]Glucose-Containing Media and Analysis of Metabolites. In biochemical experiments involving immature stages, parasites were concentrated to 12,000/ml in HS/RPMI containing antibiotics. Only mechanically transformed schistosomula were used in biochemical studies, since the skin-transformed parasites were difficult to obtain in sufficient quantities. Aliquots of 400 μl (containing 5000 schistosomula) were pipetted into the center wells of 15 ml Warburg reaction vessels. Drug-treated parasites were concentrated in incubation media containing 1 mM CN^- or 0.1 mM KSb tartrate 1 hr before testing. In experiments involving adult schistosomes, six males were placed into the Warburg vessels along with 400 μl HS/RPMI. Heat-killed controls of immatures and adults were preincubated for 15 min at 56°C immediately prior to testing.

After placement of parasites, uniformly labeled D-[^{14}C]glucose (1.4 μCi, specific activity = 348.2 Ci/mol; New England Nuclear, Boston, Massachusetts) was added to the center well of each vessel. To capture evolved $^{14}CO_2$, 1 ml of 1 N sodium hydroxide, freshly prepared in boiled distilled water, was added to the base of the Warburg vessels. The vessels were then placed on a shaker bath and maintained at 37°C for a period of 4 hr. Following incubation, the vessels were cooled on ice for 15 min prior to sample collection. For measuring evolved $^{14}CO_2$, three 200-μl aliquots of the 1 N NaOH were pipetted from the base of each vessel into scintillation vials containing 50 μl glacial acetic acid and 6 ml aqueous scintillant (Amersham Co., Arlington Heights, Illinois), and counted on a Beckman LS-7,000 liquid scintillation system (Beckman Instruments, Fullerton, California).

Acid-soluble metabolic endproducts were fractionated with a modification of the method of Tielens et al. (1981). A 350-μl sample of incubation medium was recovered from the center well and 20 μl cold 71% perchloric acid was added. The samples were centrifuged at 1000*g* for 15 min and radioactivity was determined in the supernatant by scintillation counting. Samples (50 μl) of the supernatant were loaded onto BioRad AG 1-X10, 100–200 mesh anion-exchange columns (12 × 0.75 cm; BioRad Labs, Los Angeles, California) in chloride form using 25 mM HEPES as the primary solvent. The columns were eluted with 10 ml of 25 mM HEPES (F1), followed by 5 ml of 5 mM HCl

(F2), and 5 ml of 500 mM HCl (F3). Over 99% of the radioactivity applied was recovered. Aliquots (500 μl) of the eluted material were placed into vials containing 10 ml aqueous scintillant for counting. Results were converted from disintegrations per minute (dpm, counts per minute divided by efficiency of counting) to nmol CO_2 or lactate produced. Labeled substrates from the column fractions were identified by descending paper chromatography, with butanol–water–acetic acid (25 : 10 : 4) as developing solvent. In control and CN^- experiments, only 10–12% of the glucose was degraded over the 4 hr incubation periods. Over 90% of the acid-soluble fraction comigrated with and exhibited the expected retention factor of lactate. Total protein was determined by the method of Albro (1975) using bovine serum albumin standards. All tests were run in triplicate on at least three separate occasions ($N \geq 9$), except studies involving KSb tartrate ($N \geq 4$). Variation among triplicate tests was usually less than 15%. Statistical analysis of these results were based on comparison of dpm/mg protein for each sample, using Student's nonpaired t test.

RESULTS

Volume Conducted Electrical Potentials. Endogenous electrical activity recorded from the surface of schistosomula and adult *S. mansoni* consisted of multiphasic potentials ranging from 20 to 200 μV in amplitude and 10 to 300 msec in duration (Figure 1). Under control conditions, these potentials occurred at a rate of 30–40/sec and exhibited rise times of 3–8 μV/msec. There was an exponential decrease in the frequency of potentials with increasing amplitude. Characteristics of electrical activity recorded from all stages of development were essentially identical under control conditions, although the adult parasite showed some regional variation in the frequency of potentials.

After a brief (1–3 min) episode of enhanced activity that normally followed electrode attachment, the frequency of low amplitude potentials was relatively constant over a period of up to 30 min. Large amplitude potentials (>50 μV) occurred more sporadically and appeared to correlate with peristaltic-like contractions of the parasite, suggesting that these transients may originate in underlying muscle bundles. To verify that the electrical activity originated from within the parasite, controls were conducted by recording from worms before and after addition of 0.5 ml 50% ethanol to the recording medium. This treatment immediately immobilized the parasites and eliminated nearly all electrical transients (Figure 1).

Developmental Changes in Glucose Metabolism. The results presented in Figure 2 (open bars) demonstrate that schistosomes undergo a pronounced metabolic alteration during development from the 3-hr schistosomulum to the adult stage. Most of this change appears to be complete by 24 hr after transformation, at which point CO_2 evolution is reduced by 77% from 3 hr levels, while lactate

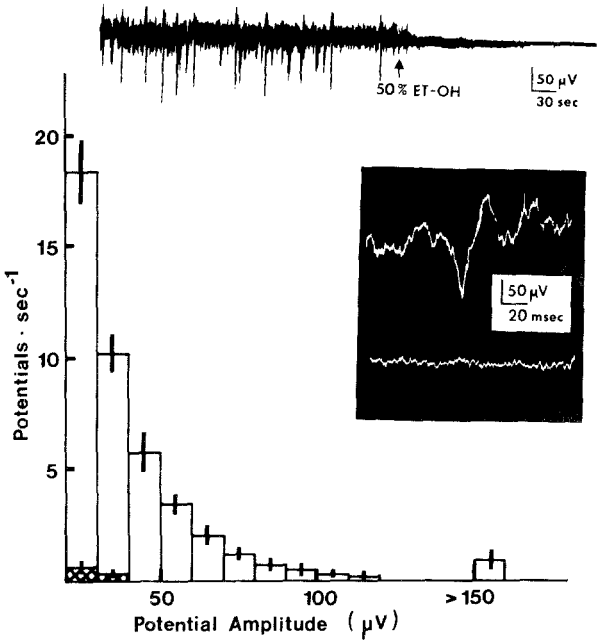

FIG. 1. Electrical activity recorded from the surface of mechanically transformed 3-hr schistosomula under control conditions and after the addition of 50% ethyl alcohol to the recording medium. Upper: slow speed chart recording showing decline in electrical activity after alcohol exposure. Middle right: high speed oscilloscope traces obtained from the same parasite before (upper) and after (lower) exposure to alcohol. Note the elimination of all large amplitude multiphasic potentials. Lower: amplitude–frequency distribution of endogenous electrical transients. Open bars represent electrical activity recorded from schistosomula incubated in HS/RPMI; hatched bars: activity from the same parasites 1 min after the addition of alcohol. Vertical lines are 1 SE; $N = 8$.

excretion nearly doubles, to a level only 18% less than that measured in the adult.

Developmental Changes in Physiological Responses to Metabolic Inhibitors. Electrophysiological recordings indicate that schistosomes undergo a significant developmental alteration in responsiveness to CN^- and rotenone, while their response to KSb tartrate is consistent throughout the course of development (Figure 3). The frequency and mean amplitude of endogenous electrical transients recorded from 3 hr mechanically and skin-derived schistosomula were significantly depressed after a 1 hr incubation in 1 mM CN^-. The degree of CN^--induced depression was essentially identical to that induced by a 1-hr incubation in 0.1 mM KSb tartrate, indicating that the 3-hr posttransformational stage may rely on a CN^--sensitive terminal oxidase for generating some of the energy used to sustain membrane electrical events.

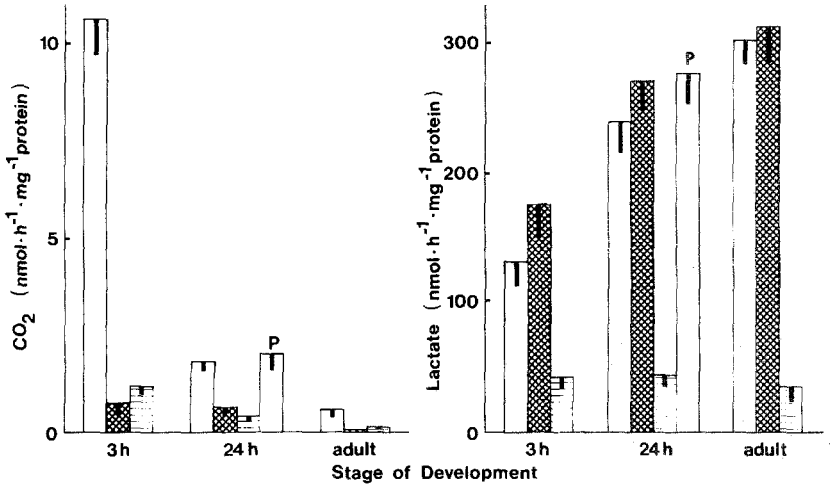

FIG. 2. Developmental changes of *S. mansoni* in the distribution of metabolic endproducts and the effects of metabolic inhibition due to 1 mM CN^- (cross-hatched bars) or 0.1 mM KSb tartrate (lined bars) on CO_2 (left) and lactate (right) produced during 4-hr incubations in D-[^{14}C]glucose-containing media. CO_2 and lactate produced by 24-hr schistosomula incubated in 200 μg/ml puromycin (P) were similar to control levels (open bars). Values for CO_2 based on dpm in NaOH aliquots; those for lactate based on dpm of acid-soluble fractions (F3) obtained from ion-exchange columns. Values presented are means of at least three separate experiments performed in triplicate ($N \geq 9$); vertical lines are 1 SE.

Electrical recordings obtained from 24-hr mechanically and skin-derived schistosomula exposed to 1 mM CN^- indicate that inhibition of the terminal oxidase no longer results in a significant depression of the spontaneous potentials. Since electrical activity in both groups was unaffected by CN^-, it appears that the method of transformation does not significantly alter this conversion process. Based on electrophysiological responses, the expression of CN^--insensitivity by 24 hr is not affected by the presence of 200 μg/ml puromycin, indicating that new protein synthesis is not required for this conversion. The apparent metabolic conversion is similarly unaffected by the presence of the cytoskeletal inhibitors colchicine or cytochalasin B, as 24-hr schistosomula exposed to either of these drugs also became resistant to cyanide-induced depression of the transmembrane electrical transients. Consistent with this finding, the 24-hr schistosomula exhibited a lower level of sensitivity to rotenone than that recorded at the 3-hr stage of development.

The adult parasite, like the 24-hr schistosomulum, exhibited no change in electrical activity in response to 1 mM CN^-, even after 24-hr incubations (Figure 3). Inhibition of glycolysis by 0.1 mM KSb tartrate, however, eliminated 75% of the potentials within 1 hr, and by 24 hr the electrical activity was totally eliminated.

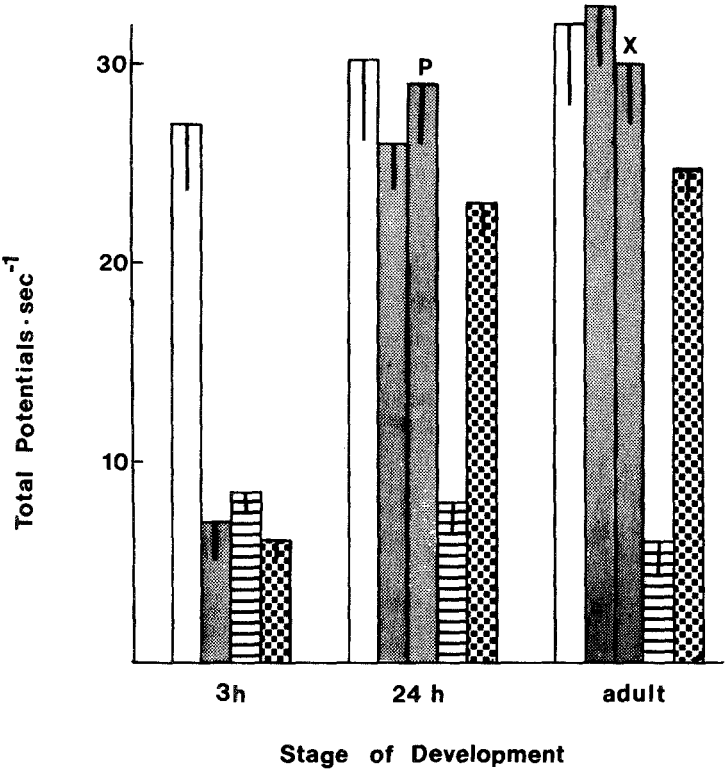

FIG. 3. Frequency of endogenous electrical potentials recorded from developmental stages of *S. mansoni* under control conditions (open bars) or after 1-hr incubations in media containing 1 mM CN^- (cross-hatched bars), 0.1 mM KSb tartrate (lined bars), or 10 μM rotenone (checkered bars). Values obtained from skin- and mechanically transformed (shown here) schistosomula were similar. The frequency of potentials recorded from 24-hr schistosomula preincubated in 200 μg/ml puromycin (P) prior to CN^- exposure was similar to control levels. Similar values were recorded from 24-hr schistosomula preincubated in 0.1 mM colchicine or 10 μM cytochalasin B (not shown). Adult parasites incubated 24 hr in 1 mM CN^- (X) exhibited control levels of electrical activity. Vertical lines are 1 SE; $N \geq 8$.

Intracellular recordings obtained from the adult schistosome revealed that the parasite's response to metabolic inhibition varies significantly with the treatment used (Table 1). In general, depolarization of the parasite's tegument was most pronounced after 24-hr preincubations under glycolytic inhibition (KSb tartrate).

Muscle tension recordings obtained from adult schistosomes indicate that energy obtained from respiration is not essential for muscle function in the mature organism. Introducing a medium containing 60 mM potassium into the recording chamber resulted in a rapid increase in longitudinal muscle tension,

TABLE I. EFFECTS OF METABOLIC INHIBITORS ON TEGUMENT POTENTIAL (E_{Teg}) RECORDED FROM ADULT MALE *S. mansoni*[a]

Control	E_{Teg} (mV)	Experimental	E_{Teg} (mV)
Aerobic medium (under air)	−52.2	Anaerobic medium (< 1% O_2 saturated)	−50.2
	−53.7	1 μM KSb-tartrate	−16.3
	−55.3	0.1 mM KCN	−54.1
	−53.2	0.1 mM FLA[b]	−48.6
	−52.4	10 μM rotenone	−31.7[c]
	−52.7	10 μM oligomycin	−35.2[c]

[a] For all treatments, experimental parasites were preincubated 24 hr in drug prior to microelectrode recordings. Incubation and recording medium consisted of RPMI-1640. All experiments were performed a minimum of three times, with recordings obtained from at least five parasites each time.
[b] FLA = fluoroacetamide.
[c] $P < 0.001$.

which occurred at approximately the same rate and was similarly sustained in parasites preincubated 24 hr in control medium or in anaerobic medium, or medium containing 1 mM CN^- (Figure 4, left). Similarly unaffected by preincubation in 1 mM CN^- was the relaxation of longitudinal muscle induced by exposure to 0.1 mM carbachol (Figure 4, right). These mechanical responses were significantly diminished, however, when adult parasites were preincubated for a period as brief as 1 hr in medium containing 0.1 mM KSb tartrate.

Kymographic recordings obtained after periods of acute exposure (15 min) to several compounds revealed that CN^-, oligomycin, and rotenone are all without effect on the longitudinal muscle tension of the adult parasite (Figure 5, right).

Effects of Metabolic Inhibitors on Substrate Utilization. Results shown in Figure 2 illustrate the effects of metabolic inhibitors on patterns of substrate utilization in various stages of the parasite. CO_2 production (left) was approximately equally inhibited by 1 mM CN^- and 0.1 mM KSb tartrate at all stages tested. Lactate excretion (right), however, was significantly inhibited only by exposure to KSb tartrate, which depressed levels by 80–90% at each stage of development. No significant CN^--induced Pasteur effect was detected in any stages of *S. mansoni* tested.

Consistent with results obtained from electrophysiological recordings, the presence of 200 μg/ml puromycin throughout the transformation process and incubation period of 24-hr schistosomula did not inhibit their conversion to a predominantly anaerobic metabolic state. This is evidenced by the low levels of CO_2 and high levels of lactate produced by these parasites.

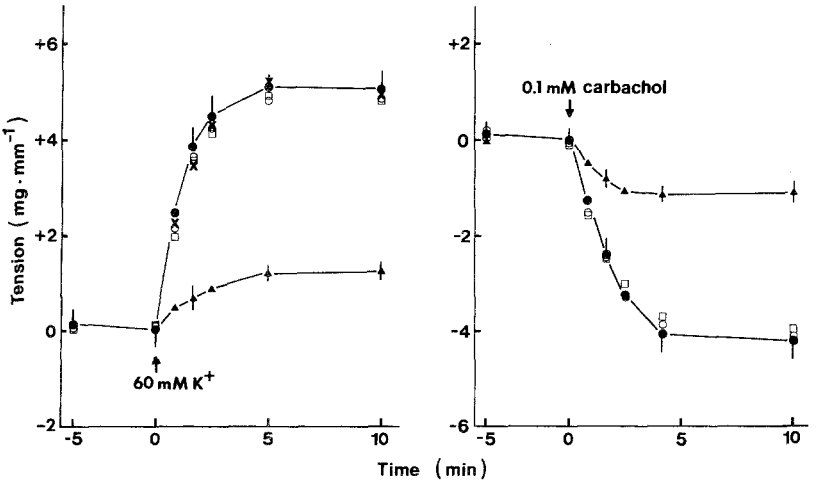

FIG. 4. Effects of incubation for 1 hr (open circles) or 24 hr (open squares) in 1 mM CN^-, 24 hr under nitrogen (Xs), or 1 hr in 0.1 mM KSb tartrate (triangles) on longitudinal muscle tension changes induced by exposure to 60 mM potassium (left) or 0.1 mM carbachol (right) in adult male *S. mansoni*. Closed circles represent muscle tension recorded in control parasites exposed to the same tension-altering treatments. For all treatments, $N \geq 5$; vertical lines are 1 SE.

DISCUSSION

Previous studies have demonstrated that measurement of endogenous electrical potentials provides a sensitive assay for agents affecting the neuromuscular system of immature and adult schistosomes (Fetterer et al., 1977; Semeyn et al., 1982; Mellin et al., 1983; Thompson et al., 1984). By this technique, the inhibitory effects of carbachol and dopamine on the schistosome can be detected at concentrations as low as 10 nM, while the stimulatory effects of serotonin are detectable at 0.1 μM. These drug concentrations are 100× lower than those required to measure detectable changes by previously used techniques. Our results indicate that a high correlation also exists between endogenous electrical activity and the metabolic state of the parasite. That is, metabolic inhibition at any stage of development is associated with a significant depression in endogenous electrical activity. Since the transmembrane electrical gradients which give rise to these potentials in schistosomes are maintained, in part, by energy-dependent processes (Fetterer et al., 1981), it is not surprising that they are sensitive to metabolic inhibition.

Based on electrophysiological recordings, 3-hr schistosomula were depressed equally by 1 mM CN^- and 0.1 mM KSb tartrate. Evolution of CO_2 by the 3-hr schistosomulum was reduced by almost 90% during 4 hr exposures to

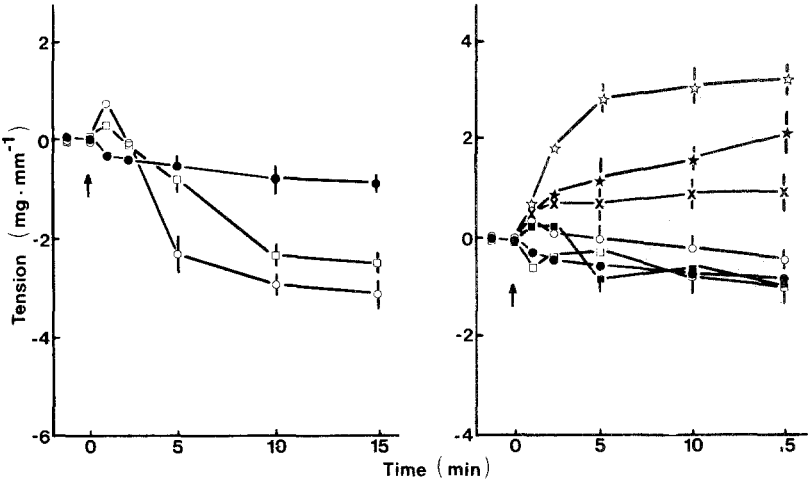

FIG. 5. Effects of acute drug exposure on longitudinal muscle tension in adult male *S. mansoni*. Left: tension-reducing effects of KSb tartrate at 0.1 mM (open circles) and 10 μM (open squares). Right: exposure to oxidative uncouplers, including 1 μM carbonyl cyanide 3-chlorophenylhydrazone (open stars), 10 μM closantel (closed stars), and 0.1 mM 2,4-DNP (Xs), induce significant increases in longitudinal muscle tension. None of the respiratory inhibitors examined, including 10 μM oligomycin (open circles), 10 μM rotenone (open squares), or 1 mM CN^- (closed squares) induced significant changes in longitudinal muscle tension over the periods examined. Closed circles represent muscle tension recorded in control parasites. For all treatments, $N \geq 5$; vertical lines are 1 SE.

these treatments. There is good evidence that the trivalent antimonials, at this concentration, inhibit schistosome phosphofructokinase both in vivo and in vitro (Mansour and Bueding, 1954; Bueding and MacKinnon, 1955; Coles and Chappell, 1979). The high degree of CN^- sensitivity obtained in electrophysiological recordings of 3-hr schistosomula was somewhat surprising, in light of findings that these parasites already excrete nearly 60% as much lactate as 24-hr schistosomula under control conditions. Apparently, the energy requirements of the 3-hr stage cannot be met entirely by lactate fermentation alone.

A number of studies suggest that the in vivo skin penetration process effects changes in schistosomula which render them less susceptible than mechanically derived parasites to the harmful effects of antibody-mediated mechanisms of immunity (McLaren et al., 1978; Van Pijkeren, 1982). During the skin-penetration process, the cercaria's tail is shed and the contents of its preacetabular glands secreted. This loss accounts for 35–50% of the parasite's total nitrogen content (Coles, 1972b). In the present study, 30% of the total protein per 5000 cercariae was lost during the mechanical transformation process. An

additional 17% was lost between the 3-hr and 24-hr stages, presumably due to delayed secretion of preacetabular gland contents and/or sloughing off of residual cercarial surface glycoproteins. It is not presently known what effects residual preacetabular gland enzymes or glycocalyx, both present to a greater extent in mechanically transformed than skin-transformed schistosomula (Cousin et al., 1981), may have on metabolic processes in the parasite. Results of our physiological assays, however, indicate that the time course of the conversion to a CN^--insensitive metabolism is similar for both groups.

In the 24-hr schistosomulum, oxidative phosphorylation appears to play a less significant role in energy production than in the 3 hr schistosomulum, since 1 mM CN^- did not eliminate a significant portion of the endogenous electrical potentials. The absence of a physiological response to CN^- in this case may be explained on the basis of the increase in lactate production which occurs between the 3-hr and 24-hr stages.

Several studies have attempted to elucidate the molecular mechanisms regulating the switch from an aerobic to an anaerobic metabolism in schistosomes. Lactate dehydrogenase was found not to change during this metabolic shift (Von Kruger, 1978). From results obtained in the present study after transforming and incubating schistosomula 24 hr in medium containing 200 μg/ml puromycin, a potent inhibitor of protein synthesis in schistosomes during all stages of development (Kusel, 1972; Nagai et al., 1977; Tavaves et al., 1980), it appears that the conversion to CN^- insensitivity does not depend on synthesis of new proteins. The metabolic changes that occur concomitantly with transformation must therefore depend on a response from enzymes already present in the emerged cercaria.

Results of experiments on the adult schistosome presented in this study are consistent with the findings of Bueding and Fisher (1982), who report that the adult parasite exhibits no Pasteur effect and no dependence on oxygen for energy formation. Although some CN^--sensitive CO_2 was evolved during incubations of the adult parasite, the amount was less than 5% (on a per mg protein basis) of that evolved by the 3-hr schistosomulum. The ratio of lactate excreted to CO_2 evolved under control conditions clearly shows that the adult parasite relies much less on oxidative phosphorylation for its energy production than the 25% reported by Coles (1972a). These findings are consistent with our observations that exposure to CN^- does not depress endogenous electrical activity or muscle responsiveness in the adult and further substantiate the principal role of homolactic fermentation in the energy metabolism of adult *S. mansoni*.

It is important to note, however, that the female parasite does rely on oxygen for egg production (Schiller et al., 1975) and that significant strain differences may exist in the metabolism of carbohydrates by schistosomes. Such differences have already been demonstrated among various strains of cestodes (Ovington and Bryant, 1981) and nematodes (Bryant and Bennett, 1983) and,

if present among *S. mansoni*, they could account for the differences between our data and that of Cole's regarding the role of aerobic energy production in the adult parasite. If there was a tight link between ATP production via this pathway and physiological processes of the parasite, we would have predicted that compounds which inhibit the electron transport chain (i.e., oligomycin, rotenone, or cyanide) would have a rapid effect on the parasite's physiology. Since these compounds did not produce a dramatic effect upon the parasite's musculature, it seems reasonable to assume that oxidative phosphorylation is not vital for the maintenance of muscle function in the parasite. Likewise, since oxygen is the common terminal electron acceptor in the electron transport chain, we should have seen, but did not, an effect on muscle tension when oxygen tension was reduced in the parasite's environment. This is not surprising since Bueding and Fisher (1982) reported that the concentration of ATP was not reduced in parasites incubated under a nitrogen atmosphere for 18 hr.

Lastly, it has been reported that uncouplers of mammalian mitochondrial oxidative phosphorylation will produce a marked decrease in parasite ATP concentration with no associated decrease in parasite lactate production (Van den Bosche, 1985). Since we did observe a clear and rapid effect of these compounds on parasite muscle function, we remain doubtful that this rapid action is due to the uncoupling of parasite mitochondrial oxidative phosphorylation. For example, these uncouplers are all known as protonophores and thus they disrupt many proton gradients, the most important of which is the pH gradient between the parasite's extracellular environment (pH 7.4) and its intracellular environment (pH 7.2). If this gradient followed the resting membrane potential (−60 mV) of the parasite's tegument, the pH should be 6.4 rather than 7.2. Thus, like all cells (except for the red blood cell) the parasite maintains a hydrogen ion concentration considerably above the predicted value (Ross and Boron, 1981). Preliminary results, using various plasma membrane proton pump inhibitors would suggest that protonophores would be highly lethal to this parasite because of their action on nonmitochondrial proton gradients.

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EFFECTS OF STEROIDS AND STEROID SYNTHESIS INHIBITORS ON FECUNDITY OF *Schistosoma mansoni* IN VITRO

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Abstract—In vitro egg production by *Schistosoma mansoni* was examined following a 72-hr incubation period in the presence of various steroids, steroid precursors, or steroid synthesis inhibitors. Mevinolin, an inhibitor of cholesterol biosynthesis, significantly decreased egg production at 10^{-6} M. However, neither cholesterol (at 10^{-5} M) nor any of its hydroxylated derivatives (at 10^{-4} M) affected *S. mansoni* fecundity. Dianisidine, an inhibitor of cholesterol metabolism to hormonal products, was also ineffective in influencing egg production. The steroid hormones progesterone, 17α-hydroxyprogesterone and medroxyprogesterone acetate, all significantly decreased egg production; however, parasite muscle tension was also significantly reduced by the concentrations of these steroids needed to produce an effect on egg laying. Various androgenic hormones (androsterone, androstenedione, testosterone) and estrogenic hormones (17β-estradiol and estrone) demonstrated no effect on in vitro egg production. Significant elevation in the rate of parasite oviposition was seen in the presence of the corticosteroid prednisolone (at 10^{-4} M), but not by dexamethasone, a corticosteroid analog.

Key Words—*Schistosoma mansoni*, steroids, fecundity, mevinolin, dianisidine.

INTRODUCTION

The effects of hormones on the viability of *Schistosoma mansoni* in vivo have been assessed in studies wherein the host was rendered hormonally imbalanced by gonadectomy and/or steroid injections (for reviews see Schwabe and Kilejian, 1968; Solomon, 1969). Unfortunately, none of the in vivo experiments

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measured the effects of host hormonal changes on the fecundity of the schistosome. It has long been known that the presence of one sex of schistosome is important for the somatic growth and sexual maturation of the other (Sagawa et al., 1928; Severinghaus, 1928). Stimulation of the development of the female reproductive tract of the parasite is brought about through a close physical relationship with the male. Although the biochemical events which mediate this process are unknown, it may be that the male schistosome secretes a hormone-like substance which triggers vitellogenesis in the female. Therefore, an examination of the effects of hormones and steroidal compounds on in vitro egg production was undertaken.

In the host, *Schistosoma mansoni* oviposits in the mesenteric venules lining the intestinal tract at a rate of over 300 eggs per day (Moore and Sandground, 1956). The pathological consequences of schistosomiasis are due to inflammation and fibrosis in the presinusoidal portal areas of the liver. Unequivocally, it is the host immune response to the schistosome eggs which instigates the pathology (Domingo et al., 1967; Warren, 1978). Hepatosplenic disease does not develop if the onset of egg-laying is prevented, and signs of amelioration of the disease are seen if egg production is halted after the onset of the disease state (Warren, 1970). Therefore, it is critical to elucidate the factors responsible for the fecundity of the female schistosome in an effort to interfere with the process of egg production.

In the present study, various steroidal compounds and steroid metabolic inhibitors were assayed for effectiveness in enhancing or inhibiting the rate of oviposition of *S. mansoni* in vitro. While egg production by the parasite differs in in vivo versus in vitro conditions, the in vitro technique allows for a direct study of these compounds on the schistosome in the absence of effects of host metabolism and host-mediated pharmacokinetic variables which affect distribution and elimination of these compounds.

METHODS AND MATERIALS

Infections. Young female white, outbred (ICR) laboratory mice were infected intraperitoneally with approximately 250 *Schistosoma mansoni* cercariae (Puerto Rican strain). Animals were communally housed with food and water ad libitum. At 50–55 days postinfection, the mice were sacrificed by cervical dislocation, and the mature parasites were removed mechanically by the method of Fetterer et al. (1977). Fifteen pairs of mated worms were transferred to 250-ml Erlenmeyer flasks containing 50 ml of media; flasks were then incubated for 72 hr at 37°C in a shaking water bath at 20 oscillations per minute. The incubation medium was a 1:1 mixture of RPMI 1640 and heat-inactivated horse serum, adjusted to pH 7.4, to which 100 μg/ml penicillin and 100 μg/ml streptomycin were added (media components from Gibco Laboratories). Mercapto-

ethanol was added to a final concentration of 5×10^{-5} M. Steroids (obtained from Sigma or Steraloids), mevinolin (obtained from Merck, Sharp, and Dohme Research Labs), or the appropriate vehicle (DMSO or ethanol) were added to the media in a volume not exceeding 100 μl. Following incubation, the condition of the parasites was noted (i.e., degree of pairing, adherence to the flask bottom), and the eggs in the entire media sample were counted under a compound microscope. Five to seven flasks were run per concentration of each test compound along with the appropriate vehicle control.

Mechanical and Electrical Activity Measurements. Recordings of schistosome longitudinal muscle tension and contractile activity were obtained from male parasites as previously described (Fetterer et al., 1977). Electrical activity recordings from the surface of parasites were determined using suction electrodes as described by Semeyn et al. (1982). Mechanical and electrical activity measurements were made on control and treated worms following the 72-hr incubation period.

RESULTS

Cholesterol or the 20α, 22-(*S*), or 22-(*R*) hydroxylated cholesterol derivatives did not significantly affect schistosome egg production. Dianisidine (3,3-dimethoxybenzidine), a compound which inhibits the metabolism of cholesterol to hormonal products (Sheets et al., 1981), was also ineffective in altering the egg production rate (unpublished observations).

The effects of pregnenolone, progesterone, 17α-hydroxyprogesterone, medroxyprogesterone acetate (MPA), and prednisolone on egg production are shown in Figure 1. Only the progestational hormones exhibited significant inhibition of egg production. However, the degree of pairing of the parasites and adhesion to the bottom of the flask were both adversely affected by these compounds at the concentrations studied. Also, recordings showed marked depression of parasite muscle tension in response to these hormones (Figure 2). These effects were not observed in response to pregnenolone; both fecundity and the physiological parameters remained unchanged in the presence of this hormone.

The corticosteroid prednisolone (10^{-4} M) caused a significant stimulation in egg production (Figure 1), whereas dexamethasone, another corticosteroid, did not. When added along with MPA, prednisolone did not reverse the effects of this compound on fecundity.

Both estrogenic hormones [17β-estradiol (10^{-4} M), estrone (10^{-4} M)] and androgenic hormones [androstenedione (10^{-4} M), testosterone (10^{-4} M), or androsterone (10^{-4} M)] proved ineffective in altering either schistosome fecundity or the physiological parameters studied (data not shown).

The nonsteroidal compound mevinolin significantly depressed egg production at 10^{-6} M (Figure 3). This inhibition was effected without a concomitant

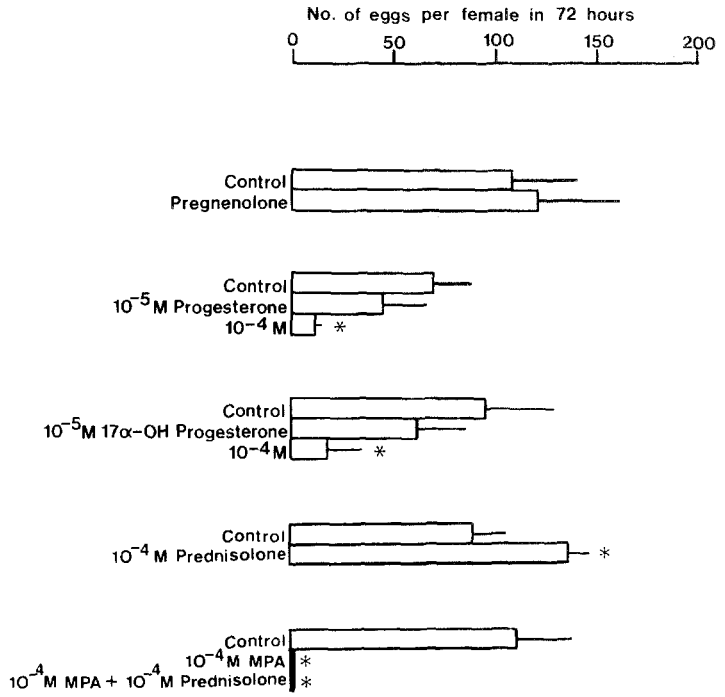

FIG. 1. Effect of various steroids on schistosome fecundity in vitro. Paired schistosomes (15 pair per 50 ml horse serum/RPMI (1:1) in 250-ml Erlenmeyer flasks) were incubated for 72 hr at 37°C in the presence of steroid hormones or their appropriate vehicle at the concentrations indicated. Egg counts were made from the entire media sample following incubation. Values were obtained from the mean egg counts in five to seven flasks run at each concentration for each compound. Asterisk = $P < 0.05$, as determined by the Student's t test; horizontal lines are 1 SE. MPA: medroxyprogesterone acetate.

depression of surface electrical activity or muscle tension. Additionally, parasite pairing and adhesion were unaffected up to concentrations of 10^{-4} M.

DISCUSSION

The factors required for the development of the reproductive tract and subsequent oviposition in the female schistosome remain largely unknown. In light of past in vivo studies wherein the effects of host hormonal imbalance on the viability of the parasite were examined, the present study assessed what effects, if any, the classical steroid hormones, as well as steroid synthesis inhibitors, had on schistosome fecundity in vitro. It could thus be determined whether these steroids played a role in modulating egg production by the schistosome.

The conversion of cholesterol to pregnenolone in *S. mansoni* was reported

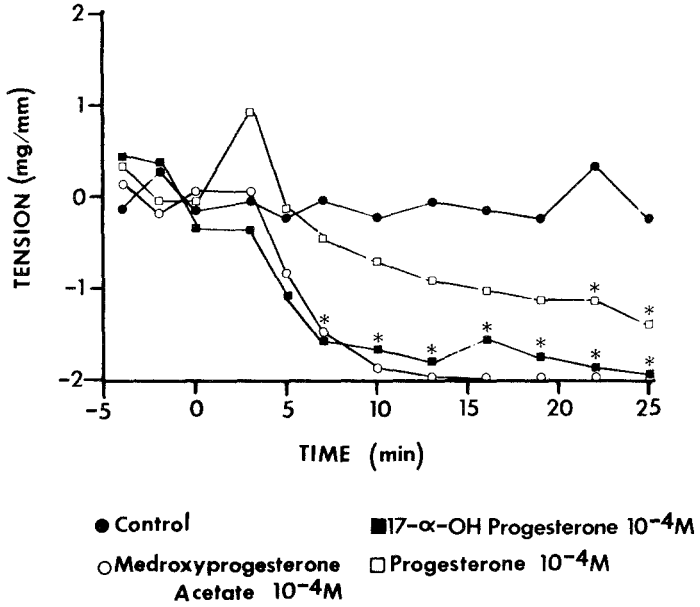

FIG. 2. Effect of progestational hormones on schistosome longitudinal muscle tension. Male schistosomes under tension were treated at time 0 with concentrations of progestational steroid hormones at which a significant effect on egg production was seen. Muscle tension was recorded using the method of Fetterer et al. (1977). Asterisk = $P < 0.05$ (Student's t test); values presented are the means of recordings from eight parasites per steroid examined.

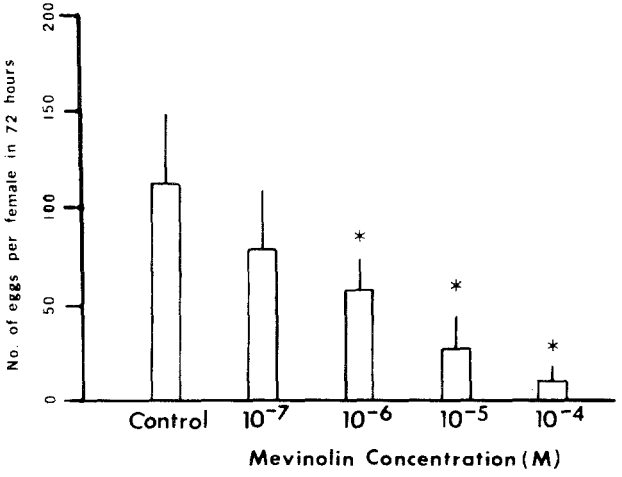

FIG. 3. Effect of mevinolin on schistosome fecundity in vitro. Paired schistosomes were incubated as described in Figure 1. Asterisk = $P < 0.05$ (Student's t test); vertical lines are 1 SE.

by Briggs (1972), although no physiological role was given for this hormone. However, the hydroxylation of cholesterol and subsequent side-chain scission necessary for the formation of pregnenolone is mediated by the cytochrome P-450 complex, which is not known to be present in schistosomes. In the present study, no effect was exhibited by cholesterol, 20α-, 22-(*R*)-, or 22-(*S*)-hydroxycholesterol, or pregnenolone on in vitro oviposition. Furthermore, dianisidine, an inhibitor of the cytochrome P-450-mediated mixed function oxidase system which catalyzes the 20α hydroxylation of cholesterol (Sheets et al., 1981), was also ineffective in altering schistosome egg production, even though the concentration of dianisidine used in this study (10^{-3} M) was in great excess of the IC_{50} for the mammalian enzyme (2.5×10^{-6} M). These data question the ability of the schistosome to form and utilize hormonal precursors under our in vitro culture conditions.

The effects of progestational hormones on the schistosome were evidenced only at concentrations much higher than those reported for most vertebrate hormone–receptor interactions (K_d 10^{-10}–10^{-8} M; Chan and O'Malley, 1978), suggesting a nonspecific physiological effect of these compounds on the parasite. It may be concluded that the decrease in schistosome fecundity caused by the progestational steroids was an effect not specific for the reproductive tract of the parasite, as other parameters of physiological functioning were also depressed.

In the present study, the corticosteroid prednisolone enhanced egg production while dexamethasone, a corticosteroid analog, had no effect. This is of interest, since it was shown that cortisone-treated mice infected with *S. mansoni* generally yield fewer worms than do control infected mice (Coker, 1957; Lewert and Mandlowitz, 1963). Also, both Lagrange (1963) and Newsome (1963) noted effective, long-term antischistosomal activity with dexamethasone and several steroid derivatives of the pregnane series on in vivo treatment. The basis for the stimulation of egg production by prednisolone in vitro is unknown, but it may not be due to a selective effect on the reproductive tract of the parasite. However, at the concentrations tested, prednisolone had no effects on the other physiological parameters examined after incubation. Therefore, the effect of prednisolone on egg production may be the result of a generalized stimulation of the anabolic processes of the parasite.

The inability of either androgenic or estrogenic steroids to alter in vitro fecundity in the present study is interesting considering the results of Bussolati et al. (1967) and Barrabes et al. (1979), who reported a significant reduction in worm burden following dosing of the host with androgenic or estrogenic compounds. The comparison between the in vitro and in vivo data with these hormones suggests that the results obtained in vivo may reflect host-mediated effects on metabolism or distribution of these compounds, rather than direct effects of the hormones on the parasites, since neither group of hormones had an adverse effect on physiological parameters of the parasites.

Finally, the effects of mevinolin on egg production by *S. mansoni* were of great interest. Mevinolin prevents the de novo synthesis of cholesterol by inhibiting hydroxymethylglutaryl-CoA reductase (Alberts et al., 1980), the enzyme which is an important control point in the regulation of cholesterol biosynthesis. Adult schistosomes are incapable of de novo cholesterol formation (Meyer et al., 1970; Smith et al., 1970), and, further, the inhibitory effect of mevinolin on fecundity was not reversed by coincubation with 10^{-4} M cholesterol (unpublished observation), suggesting that the inhibition of egg production caused by this agent is not due to a steroid-mediated effect. The ability of mevinolin to decrease egg production without altering either muscle tension or surface electrical activity of the parasite have warranted our further investigation into the mechanism of action of this agent.

As the data indicate, there are differences between earlier in vivo reports and this in vitro study on the effects of steroid hormones on *Schistosoma mansoni*. Host-mediated processes, such as metabolism and differential distribution of these hormones, could play a role in reducing the worm burden, as was seen in the in vivo studies. Unfortunately, the fecundity of the parasites retrieved after the in vivo studies of Bussolati et al. (1967), Barrabes et al. (1979), and others (Coker, 1957; Lewert and Mandlowitz, 1963) was not examined, making it difficult to compare these in vivo effects of hormones with the present results. However, in the absence of host-mediated processes, as in the in vitro system, the data suggest that the "classical" steroid hormones probably exert little or no effect on the modulation of egg production by *S. mansoni*.

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SEX PHEROMONE OF FALL ARMYWORM, *Spodoptera frugiperda* (J.E. SMITH)[1]
Identification of Components Critical to Attraction in the Field

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Abstract—Analyses of extracts of pheromone glands and of volatiles from calling female fall armyworm moths, *Spodoptera frugiperda* (J.E. Smith), revealed the presence of the following compounds: dodecan-1-ol acetate, (Z)-7-dodecen-1-ol acetate, 11-dodecen-1-ol acetate, (Z)-9-tetradecenal, (Z)-9-tetradecen-1-ol acetate, (Z)-11-hexadecenal, and (Z)-11-hexadecen-1-ol acetate. The volatiles emitted by calling females differed from the gland extract in that the two aldehydes were absent. Field tests were conducted with sticky traps baited with rubber septa formulated to release blends with the same component ratios as those emitted by calling females. These tests demonstrated that both (Z)-7-dodecen-1-ol acetate and (Z)-9-tetradecen-1-ol acetate are required for optimum activity and that this blend is a significantly better lure than either virgin females or 25 mg of (Z)-9-dodecen-1-ol acetate in a polyethylene vial, the previously used standard. Addition of the other three acetates found in the volatiles did not significantly increase the effectiveness of the two-component blend as a bait for Pherocon 1C or International Pheromones moth traps.

Key Words—*Spodoptera frugiperda*, fall armyworm, Lepidoptera, Noctuidae, (Z)-7-dodecen-1-ol acetate, (Z)-9-tetradecen-1-ol acetate, pheromone, attractant, sex pheromone.

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

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INTRODUCTION

The genus *Spodoptera* includes several insect species that cause substantial damage to agricultural crops throughout the world. Three species, *S. exigua* (Hübner), *S. frugiperda* (J.E. Smith), and *S. eridania* (Cramer), are serious pests of many crops in the United States. The fall armyworm (FAW), *S. frugiperda*, is an important pest of corn and other grass crops in the United States and occurs throughout the Caribbean basin, where it is known as the whorlworm, and attacks a variety of crops (Andrews, 1980).

Components of the FAW pheromone have been identified in previous investigations. Sekul and Sparks (1967) used a laboratory bioassay in which a mating response was evoked in FAW males to monitor the isolation and identification of a sex pheromone, (*Z*)-9-tetradecen-1-ol acetate (*Z*9–14 : Ac), from FAW females. Subsequent tests showed this compound to be an ineffective lure for FAW males in the field (Mitchell and Doolittle, 1976; Sparks, 1980). However, this compound did reduce mating by FAWs when it was evaporated into the atmosphere in disruption tests (Mitchell and McLaughlin, 1982). A second compound, (*Z*)-9-dodecen-1-ol acetate (*Z*9–12 : Ac), was isolated and identified from FAW females (Sekul and Sparks, 1976), and this compound either alone or with small quantities of *Z*9–14 : Ac added is a good practical lure for fall armyworm males when used in sticky traps (Mitchell, 1978; Jones and Sparks, 1979). However, fairly large quantities of *Z*9–12 : Ac are required for effectiveness (5–10 mg on a rubber septum), and the baits are effective in the field for only one to two weeks (Mitchell et al., 1983). Sparks (1980) reported that two additional compounds had been identified from washes of FAW female ovipositors, but he did not give their identity and he stated that they did not improve the effectiveness of *Z*9–12 : Ac as a lure.

Descoins and coworkers (personal communication) analyzed the FAW female-produced pheromone and found (*Z*)-11-hexadecen-1-ol acetate (*Z*11–16 : Ac), in addition to the two compounds already reported. However, in field tests conducted in Florida, we could not find a blend of these three compounds that was significantly better than *Z*9–12 : Ac alone in luring FAW males to sticky traps (Mitchell et al., 1983).

Although *Z*9–12 : Ac can be used as a lure for monitoring FAW males, it is obvious that our knowledge of the pheromone system of this insect is incomplete and that the complete pheromone is needed to achieve maximum effectiveness in pheromone monitoring and control programs. Therefore, we decided to reinvestigate the pheromone system of this species to determine the precise blend emitted by calling FAW females and the optimum blend for trapping FAW males in the field.

METHODS AND MATERIALS

Rearing. The majority of the fall armyworm females from which pheromone was obtained were reared in the laboratory on an artificial diet (Burton, 1969). Insects were sexed in the pupal stage and placed in 0.5-liter paper cartons that were then placed in 23 × 23-cm Plexiglas cages for eclosion. Male and female pupae were maintained in separate rooms under a reversed light cycle of 14 hr light and 10 hr dark (65% relative humidity, 26°C). Daily sequential transfers of uneclosed pupae were made to uninhabited cages so each holding cage contained adult insects of a single age and sex.

Additionally, fourth- to sixth-instar fall armyworm larvae were collected in the field from sorghum plants and reared to pupation in the laboratory on artificial diet. Subsequent sexing and handling of these insects were conducted the same as for the laboratory-reared insects.

Pheromone Extraction and Collection. Actively calling 2- to 3-day-old FAW females were used for all pheromone extractions and volatile collections. In the laboratory the calling activity of the female FAW peaked about 4 hr after the beginning of the dark period. Sex pheromone glands from both laboratory-reared and wild females were excised and extracted. Excision of the sex pheromone glands was performed during the dark period by gently squeezing the lateral–posterior abdominal section of the female, causing protrusion of the terminal abdominal segments, which were then clipped as a unit. The excised glands were then placed in 50 μl of isooctane (Fisher Scientific Co., 99 mol % pure) in a microvial. Typically, when 25 glands were accumulated in the solvent (about 15 min), the walls of the microvial were rinsed with an additional 20 μl of isooctane and the solvent was removed from the glands and transferred to a clean vial. The extract was then concentrated by careful evaporation with gentle warming to a final concentration of about five female equivalents (FE) per microliter. The number of glands excised during any collection period varied with availability of calling females within a particular batch. Gland extract was stored at −60°C in microvials with Teflon-lined screwcaps until analysis.

An apparatus similar to the ones described by Cross (1980) and Tumlinson et al. (1982) was used for all volatile collections. Zero-grade nitrogen (99.99% minimum purity, 0.5 ppm maximum hydrocarbons) or, when live females were aerated, "hydrocarbon-free" compressed air, was delivered through a stainless-steel regulator, a gas purifier (Alltech Associates), a methylene chloride-extracted charcoal (6–14 mesh, Fisher Scientific Co.) prefilter (9 cm × 2 cm OD), and then through a glass Y-tube to two aeration chambers simultaneously. Each aeration chamber was constructed from two glass tubes (7 cm × 2.8 cm OD) connected by a ball joint (35/25). A coarse-glass frit was sealed into the upwind

tube to provide a laminar flow of air (or N_2) over the aeration subject. The downwind tube was the aeration chamber, and its exit narrowed to a tube 0.6 cm in outside diameter to which was connected a small charcoal filter similar to the one described by Grob and Zürcher (1976). With this apparatus, volatiles can be collected from any desired subject in one chamber and simultaneously a "system blank" can be collected in the other chamber under identical conditions.

Four or five FAW females were selected during their peak calling activity and placed in an aeration chamber. An airflow of about 1.0 liter/min was maintained over the females for 1.5 hr. When the aeration was complete, the collected volatiles were eluted from the charcoal collection filters with three aliquots (20, 15, and 15 μl) of redistilled dichloromethane (HPLC grade, J.T. Baker), and the eluates from two aerations, representing 9–10 females, were combined.

The initial collections were concentrated by gentle warming to a volume of 2–5 μl. Then 5 μl of isooctane was added to rinse the walls of the microvial and the solution was concentrated to a final volume of 1–2 μl for analysis by capillary gas chromatography. After the initial analyses to establish the location of the peaks of interest on a particular stationary phase, 5 ng of an internal standard in 1 μl of isooctane was added to the CH_2Cl_2 filter eluate before concentration. Pentadecan-1-ol acetate (*S*-15 : Ac) and hexadecan-1-ol acetate (*S*-16 : Ac) were used as internal standards for analyses on OV-1 and cyanosilicone columns, respectively. The system blank filters were extracted and the extract concentrated and analyzed in an identical manner on the same column and on the same day as that of the companion filters containing female volatiles.

Chemical Analyses. Gas chromatographic (GC) analyses were conducted on a Varian model 3700 GC and a Hewlett-Packard model 5710A GC, both equipped with splitless capillary injector systems and flame ionization detectors. A Perkin Elmer chromatographic data system was used for data collection, storage, and subsequent analysis. Either nitrogen (linear flow velocity 9.8 cm/sec) or helium (linear flow velocity 19 cm/sec) was used as a carrier gas. Columns used for initial analyses of gland extracts and volatiles and conditions for each column were: 42-m × 0.25-mm ID glass capillary column coated with SP-2340, operated at 80°C for 2 min, then temperature programmed (TP) at 30°/min to 170°; 60-m × 0.25-mm ID fused silica column coated with 0.20 μm film of SP-2330, initial temp 80°C for 1 min, TP at 30°C/min to 170°C; 29-m × 0.25-mm ID glass column coated with 0.4% OV-1 over 1% Superox, initial temp 80° for 2 min, TP at 32°C/min to 180°C.

Although the three columns initially used for these analyses were adequate for separation of most lepidopteran pheromones, they did not provide the separation and resolution required to unequivocally assign the double bond posi-

tions in the 12-carbon acetates present in volatiles collected from FAW females. Therefore, three columns with superior resolution and with three different, but complementary, separation mechanisms were selected for analysis of the volatiles. They were: 50-m × 0.25-mm ID fused silica CPS-1 (cyanopropyl methyl silicone, Quadrex Corp., New Haven, Connecticut), initial temperature 80°C for 1 min, TP at 10°C/min to 165°C; 50-m × 0.25-mm ID fused silica OV-101, initial temperature 80°C for 1 min, TP at 10°C/min to 180°C; 13.6-m × 0.25-mm ID glass cholesteryl *p*-chlorocinnamate (liquid crystal) (Heath et al., 1979; Heath and Doolittle, 1983) column coupled to 0.46-m × 0.33-m ID fused silica BP-1 (SGE) precolumn, initial temperature 60°C for 1 min, TP at 30°C/min to 150°C. All injections were made in the splitless mode.

Samples also were analyzed by GC–mass spectrometry (MS) with either a Finnigan model 3200 chemical ionization mass spectrometer or a Nermag model R1010 mass spectrometer in the chemical ionization mode. The SP-2340 and OV-101 capillary columns used in previous analyses were used in the GC-MS analyses with He carrier gas. Methane and isobutane were used as the reagent gases in the mass spectrometers. Spectra of the natural products were compared with those of candidate synthetic compounds.

All synthetic standards used in this study were obtained from commercial sources and were purified by high-performance liquid chromatography on a 25 × 2.5-cm (OD) $AgNO_3$-coated silica column eluted with toluene (Heath et al., 1977). These compounds were analyzed on both polar and nonpolar capillary GC columns described previously and determined to be greater than 99% pure.

Formulation. Synthetic blends and individual synthetic compounds were formulated on 5 × 9-mm rubber septa (A.H. Thomas Co.) for all biological tests. Septa were Soxhlet extracted with CH_2Cl_2 for 24 hr and air dried prior to loading. Desired release ratios of the components of blends were obtained by loading the septa with mixtures containing calculated percentages by weight of each component of the blend. The percentage of a component in the loading mixture was calculated on the basis of its relative volatility determined from retention indices on liquid crystal capillary GC columns (Heath and Tumlinson, 1986) and a method developed to predict release ratios of components of a blend from rubber septa (Heath et al., 1986). Each septum was loaded with 200 μl of a hexane solution of the blend pipetted into the well on the large end of the septum. Septa were aired for two days at room temperature before use and were then used for a maximum of 14 days in the field.

In field tests conducted in 1982, the quantity of Z9–14:Ac, the major component, in all blends was 2 mg/septum. In these initial tests, which compared the effectiveness of four-, five-, and six-component blends with live females and Z9–12:Ac in a polyethylene vial, the relative amount of each component was adjusted to keep the release ratio of the blend components as constant

as possible for all blends. In the 1983 and 1984 field tests, each septum was loaded with 2 mg of the total blend dissolved in 200 μl of hexane, and after airing for two days at room temperature, was used for 4–11 days.

To verify that ratios released from the septa were the same as those calculated for a blend, septa loaded with the various blends and aired for two days were aerated in the same apparatus used to collect volatiles from glands and live females, and the volatiles collected on charcoal were eluted and analyzed by capillary GC on SP-2330. Two septa were placed in the aeration chamber of the apparatus, and zero-grade nitrogen was blown over them at flow rates of 100, 200, 400, 600, and 800 cc/min for 1.5 hr. An internal standard, *S*-16:Ac, was added to the eluate from the charcoal filter for quantitative analysis.

Field Tests. Trapping experiments were conducted in Alachua County, Florida, in late summer 1982. The rubber septa containing the test blends were placed in Pherocon 1C sticky traps supported ca. 1 m above the ground on metal poles. Treatments were arranged in three randomized complete blocks in and around corn fields. The traps were set ca. 30 m apart in lines perpendicular to the prevailing wind and were checked every one to two days for captured FAW moths. Trap liners were replaced when more than five moths were captured or on every second visit. Whenever a trap had five or fewer moths, the insects were removed carefully to leave the sticky trapping surface intact; traps were rerandomized after every collection; thus, each collection was considered a separate replicate. For statistical analysis, the data were transformed to $\sqrt{X + 0.5}$ and subjected to analysis of variance (Steel and Torrie, 1960). Mean separations were achieved by Duncan's (1955) multiple-range test.

In 1983, field-trapping experiments were conducted in late September with blends and ratios found in volatiles collected from calling females. The experimental design and data analysis were as described for the 1982 experiment.

In 1984, the field trapping studies were conducted in August and September using International Pheromones moth traps (IPM traps) positioned ca. 1 m above the ground in and around sorghum fields. The IPM traps capture moths using a funnel–bucket system. Moths entering the bucket receptacle through the funnel were killed with a volatile insecticide, Vapona. The switch to the IPM trap was based upon preliminary experiments which indicated that the IPM trap was more effective in capturing large numbers of FAW moths than the Pherocon 1C sticky traps used in 1982 and 1983. The experimental design was as described for the 1982 and 1983 field tests. Due to the large numbers and extreme variations in total numbers of moths captured in different blocks on the same or different dates in the 1984 field tests, the data were converted to percentages using the formula:

$$\frac{\text{Number of moths caught in treatment}}{\text{Total number of moths caught in block}} \times 100$$

The data then were converted to arcsin $\sqrt{X}$ and subjected to analysis of variance (Steel and Torrie, 1960). Means were separated using Duncan's (1955) multiple-range test.

RESULTS AND DISCUSSION

Gland Extracts. Initially the pheromone glands from calling FAW females were excised and extracted to determine what compounds were present, although we realized from previous experience and from literature reports (Cross et al., 1976; Hill et al., 1975) that the components and ratios present in the gland may differ from those released by calling females. Analysis of extracts of pheromone glands excised from laboratory-reared and wild, calling female FAW, by capillary GC on SP-2330 and SP-2340, indicated that there were five peaks consistently present above the background peaks of impurities in the solvent. Four of these peaks coincided in retention times with the following authentic standards: Z7–12 : Ac, Z9–14 : Al, Z9–14 : Ac, and Z11–16 : Ac. The other peak was very close in retention time to that of Z9–12 : Ac. Furthermore, a peak coincidental in retention time with Z11–16 : Al on SP-2330 also appeared, but it was not present consistently in quantities large enough to distinguish it from background impurities. Analysis on OV-1 confirmed the presence of Z9–14 : Ac, the major peak, and Z11–16 : Ac. Additionally, peaks coincidental in retention time with Z7–12 : Ac and Z9–14 : Al were present, but since Z9–12 : Ac coeluted with Z9–14 : Al on this column, confirmation of the presence of the latter two was not possible.

Methane ionization mass spectral analysis of gland extracts from laboratory-reared FAW females confirmed that the major peak, coincidental in retention time with Z9–14 : Ac on SP-2340, SP-2330, and OV-1 capillary columns, was a monounsaturated 14-carbon acetate with diagnostic ions at *m/e* 255 (M + 1), 195 (M + 1 − 60), 89, and 61. Also, the presence of peaks consistent in retention time and mass spectral diagnostic ions with Z7–12 : Ac, Z9–14 : Al, and Z11–16 : Ac was confirmed. Furthermore, small peaks, eluting just prior to and just after Z7–12 : Ac on the SP-2340 column, had mass spectra consistent with dodecan-1-ol acetate (*S*-12 : Ac) and a monounsaturated 12 : Ac, respectively.

Thus the presence of Z7–12 : Ac, Z9–14 : Al, Z9–14 : Ac, and Z11–16 : Ac in pheromone glands of calling FAW females was firmly established by GC-MS and by retention times on OV-1, SP-2340, and SP-2330 capillary GC columns; the cyanosilicone GC phases have previously been demonstrated to separate both geometrical and positional isomers of most olefinic aliphatic primary acetates (Heath et al., 1980; Tumlinson et al., 1982). Additionally, *S*-12 : Ac, Z9–12 : Ac, and Z11–16 : Al appeared to be present in small quantities, but this could not be confirmed.

Gland extracts were prepared from five batches of laboratory-reared FAW females and from two batches of wild FAW females from each of two locations in Alachua County, Florida. There was considerable variability in gland contents, in both ratios and total quantities between laboratory-reared and wild females and also among batches of wild females collected from different locations. The relative amounts of Z9–14 : Al, Z11–16 : Al, and Z11–16 : Ac in particular varied more than those of the other components. Thus, "the most representative ratio" of components found in the glands was 4 : 2 : 13 : 69 : 3 : 9 for Z7–12 : Ac, Z9–12 : Ac (subsequently identified as 11–12 : Ac), Z9–14 : Al, Z9–14 : Ac, Z11–16 : Al, and Z11–16 : Ac, respectively, but the relative amounts of the two aldehydes and Z11–16 : Ac varied from these values by as much as 100%. However, as noted earlier, the actual pheromone blend emitted by the female often differs significantly, both in compounds present and in ratio, from the contents of the gland. Therefore, we proceeded to collect volatiles to more accurately assess the composition of the FAW pheromone.

Volatile Collections. Analysis on the SP-2330 capillary column of the volatiles collected from laboratory-reared, calling FAW females indicated the presence of five peaks not present in the system blank analyzed under identical conditions (Figure 1). Four of these peaks coincided in retention times on this column with peaks representing *S*-12 : Ac, Z7–12 : Ac, Z9–14 : Ac, and Z11–16 : Ac. Again, the third peak in the chromotogram (Figure 1) had a retention

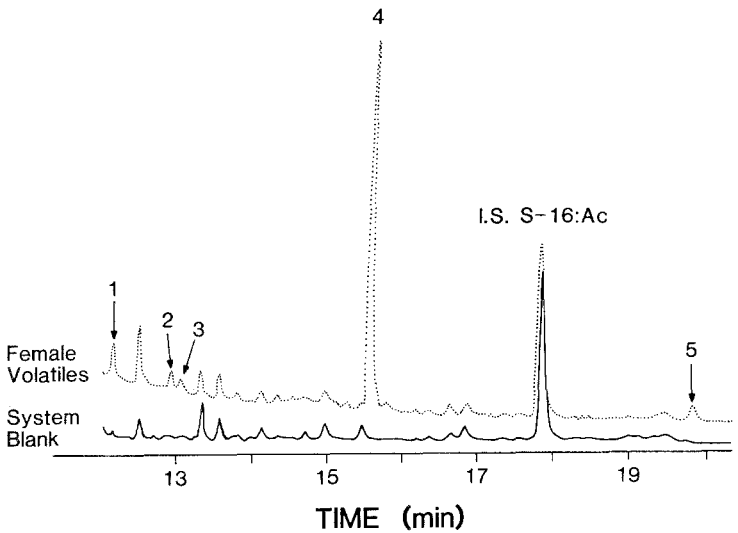

FIG. 1. Analysis of volatiles collected from calling, laboratory-reared fall armyworm female moths on a 60-m fused silica SP-2330 capillary gas chromatographic column. Peaks not present in the blank correspond in retention time to: (1) *S*-12 : Ac, (2) Z7–12 : Ac, (3) 11–12 : Ac, (4) Z9–14 : Ac, and (5) Z11–16 : Ac.

time very close to, but not identical with that of Z9–12 : Ac. The ratio (mean of three replicates, ca. 10 females per replicate) of these compounds present in the volatiles was 4.9 : 3.1 : 1.7 : 86.9 : 3.5, respectively.

Although Z9–12 : Ac was previously reported to be a component of the FAW pheromone, its presence in the volatiles released by calling females could not be confirmed. Additionally, the quantity of *S*-12 : Ac in the volatiles could not be accurately determined because of interference of an impurity in the system. Therefore, an analytical procedure was devised, employing three high-resolution capillary GC columns and mass spectrometry, that was capable of resolving all possible 12-carbon acetate candidate compounds and confirming their identities. The CPS-1 (cyanosilicone) column separated the *S*-12 : Ac from the interfering impurity and resolved the other isomers, although 11–12 : Ac was not completely resolved from Z9–12 : Ac (Figure 2A). However, the OV-101 column completely resolved 11–12 : Ac from Z9–12 : Ac, although *S*-12 : Ac was now incompletely resolved from Z9–12 : Ac (Figure 2B). The liquid crystal column resolved all candidate compounds except 11–12 : Ac and *S*-12 : Ac (Figure 2C).

Volatiles were collected from 44 batches of laboratory-reared, calling FAW females (five females per batch) and pooled for analysis. Aliquots of this sample were analyzed on all three high-resolution capillary columns and by GC-MS. As indicated in Figure 2, the volatile components not present in the system blank were coincidental on all three columns with *S*-12 : Ac, Z7–12 : Ac, and 11–12 : Ac. Z9–12 : Ac did not coincide in retention time with any of the candidate pheromone peaks on any of the these columns. Methane/isobutane ionization mass spectra of the volatile peaks were consistent with these identities. Z9–14 : Ac and Z11–16 : Ac also were present in these volatiles and confirmed by all the data. The ratio of the components in the volatiles was 1.9 : 3.2 : 2.2 : 90.1 : 2.6 for *S*-12 : Ac, Z7–12 : Ac, 11–12 : Ac, Z9–14 : Ac, and Z11–16 : Ac, respectively. The quantity of Z9–14 : Ac collected per hour per female was about 2 ng.

Formulation. Volatiles released from rubber septa formulated for field tests were collected and analyzed by capillary GC to verify that actual release ratios were approximately the same as the calculated release ratios. Examples of release ratios measured in this way for the five-component blend and a four-component blend are given in Table 1. Release rates, but not blend ratios, from the septa varied with the flow rate through the aeration chamber. For example, the mean release rates of Z9–14 : Ac from a septum loaded with 200 μg of the five-component blend at flows of 100, 200, and 400 cc/min were 1.7, 2.5, and 4.3 ng/hr/septum, respectively. Because release rates from septa vary with the velocity of airflow over the septa, as well as temperature, it is very difficult to control release rates in the field. Therefore, it is more practical to compare captures of septa loaded with different doses of pheromone (see later) whose relative release rates can be measured accurately.

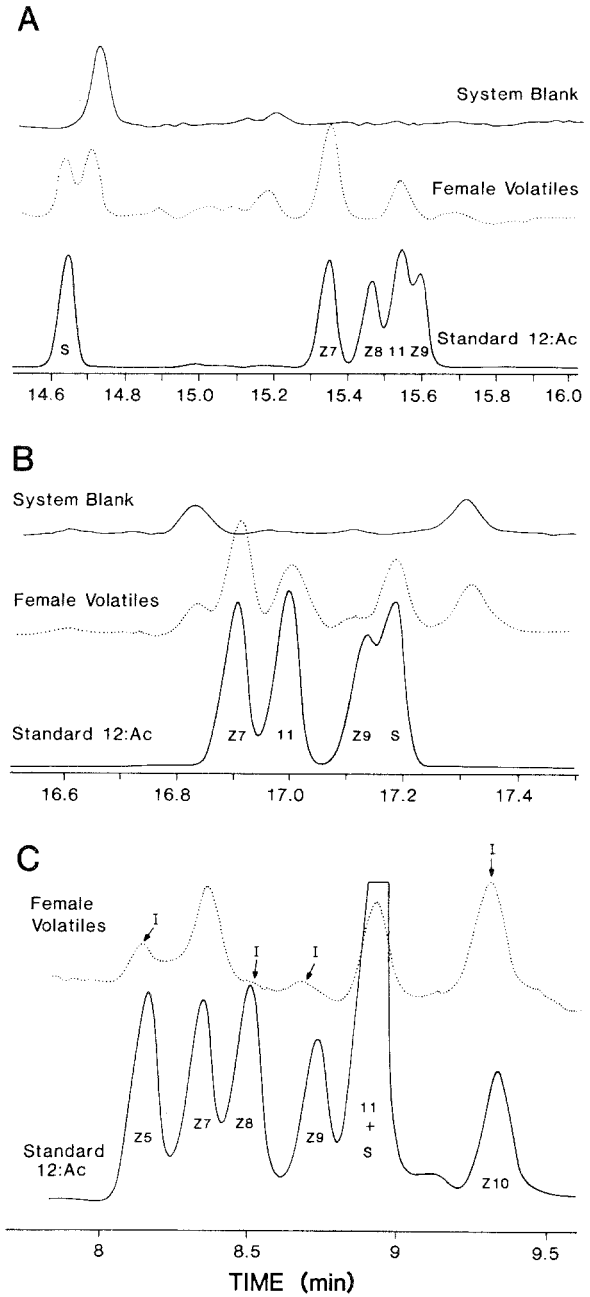

FIG. 2. Sections of chromatograms showing separation of 12-carbon acetates and analysis of volatiles collected from calling, laboratory-reared fall armyworm female moths on three-capillary GC columns (A) CPS-1; (B) OV-101; (C) cholesteryl-*p*-chlorocinnamate (liquid crystal). In (C), the symbol I indicates impurities found in the system blank.

TABLE 1. CHROMATOGRAPHICALLY MEASURED RELEASE RATIOS OF COMPONENTS IN TWO PHEROMONE BLENDS FORMULATED ON RUBBER SEPTA

	Blend 1			Blend 2		
	Load (%)	Theoretical release (%)	release (%, $\bar{X} \pm$ SD)[a]	Load (%)	Theoretical release (%)	release (% $\bar{X} \pm$ SD)[a]
S-12:Ac	1.0	4.9	6.2 ± 0.4	—	—	—
Z7-12:Ac	0.5	3.1	3.0 ± 0.2	0.5	3.0	4.0 ± 1.3
Z9-12:Ac	0.3	1.7	1.9 ± 0.1	0.3	1.7	1.1 ± 0.1
Z9-14:Ac	79.5	86.9	86.9 ± 0.6	81.6	91.9	90.8 ± 3.4
Z11-16:Ac	18.8	3.5	2.0 ± 0.2	17.7	3.4	4.1 ± 2.2

[a] Mean of three replications. Each septum loaded with 2 mg of the total blend.

Field Tests. The field tests conducted in 1982 were designed to evaluate blends of the compounds identified in pheromone gland extracts. All experiments included 25 mg of Z9–12:Ac dispensed from a polyethylene vial, the previously used standard (Mitchell, 1978), and three virgin females for comparison. In the first experiment in which four different ratios of components in the six-component blend (Z7–12:Ac, Z9–12:Ac, Z9–14:Al, Z9–14:Ac, Z11–16:Al, Z11–16:Ac) and two different ratios in a four-component blend (Z9–14:Al, Z9–14:Ac, Z11–16:Al, Z11–16:Ac) were tested (40 replicates/treatment), the ratios of components in these blends were selected to represent ratios found in gland extracts from laboratory-reared and different batches of wild FAW females. All the six-component blends tested were equivalent in luring FAW males to traps (means of 21.5–23.2 males/trap per night), and they were not significantly different from the standard 25 mg of Z9–12:Ac in a polyethylene vial (mean of 26.5). However, it is very unlikely that the attractancy of these blends was due solely to the presence of Z9–12:Ac because the qauntity of Z9–12:Ac loaded on a septum was only 68 μg at the most. Traps baited with both four-component blends tested captured significantly fewer males (mean of 7.4) than either the standard Z9–12:Ac or females (mean of 13.8) (5% level, Duncan's multiple range test). Thus either Z7–12:Ac or Z9–12:Ac or both appeared to be necessary for optimum activity.

In a subsequent experiment, we compared a six-component blend with five-component blends, each of which was missing a different component. The results (Table 2) clearly establish that Z7–12:Ac is necessary for activity and suggested that none of the other components except Z9–14:Ac are required. A separate experiment established that deletion of Z9–14:Ac from the blends resulted in trap captures not significantly different from a hexane blank. It also was interesting to note that deletion of Z9–14:Al increased the trap capture above that of all other blends and of the standard Z9–12:Ac. This suggests that

Table 2. Comparison of Sticky Trap Catches of *Spodoptera frugiperda* Males with Five- and Six-Component Blends of Synthetic Compounds Formulated on Rubber Septa, Gainesville, Florida, September 15–29, 1982[a]

Z7–12 : Ac	Z9–12 : Ac	Z9–14 : Al	Z9–14 : Ac	Z11–16 : Al	Z11–16 : Ac	Mean/trap per night (± SE)[b]
4 (0.5)	2 (0.3)	13 (2.5)	69 (52.8)	3 (3.5)	9 (40.4)	14.5 ± 1.7 b
0	2 (0.3)	14 (2.5)	72 (53.0)	3 (3.5)	9 (40.6)	1.2 ± 0.3 d
4 (0.5)	0	13 (2.5)	70 (53.0)	3 (3.5)	9 (40.5)	13.0 ± 1.6 b
5 (0.5)	2 (0.3)	0	79 (54.0)	3 (3.6)	10 (41.5)	20.7 ± 2.2 a
4 (0.5)	2 (0.3)	13 (2.6)	71 (55.0)	0	9 (41.9)	14.9 ± 1.8 b
4 (0.9)	2 (0.4)	14 (4.2)	76 (88.6)	3 (5.9)	0	16.2 ± 2.0 b
	25 mg[c]					14.4 ± 1.8 b
3 Females						7.0 ± 1.3 c

[a] Numbers under each compound in each row indicate the approximate percentage of that component released in the blend. Numbers in parentheses indicate the percent of each compound loaded to obtain the desired release ratio. The quantity of Z9–14 : Ac loaded onto the septum was 2 mg for all blends.

[b] Forty replicates/treatment; means not followed by the same letters differ significantly at the 1% level, Duncan's multiple-range test.

[c] In a polyethylene vial.

Z9–14 : Al may be an inhibitor and that, although it is present in the pheromone gland, it may not be a pheromone component.

Since the volatiles collected from calling females contained only five acetates and did not contain either of the aldehydes, we designed our 1983 field experiments to evaluate specific blends of the acetates (Table 3). Our primary objective was to determine whether all five compounds were required for optimum trap captures and whether any of the components were critical for biological activity. At this point Z9–12 : Ac was included as a blend component, although we had not yet confirmed whether or not it was released by females.

Blends of two, three, four, and five components were all equally attractive in our 1983 field trapping experiments (Table 3). As demonstrated in our 1982 field tests, both Z7–12 : Ac and Z9–14 : Ac were required for maximum activity. However, in the 1983 tests with blends formulated to release the same ratio emitted by calling females, all the blends were significantly more active than 25 mg of Z9–12 : Ac dispensed from a polyethylene vial. In fact Z9–12 : Ac, formulated in the same manner and quantity as the blends, was not different significantly from the blank.

Table 3. COMPARISON OF STICKY TRAP CAPTURES OF *S. frugiperda* MALES WITH BLENDS OF SYNTHETIC COMPOUNDS FORMULATED ON RUBBER SEPTA TO RELEASE RATIOS CORRESPONDING TO THOSE FOUND IN VOLATILES COLLECTED FROM CALLING FEMALES, GAINESVILLE, FLORIDA, SEPTEMBER 19–27, 1983[a]

S12 : Ac	Z7-12 : Ac	Z9-12 : Ac	Z9-14 : Ac	Z11-16 : Ac	Mean/trap per night (±SE)[b]
0	3.4 (0.6)	0	96.6 (99.4)	0	14.5 ± 1.5 a
0	3.3 (0.5)	0	92.9 (80.3)	3.8 (19.2)	15.3 ± 1.6 a
0	3.0 (0.4)	1.7 (0.3)	91.9 (81.6)	3.4 (17.7)	14.9 ± 1.6 a
5.0 (1.0)	3.2 (0.5)	0	88.3 (79.5)	3.6 (19.0)	14.4 ± 1.8 a
4.9 (1.0)	3.1 (0.5)	1.7 (0.3)	86.9 (79.5)	3.5 (18.7)	12.3 ± 2.0 a
		25 mg[c]			3.9 ± 0.8 b
			100.0		1.8 ± 0.7 c
		100.0			0.9 ± 0.6 cd
Hexane blank					0.1 ± 0.1 d

[a] Numbers under each compound in each row indicate the approximate percentage of that component released in the blend. Numbers in parentheses indicate the percent of each compound loaded to obtain the desired release ratio. Each septum was loaded with 2 mg of the total blend dissolved in 200 μl of hexane.

[b] Eighteen replicates per treatment; treatments in blocks ($N = 3$) were randomized after each collection ($N = 6$); means not followed by the same letters differ significantly at the 5% level, Duncan's (1955) multiple-range test.

[c] Twenty-five mg of Z9–12 : Ac in a polyethylene vial.

Table 4. CAPTURE OF MALE FALL ARMYWORM MOTHS IN TRAPS BAITED WITH DIFFERENT BLENDS OF SYNTHETIC COMPOUNDS FORMULATED ON RUBBER SEPTA TO RELEASE RATIOS CORRESPONDING TO THOSE FOUNDS IN VOLATILES COLLECTED FROM CALLING FEMALES, GAINESVILLE, FLORIDA, 1984[a]

	Release ratios (%) of pheromone components					Total catch (% ±SE)[b]	
S-12 : Ac	*Z*7-12 : Ac	*Z*9-12 : Ac	Δ11-12 : Ac	*Z*9-14 : Ac	*Z*11-16 : Ac	Test 1 (August, 9-20)	Test 2 (August, 21-29)
	3.3 (0.5)			92.9 (80.3)	3.8 (19.2)	19.7 ± 1.8 a	
1.9 (0.4)	3.2 (0.5)		2.2 (0.5)	90.1 (84.4)	2.6 (14.2)	18.2 ± 2.3 a	16.9 ± 2.2 a
	3.2 (0.5)		2.2 (0.5)	91.9 (84.5)	2.7 (14.5)	15.5 ± 1.7 a	16.9 ± 1.0 a
	3.4 (0.6)			96.6 (99.4)		15.3 ± 1.8 a	12.6 ± 1.8 a
	3.0 (0.4)	1.7 (0.3)		91.9 (81.6)	3.4 (17.7)	15.0 ± 1.8 a	17.2 ± 2.9 a
	3.3 (0.6)		2.3 (0.5)	94.4 (98.9)		13.4 ± 1.8 a	13.5 ± 1.3 a
	3.3 (0.6)	2.3 (0.4)		94.4 (99.0)			8.6 ± 1.2 a
1.9 (0.4)	3.2 (0.5)	2.2 (0.4)		90.1 (84.5)	2.6 (14.2)		12.8 ± 1.9 a
Hexane control						0.0 b	0.0 b

[a] Numbers under each compound in each row indicate the approximate percentage of the component released in the blend. Numbers in parentheses indicate the percent of each compound loaded to obtain the desired release ratio. Each septum was loaded with 2 mg of the total blend dissolved in 200 μl of hexane.

[b] For analysis of variance, percentages were transformed to arcsin $\sqrt{X}$ and mean separations were achieved using Duncan's (1955) multiple-range test. Treatments were replicated 21 (7 randomizations) and 15 (5 randomizations) times in tests 1 and 2, respectively. Means (reconverted to percentages) followed by different letters differ significantly at the 1% level. Totals of 10,077 and 28,114 moths were captured in tests 1 and 2, respectively.

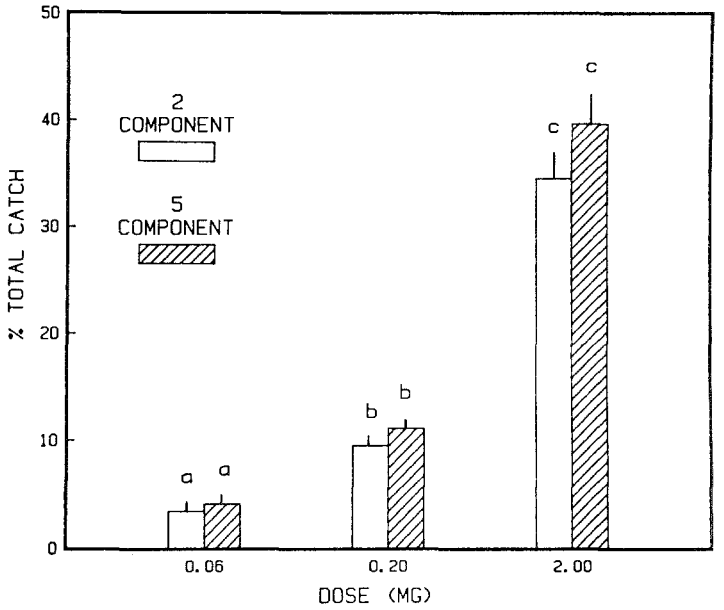

FIG. 3. Capture of male FAW moths in IPM traps baited with different dosages of two blends of synthetic sex pheromone on rubber septa, September 10–17, 1984 (14 replicates per treatment; treatments randomized four times per test period). Two-component blend and approximate release ratios: Z7–12:Ac, 3.4% and Z9–14:Ac, 96.6%. Five-component blend and approximate release ratios: *S*–12:Ac, 1.9%, Z7–12:Ac, 3.2%, 11–12:Ac, 2.2%, Z9–14:Ac, 90.1%, and Z11–16:Ac, 2.6%. Percentages were converted to arcsin $\sqrt{X}$ for statistical analysis. A total of 4668 FAW males were captured. Bars (reconverted to percentages) with different letters differ significantly at the 1% level, Duncan's (1955) multiple-range test. Narrow lines above bars represent SE of the mean.

Our 1984 field trapping tests were designed to evaluate blends containing the compounds and ratios found in our latter analyses and to compare these blends with some of the previously tested blends containing Z9–12:Ac rather than 11–12:Ac. The blends tested and the results are given in Table 4. The numbers of moths captured per trap per night were much greater than in 1983. However, as the data in Table 4 illustrate, it is still difficult to determine which blend, if any, is optimum as a trap bait. There are indications that some blends are better, although they cannot be validated statistically. For example, the five-component blend containing 11–12:Ac, which is identical to the volatile blend collected from calling females, is consistently near the top in percentage of males captured. Also, the two-component blend is always in the middle to low range in percent captured although, statistically, it is always equal to the best blend.

Thus a field trapping test was conducted in 1984 to compare the effectiveness of the two-component and five-component blends. IPM traps, each baited with a rubber septum containing 0.06, 0.2, or 2 mg of either the two-component or the five-component blend, were deployed in randomized complete blocks as previously described (14 replicates). Additionally, volatiles were collected from septa loaded with each of these blends at the doses indicated and analyzed by capillary GC to determine the release rates of the blends. The septa were aerated at flow rates of 1 and 2 liters/m. At any given dose and flow rate, the release rate of the two-component blend from a septum was the same as that of the five-component blend within experimental error. Additionally, the results of the field trapping experiment (Figure 3) indicate that both blends are equivalent as trap baits at each of the three doses tested.

CONCLUSIONS

These data support the conclusion that the sex pheromone of the FAW consists of at least two components, Z7–12 : Ac and Z9–14 : Ac. Three other components also may have a role in the pheromonal communication system of this species, but behavioral studies will be required to define this pheromone system more accurately and precisely.

The reason for the capture of male FAW in traps baited with Z9–12 : Ac is not known. Although we could not detect this compound in gland extracts or volatiles from FAW females, Jones and Sparks (1979) found that traps baited with 500 μg of Z9–12 : Ac on a dental wick captured twice as many FAW males as did FAW females. However, we found that traps baited with 2 mg of Z9–12 : Ac on a rubber septum captured no more FAW males than traps baited with a hexane blank and that much higher doses of Z9–12 : Ac are required for attraction equal to 2 mg of the blends containing both Z7–12 : Ac and Z9–14 : Ac. It is likely that cotton wicks release these compounds at a much higher rate than rubber septa (although we have no data to substantiate this), and that Z9–12 : Ac is active only at concentrations much higher than that of the pheromone produced by a calling female. Thus, we now have identified a pheromone blend that is a more powerful attractant in the field than anything available previously. It is already proving useful in monitoring programs and for studying migration by the fall armyworm (Mitchell et al., 1985).

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ODOR MIMETISM? Key Substances in *Ophrys lutea–Andrena* Pollination Relationship (Orchidaceae: Andrenidae)

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Abstract—The hypothesis of chemical mimesis in the relationship between *Ophrys* orchids and their pollinators was tested experimentally using preparations of *Ophrys lutea* var. *lutea* odor and the males of the solitary bee *Andrena fuscipes*, a species constellation that does not coexist under natural conditions. Correspondence in odor production was shown to occur. Identification of the compounds in the cephalic secretion of *A. fuscipes*, males and females, and the flower labella extracts, was performed by gas chromatography and mass spectrometry. Geraniol, nerol, citral, *E,E*-farnesol and 6-methyl-5-hepten-2-one were present in the extracts of both the orchid and one or both sexes of the bee. Extracts, TLC fractions of extracts, blends, and separate compounds were tested in the field in the mating flight areas of *A. fuscipes* males. Extracts and blends containing *E,E*-farnesol, geraniol, and geranial showed the highest attractivity to the male bees. These compounds seem to be responsible for the release of the odor-guided mating behavior at the *O. lutea* labellum and can be regarded as general attractants for many species of *Andrena*.

Key Words—Orchidaceae, Andrenidae, Apoidea, Hymenoptera, geraniol, farnesol, chemical mimetism, odor attraction, gas chromatography, mass spectrometry, semiochemical.

INTRODUCTION

Ophrys lutea var. *lutea* Cav. belongs to a group of *Ophrys* L. species that are pollinated by *Andrena* F. male bees (Kullenberg, 1961, 1973a,b). The polli-

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nation is a result of the male "pseudocopulatory" behavior on the flower labellum and his subsequent visits to conspecific flowers. The bees are attracted by chemical, visual, and tactile cues. The odor of *Ophrys lutea* attracts members of many, if not all, *Andrena* species and seems not to have a specialized pollinator relationship with one or a few species. Male and female andrenid bees are attracted to one another by their mandibular gland secretion (Tengö, 1979). The composition of this secretion is complex and closely similar between the sexes (Tengö and Bergström, 1976, 1977). For the *Andrena* species that have been tested for their response to *O. lutea* odor, (Kullenberg, 1961; Tengö, 1979), a difference in the intensity of responses between the species has been noted. Also bees of species that are too small to be able to pollinate the *Ophrys* flowers are attracted by the odor (Kullenberg, 1961). It has, therefore, been postulated that *O. lutea* produces a set of substances with properties that attracts a wide array of *Andrena* species. We tested this hypothesis with *Andrena fuscipes* K., a species not coexisting with *O. lutea* under natural conditions, as they are separated both phenologically and geographically. Another purpose of the present study was to identify the substance, or substances, that cause the attraction of a whole set of *Andrena* species to *O. lutea*. These key compounds have so far remained unknown.

METHODS AND MATERIALS

Chemical Analyses. Heads of *Andrena fuscipes* males were extracted in pentane (P) for 24 hr. Three different extracts of *Ophrys lutea* var. *lutea* (Nelson, 1962) flower labella (Montbazin, Hérault, Southern France), were prepared: M, methanol extract of 62 labella/ml solvent (pa. Merck); H, hexane extract of 5 labella/ml solvent (redistilled pa. grade, Merck); MWP, a methanol extract of 800 labella was used for preparation of the TLC (thin-layer chromatography) fractions. The extracts were diluted five times with water. Sodium chloride was added until saturation. The methanol–water–sodium chloride mixture was extracted with pentane (4× 20 ml) and the pentane phase MWP) was carefully concentrated by evaporation at 42°C.

High-performance TLC-plates (Merck, HPTLC plates) were used for the separation. The HPLC plates were rinsed in methanol before use, and 40% diethyl ether in hexane was used as eluent. R_f values were measured between the concentration zone and the solvent front. Seven fractions (1–7) were obtained with the help of a colored standard mixture. The R_f values obtained for the fractions were; 1 = 0.77–1.00; 2 = 0.54–0.77; 3 = 0.42–0.54; 4 = 0.35–0.42; 5 = 0.18–0.35; 6 = 0.09–0.18; 7 = 0.00–0.09. The various fractions were extracted from the TLC plates with methanol (3× 1.5 ml) and treated as described above under MWP, to obtain the odoriferous compounds in a nonpolar volatile solvent.

The concentration of the *O. lutea* extracts was determined in terms of labellum equivalents (LE), which is the number of labella extracted/ml solvent. The calculated concentrations of labella or authentic compounds presented at each test period are listed in Table 1. The identifications were made on an LKB 2091 mass spectrometer (MS) in combination with a PYE gas chromatograph (GC) and/or a Finnigan 4021 GC-MS instrument. A WG11 (FFAP-type) 50-m glass capillary column (ID 0.25 mm) was used for the separation. A typical temperature programming was 50°C in 4 min, followed by 4°C/min to 200°C. Mass spectral data and retention values were compared with authentic compounds, and in a few cases coinjection was performed. Voucher specimens together with gas chromatograms and mass spectra, are deposited at the Ecological Research Station on Öland.

Behavior Tests. The tests took place in the field during the period July 31–August 26, 1980, on Öland, an island in the Baltic sea close to the mainland of southern Sweden. The *A. fuscipes* male mating flight area used was situated at the sandy border to a small *Pinus silvestris* L. forest with heather, *Calluna vulgaris* (L.) Hull, occurring in abundance.

The tested extracts, TLC fractions, blends, and individual compounds (Table 1) were applied to a 5 × 10-mm piece of black velvet (the dummy) that was put out in the mating flight routes patrolled by the *A. fuscipes* males. The dummy was placed either on a food plant bush (heather) or at a branch of a pine. The heights of flight routes above the ground depended on the temperature and varied from 0.1 to 5 m. On days when the temperature was between 20 and 25°C at 1.5 m, the males flew at heights of 3–4 m.

The choice of the test samples was based on the results of the chemical analyses of the mandibular gland secretion of *A. fuscipes* bees (Table 3) and the *O. lutea* flower labella (Table 2). In all, 25 different samples (extracts, TLC fractions of extracts, blends, and single compounds) were tested (Table 1). The proportions of the authentic compounds in the blends were based on the GC-MS chromatograms.

The tests estimated relative attraction and behavior-releasing qualities of the samples for bees passing within 0.1 m of the source. Approach flights were classed into one of four types ranked by increasing attraction and following Kullenberg (1973b), modified by Tengö (1979). Each test lasted for 5 min. The test samples were not presented to the males in a randomized manner but were repeatedly tested during the whole flight period of the males. All approaches towards the scented dummy were counted and classified into behavior types p, 1, 2, and 4 (Figure 1). Assuming homogenity for each test sample {e.g., for each test occasion of a certain test sample, the theoretical frequency [(2 + 4)/all events] should be the same}, the chi-square test for contingency tables was considered to be appropriate for our test data. In addition, Kruskal-Wallis (KW) and Wilcoxon (W) tests were used when comparing pairwise and groups of samples. When using the chi-square test, the frequency of the total number of

Table 1. Relative Concentration of *O. lutea* Extracts, TLC Fractions of Extracts, Blends, and Single Compounds in Labellum Equivalents or Nanograms per Test[a]

	Concentration	
	Base extract (LE)	Amount presented to the insects at each test
Extracts:		
O. lutea MWP	50	5 labella
O. lutea M	62	6 labella
O. lutea H	5	0.5 labella
O. lutea fr 1	350	35 labella
O. lutea fr 2	350	35 labella
O. lutea fr 3	350	35 labella
O. lutea fr 4	350	35 labella
O. lutea fr 5	350	35 labella
O. lutea fr 6	350	35 labella
O. lutea fr 7	350	35 labella
A. fuscipes male P		0.15 heads/testoccasion
	ng/test occasion	% of compounds in the blend
Blends:		
O. lutea blend		
1-Octanol	83	(67)
2-Nonanol	8	(7)
Geraniol	18	(13)
Nerol	9	(7)
Citronellol	9	(6)
Geranial	45	(49)
Neral	44	(48)
6-Methyl-5-hepten-2-one	3	(3)
Geranial	45	(34)
Neral	44	(33)
E,E-Farnesol	44	(33)
Geraniol	89	(90)
Nerol	9	(10)
Geranial	89	(50)
Neral	89	(50)
Geranic acid	45	(50)
Nerolic acid	45	(50)
Single compounds:		
Geraniol	84	(95)
Nerol	84	(95)
E,E-Farnesol	88	(99)
E,Z-Farnesol	84	(95)
Z,E-Farnesol	84	(95)
Z,Z-Farnesol	84	(95)
1-Octanol	83	(99)

[a] For each test 0.1 ml of the solution was used. The percentage of individual compounds in the blends are given in brackets.

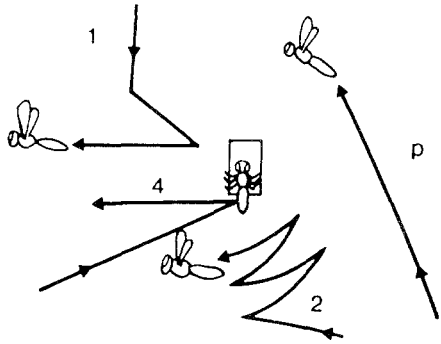

FIG. 1. The four different types of approaching behavior towards the dummy used in the field tests: (1) quick inspection; (2) hovering inspection (short-lasting attraction < 1 sec and long-lasting attraction > 1 sec); (4) Alighting or quick visit with or without an attempted copulation; (p) uninfluenced passage of the dummy.

high attractive behavior (2 + 4) in relation to the total number of low attractive (1 + p) behaviors for each test sample was calculated. When the Kruskal-Wallis or Wilcoxon tests were used, the frequencies [(2 + 4)/all events] for each test occasion were calculated and ranked (Sokal and Rohlf, 1981).

RESULTS

Chemical Analyses. The methanol extracts of *Ophrys lutea* flower labella consisted of a large number of components. It was possible to distinguish about 150 components at a concentration of 5 labellum equivalents (LE). Major compounds were aliphatic alcohols and terpenols with 1-octanol (60% of the total amount), geraniol (5%), 1-nonanol (5%), and 1-decanol (5%) as the most prominent ones. A minor amount of the sesquiterpene alcohol *E,E*-farnesol was also present (2%).

Seven fractions from the preparative TLC of the *O. lutea* MWP extract were analyzed by GC-MS. The results are given in Table 2.

The composition of volatile compounds in pentane extracts of female and male *A. fuscipes* heads is shown in Table 3. *E,E*-Farnesol, large amounts of citral, as well as geranic and nerolic acids, are found in both female and male head extracts.

Behavior Experiments. The test samples 1–25 are ordered in increasing degree of attractivity to the *Andrena fuscipes* males (Figure 2). The numbers in each column are percentage values of the behavior types p, 1, 2, or 4 in relation to all events (N) for a certain sample. Long-lasting behaviors (>1 sec) are calculated in percentage of the behavior type 2. Copulatory attempts to the dummy as well as long-lasting events (>1 sec) are considered to be the most

TABLE 2. VOLATILE COMPOUNDS IDENTIFIED IN TLC-FRACTIONS OF METHANOL EXTRACTS OF *O. lutea* var. *lutea* FLOWER LABELLA[a]

Substances		Fractions						
		1	2	3	4	5	6	7
Aliphatic compounds								
Hydrocarbons								
Nonane		X			XX			
Decane		X	XX		X	X	X	
Undecane		X	X		X	X	X	X
Dodecane		X	X					
Tridecane		X	X					X
Tetradecane		XX	X					
Pentadecane		XX	X	X				X
Hexadecane		XXX	X				X	X
Heptadecane	A	XX	X	X			X	X
A heptadecene	A							X
Octadecane	A						X	X
An octadecene	A		X					X
Heneicosane	A		X					
Docosane	A		X					
Tricosane	A		X					
Alcohols								
1-Hexanol						X	X	
1-Heptanol						X	X	
2-Heptanol						X		
1-Octanol			X		X	XXX	XXX	X
An octen-1-ol					X	X		
1-Nonanol						XX	X	
A nonen-1-ol						X	X	
2-Nonanol						X	X	
1-Decanol						XX	X	
1-Undecanol						X		
2-Undecanol						X	X	
1-Dodecanol						X	X	
1-Tridecanol						X		
1-Tetradecanol						X		
Aldehydes								
Pentanal					XX			
Hexanal					X	X		
Heptanal					XXX	X		
Octanal				X	XXX	X		
An octanal					X			
Nonanal				XX	XXX	X	X	
Decanal		X		X	XXX	X	X	
Undecanal		X		XXX	XXX	X		
Dodecanal		X		X	X			

TABLE 2. (Continued)

Substances	Fractions						
	1	2	3	4	5	6	7
Tridecanal	XX		XX	XX			
Tetradecanal			X				
Pentadecanal			X				
Ketones							
2-Octanone			X				
Esters							
Methyl hexanoate		XXX	XXX				
Methyl heptanoate		X	X				
Methyl octanoate		XXX					
Methyl nonanoate		XXX	X				
Methyl nonenoate		X	X				
Methyl decanoate		XX	X				
Methyl undecanoate		XX					
Methyl dodecanoate		XX					
Methyl tridecanoate		X					X
Methyl tetradecanoate		XX				X	X
Methyl tetradecenoate		X					
Methyl pentadecanoate		X					
Methyl hexadecanoate		XXX					X
Methyl hexadecenoate		X					
Methyl hexadecadienoate		X					
Methyl octadecanoate		XXX					X
Methyl octadecanoate		X					
Methyl octadecadienoate		XXX					X
Methyl octadecatrienoate		X					X
Ethyl hexdecanoate						X	
Hexyl acetate		X	X				
Octyl acetate		X					
Undecyl acetate		X					
Terpenes							
α-pinene						X	
Myrcene						X	
Limonene					X	X	
Neral	A		X	X			
Geranial	A		X	X			
Nerol	A			X	X		
Geraniol	A			X	XXX	XX	
Linalool				X			
Geranyl acetone	XX		X				
Geranyl hexanoate					X		
Cyclosativene	X	X					

TABLE 2. (Continued)

Substances		Fractions 1	2	3	4	5	6	7
γ-Cadinene			X					
Cubenol				XX		X		
t-Muurolol				X	XX	X		
A farnesal isomer	A					X		
E,E-Farnesol	A					X		
Aromatic compounds								
Toluene		X	X	X		X	X	X
o,m,p-xylene		X	X	X	X	X	X	X
Alkyl benzenes (M = 120)		X	X		X		X	
Alkyl benzenes (M = 134)		X	X				X	
Naphthalene		X	X					
Styrene						X	X	
trans-Methyl cinnamate				X				
Benzaldehyde		X	X				X	

[a] Main compound = XXX, compound in major amounts = XXX, compound in medium amounts = XX and compounds in minor amounts = X. A = compounds identified also in the mandibular gland secretion of *A. fuscipes*. The R_f values obtained for fractions 1–7 were: 1 = 0.77–1.00; 2 = 0.54–0.77; 3 = 0.42–0.54; 4 = 0.35–0.42; 5 = 0.18–0.35; 6 = 0.09–0.18; 7 = 0.00–0.09.

powerful expression of male behavior and are elicited only by highly excited males.

Extracts. The pentane extract of *A. fuscipes* males heads (No. 24 in Figure 2) was extremely attractive to patrolling conspecific males. Less than one fifth of one head was enough to attract 95% of the males patrolling within a meter of the odor source. The male extract (24), which contained *E,E*-farnesol was comparable in attractivity to the *O. lutea* blend in combination with *E,E*-farnesol (25), the mixture of citral + *E,E*-farnesol (22), and the *E,E*-farnesol (19) itself. The authentic samples of geraniol + nerol (13) and citral + 6-methyl-5-hepten-2-one (6) were less attractive than the male extract ($P < 0.01$), which contained all these compounds.

The three *O. lutea* extracts, M (21), MWP (20), and H (15), showed high levels of attractivity despite large differences in the composition of major compounds (Borg-Karlson et al., 1985). The attraction capacity of M and MWP is as much stronger than 1-octanol 5, $P < 0.01$) and *O. lutea* blend (10, $P < 0.01$), although 1-octanol is the main compound in these extracts (65% of the total amount in the volatile part of the M extract). Because of the scarcity of female bees during the test period, no female extract was tested.

TABLE 3. VOLATILE COMPOUNDS IDENTIFIED IN HEAD EXTRACTS OF *Andrena fuscipes* FEMALES AND MALES[a]

Substances	Males	Females
3-Methyl-1-butanol		X
6-Methyl-5-hepten-2-one	X	
Neral	XX	XX
Geranial	XX	XXX
Nerol	X	X
Geraniol	X	X
Nerolic acid	XXX	X
Geranic acid	XXX	X
A-Farnesene		X
A-Farnesal		XX
E,E-Farnesol	XX	XXX
2-Phenyl ethanol	XX	X
Heptadecane	X	X
A heptadecene	XX	
Octadecane	X	
An octadecene	X	
Nondecane	XX	
A nondecene	X	
Eicosane	X	
Heneicosane	X	X
Docosane	X	
Tricosane	X	XX
Pentacosane	XX	XX
A pentacosene		X

[a]X = small amounts, XX = medium amounts, XXX = major amounts.

Ophrys lutea TLC Fractions. A comparison between all fractions showed that numbers 4 (23) and 5 (18) had the largest attracting capacity. They differed, however, in their chemical composition (Table 2). In fraction 4, 1-octanol, citral, geraniol, and nerol constituted only a few percent, while fraction 5 contained 1-octanol and geraniol in large amounts and, in addition, *E,E*-farnesol. A blend containing these latter compounds has a characteristic smell that closely resembles the fragrance of *O. lutea*.

Fractions 4 (23) differed in attraction capacity from fractions 1 (2), 2(12), and 6 (8, $P < 0.01$). Fraction 6 had only a weak attracting capacity, but the frequency of long-lasting behaviors was high (25% of the approach being of high excitation status). It is probable that only the males in a certain, highly motivated state, reacted.

TLC fractions 1 and 2 showed only weak attracting capacity. Only these fractions contained the sesquiterpenes cyclosativene and γ-cadinene.

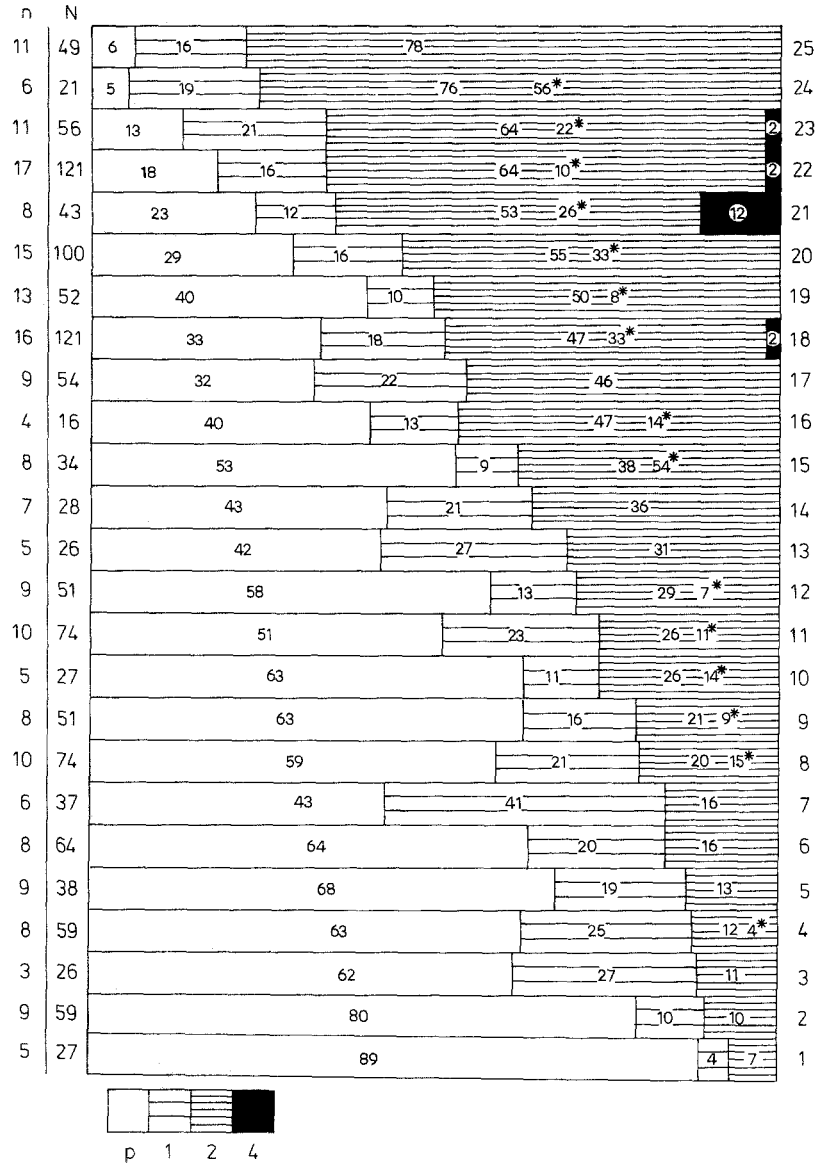

FIG. 2. The figure demonstrates the various types of attraction released by the 25 samples tested. The samples are listed in increasing order of attractivity. The numbers in each column are the percentage values of the behavior type p, 1, 2, or 4 in relation to all events (N) for a certain sample. Long-lasting behavior (>1 sec) is calculated in percentage of behavior type 2 (Figure 1). Sample No.: 1, *O. lutea* fr. 7; 2, *O. lutea* fr. 2; 3, geranic/nerolic acid; 4, *Z,Z*-farnesol; 5, 1-octanol; 6, geranial/neral + 6-methyl-5-hepten-2-one; 7, nerol; 8, *O. lutea* fr. 6, 9, *E,Z*-farnesol; 10, *O lutea* blend; 11, *O. lutea* fr.3; 12, *O. lutea* fr. 2; 13, geraniol/nerol; 14, *Z,E*-farnesol; 15, *O. lutea* H; 16, geranial/neral; 17, geraniol; 18, *O. lutea* fr. 5; 19, *E,E*-farnesol; 20, *O. lutea* MWP; 21, *O. lutea* M; 22, geranial/neral + *E,E*-farnesol; 23, *O. lutea* fr. 4; 24, *A. fuscipes* male; 25, *O. lutea* blend + *E,E*-farnesol.

TABLE 4. NUMERICAL VALUES OF FREQUENCY OF BEHAVIOR TYPES 2 + 4 IN RELATION TO ALL EVENTS (f)[a]

Sample	f	N	n
Extracts			
21 *O. lutea* M	0.65	43	8
20 *O. lutea* MWP	0.55	100	15
15 *O. lutea* H	0.38	34	8
24 *A. fuscipes* male P	0.76	21	6
Fractions of extracts			
2 *O. lutea* fr. 1	0.10	59	9
12 *O. lutea* fr. 2	0.29	51	9
11 *O. lutea* fr. 3	0.26	74	10
23 *O. lutea* fr. 4	0.67	56	11
18 *O. lutea* fr. 5	0.48	121	16
8 *O. lutea* fr. 6	0.20	74	10
1 *O. lutea* fr. 7	0.07	27	5
Blends and single compounds			
10 *O. lutea* blend	0.26	27	5
25 *O. lutea* blend(12) + *E,E*-farnesol(19)	0.78	49	11
22 Geranial/neral + *E,E*-farnesol	0.65	121	17
6 Geranial/neral + 6-methyl-5-hepten-2-one	0.16	64	8
16 Geranial/neral (1/1)	0.43	16	4
3 Geranic/nerolic acid (1/1)	0.11	26	3
7 Nerol	0.16	37	6
17 Geraniol	0.46	54	9
13 Geraniol/nerol (10/1)	0.31	26	5
19 *E,E*-Farnesol	0.50	52	13
9 *E,Z*-Farnesol	0.21	51	8
14 *Z,E*-Farnesol	0.36	28	7
4 *Z,Z*-Farnesol	0.12	59	8
5 1-Octanol	0.13	38	9

[a] N = the number of all events for each test sample. n = the number of test occasions for each test sample. The numbers 1–25 in front of the test samples refer to sample No. in Figure 2.

Authentic Samples. Tengö (1979) has shown that *E,E*-farnesol is strongly attractive to the *A. fuscipes* males. Also in this study all samples and extracts containing *E,E*-farnesol had a high behavior-releasing capacity (Table 1, Figure 2). The *O. lutea* blend (12), *E,E*-farnesol (10), and geraniol (17) could not be distinguished as regards their attraction of males. However, when *E,E*-farnesol (19) was tested in combination with the *O. lutea* blend (25: 10 + 19), which also contained geraniol and 1-octanol, a significantly higher attractivity occurred (χ^2, $P < 0.01$; W, $P < 0.05$) in relation to both the blend (10; W, $P < 0.05$; χ^2, $P < 0.01$) and the *E,E*-farnesol (19; W, $P < 0.05$; χ^2, $P < 0.01$).

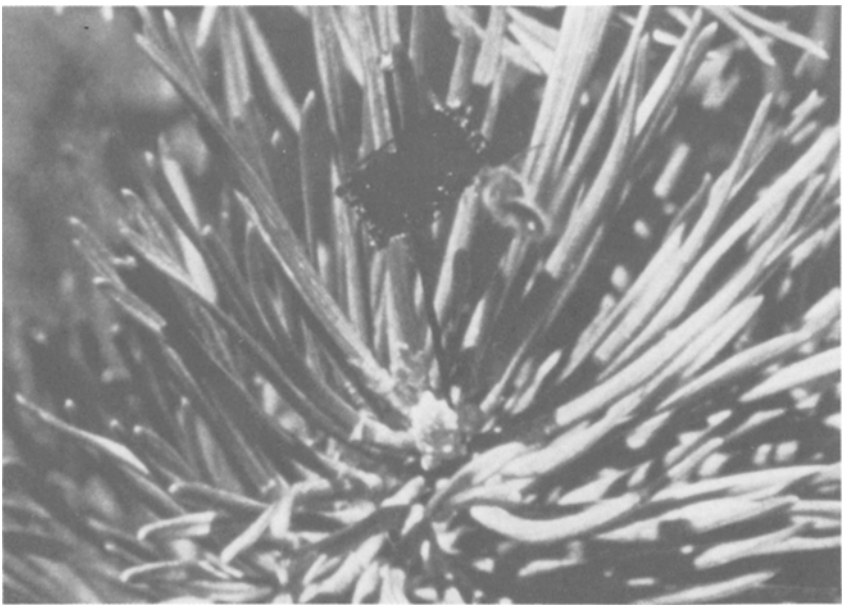

FIG. 3. *Adrena fuscipes* male approaching a dummy scented with *E,E*-farnesol.

When the four isomers of farnesol were compared, significant differences were observed (KW, $P < 0.05$). The *E,E*-farnesol (19) differed significantly from the *E,Z* (9) and *Z,Z* (4) isomers (χ^2, $P < 0.05$). The *Z,E* sample was found to be contaminated with 3% of the *E,E* isomer, which might explain the correspondence observed in attractivity between *E,E* and *Z,E*. Also the behavior-releasing capacity of geraniol (17) was considerably higher than that of the *Z* isomer nerol (7; χ^2, $P < 0.01$; W, $P < 0.05$).

The amounts of geranic and nerolic acids in the extracts of the bees seemed to increase during aging of the extract. Preliminary tests with a mixture (1 : 1) of these acids (3) elicited only weak attraction, which may indicate that these compounds probably are of minor importance in the pollination relationship.

DISCUSSION

Chemical mimesis as a factor in *Ophrys* pollination was proposed by Kullenberg (1961). The compounds hitherto identified and found to be similar for both the *O. lutea* flowers and the cephalic secretion of the *A. fuscipes* bees are, besides the aliphatic hydrocarbons, geraniol, nerol, geranial, neral, 6-methyl-5-hepten-2-one, *E,E*-farnesol, and an isomer of farnesal. These compounds are marked with an A in Table 2. Previously, no single compound or any specific combination of compounds has been reported to be responsible for the attraction

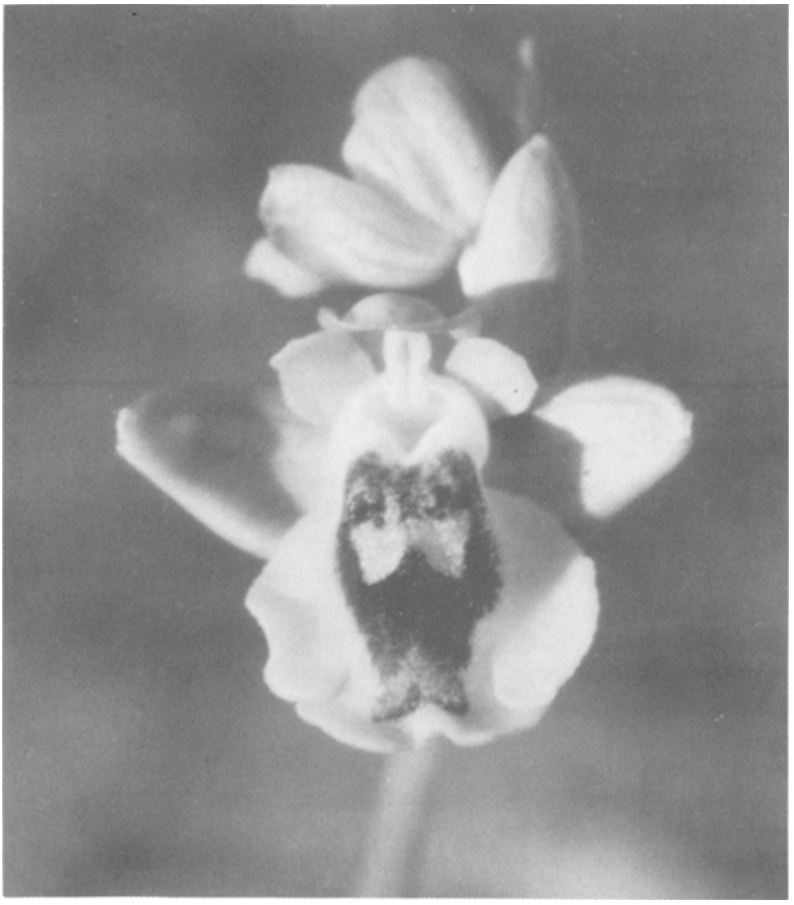

FIG. 4. *Ophrys lutea* flower, Spain.

of the visiting males. However, the strong behavior-triggering effect of the *E,E*-farnesol demonstrated in this study might be interpreted as a hint of such a condition. The females of several *Andrena* species also produce *E,E*-farnesol in their abdominal Dufour's gland (Bergström and Tengö, 1974). Several aliphatic primary and secondary alcohols and a few terpene alcohols present in the *O. lutea* labella extracts also have been found in the gland secretion of other *Andrena* bee species (Tengö and Bergström, 1976, 1977; Francke et al., 1981; Bergström et al., 1982). The odor similarities between the *Ophrys* group *Fusci-Luteae* and their pollinators are further discussed by Borg-Karlson et al. (1985).

The sesquiterpene hydrocarbons found in *O. lutea* have previously been proposed to be responsible for the odor attraction of the pollinator bees to the flowers (Kullenberg and Bergström, 1976). So far these compounds have not

been found in *Andrena* bees. Accepting the condition that the odor of *Ophrys lutea* is generally attractive to many species of *Andrena*, we note that the sesquiterpene hydrocarbons seem to have less influence in the pollination relationship. The large amounts of aliphatic hydrocarbons, aldehydes, and esters in fractions 1, 2, and 3 also seem to be of minor importance in releasing the odor-guided copulatory behavior.

Samples that elicited copulatory attempts on the dummies were the methanol extract (21), the TLC fractions 4 (23) and 5 (18), and the mixture of citral and *E,E*-farnesol (22). No single compound had comparable excitatory capacity. These observations indicate that a complex mixture of odor compounds might have an additive or synergistic function in the behavior chain.

The extracts and the TLC fractions differed from the authentic blends and single substances in their ability to elicit long-lasting approach flights (lasting more than 1 sec) towards the odor source. The *A. fuscipes* male extract (56% of behavior type 2), the *O. lutea* extracts (M = 26%, MWP = 33%, H = 54%), and fractions 4 (22%) and 5 (33%) frequently elicited long-lasting approaches, while the mixture of citral and *E,E*-farnesol as well as the *E,E*-farnesol alone, although being strongly attractive, with many approaches and in some cases copulation attempts, only elicited a few long-lasting behavioral events (10% and 8%, respectively).

A comparison of the *O. lutea* blend (10), the *E,E*-farnesol sample (19), and the combination of these (25) in the behavior experiments may indicate a synergistic effect, but also the importance of a multicomponent secretion to release high-level excitation in the male bees. A comparison of the attractivity of single compounds and blends shows the *E,E*-farnesol is of great importance when a powerful artificial male or female *A. fuscipes*, as well as an *O. lutea*, blend is to be constructed.

The large proportion of citral present in the mandibular gland secretion of *A. fuscipes* (Table 3) (cf. also Tengö and Bergström, 1976) is characteristic for several species of *Andrena* bees (Tengö and Bergström, 1976, 1977; Bergström et al., 1982) The odor marks made by the *A. fuscipes* males during their patrolling flights smell strongly like citral to the human nose. Since the bees continuously odor-mark during their flights, and the gland secretion thus evaporates from all perfume spots within the whole flight area, geranial and neral probably function as a general flight area attractant rather than as an close-range excitant. The occurrence of citral in *O. lutea* (although in relatively small amounts) might attract the pollinators to the orchids' habitats. Geranial and neral may also be formed from the corresponding alcohols during the test period, thus increasing the attractivity of the extract samples.

Tengö (1979) showed that two North American *Andrena* species are attracted to the odor of *O. lutea* hexane extract, thus supporting the concept that *O. lutea* emits a composition of odoriferous compounds that appeals to *Andrena* male bees in general. Our present tests, using a noncoexisting *Ophrys–Andrena*

constellation, confirms this view. It is conceivable that the orchid, by producing a large array of compounds, has evolved and retained the ability to attract males of many or most of the *Andrena* species. It must be advantageous for the *Ophrys* orchids to have an odor that is attractive to *Andrena* species in general. This may facilitate a wider geographical distribution and make the pollination of the flowers less dependent on exact correlation of blossom time and swarming period of the bee.

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FEMALE-PRODUCED SEXUAL PHEROMONE OF *Sceliodes cordalis* (LEPIDOPTERA: PYRALIDAE)

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Abstract—(*E*)-11-Hexadecen-1-yl acetate and (*E*)-11-hexadecen-1-ol were identified in extracts from the abdominal pheromone gland of female *Sceliodes cordalis* and in a 1:1 ratio acted as a potent field attractant for males. Sustained upwind flight by males to this mixture in a wind tunnel confirmed the identity of these compounds as major components of the natural sex pheromone of this species. The synthetic pheromone was used to define the annual limits of male flight activity. In the presence of 1–10 μg of pheromone per liter of air, the mating rate of laboratory populations was halved. The *Z* isomers of both pheromone components decreased the numbers of males captured in traps baited with virgin females in the field and inhibited upwind flight in the wind tunnel. The attraction of males of *Mnesictena flavidalis* to virgin females of *S. cordalis* and synthetic mixtures was demonstrated to be due to the acetate component alone. Male *Eudonia* sp. nr *linealis* were captured by (*Z*)-11-hexadecen-1-yl acetate.

Key Words—Sex pheromone, *Sceliodes cordalis*, *Mnesictena flavidalis*, Lepidoptera Pyralidae, Pyraustinae, *Eudonia* sp nr *linealis*, Scopariinae, (*E*)-11-hexadecen-1-yl acetate, (*E*)-11-hexadecen-1-ol, (*Z*)-11-hexadecen-1-yl acetate, communication disruption, wind tunnel.

INTRODUCTION

Sceliodes cordalis (Doubleday) (Pyralidae, Pyraustinae) has been recorded as feeding on a variety of solanaceous plants in New Zealand and Australia. Win-

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ter crops of eggplant (*Solanum melongena* L.) are seriously affected, but only minor damage to tomatoes and capsicums in Queensland has been recorded (Davis, 1964). New Zealand Ministry of Agriculture and Fisheries records (1980–1981) suggest that *S. cordalis* has the potential to limit the production of pepino (*Solanum muricatum* Aiton) in New Zealand. Large commercial plantings of the New Zealand native host, poroporo (*Solanum aviculare* G. Forster), are relatively little troubled (N. Martin, personal communication).

The adult moth is rarely attracted to light, and no specific baits are known. Our study aimed to identify the sexual pheromone to provide a tool for surveys and possibly as a component of future integrated control programs.

METHODS AND MATERIALS

Insects. Larvae were collected from infested poroporo berries at the Pukekohe Research Station, South Auckland. Adults emerged, mated, and laid eggs on paper towels. The calyx was removed from a poroporo berry, and a freshly emerged first instar larva was placed on the moist surface. The berries were placed on sand-lined trays and held at 25°C in the dark. Pupae were sexed on the basis of their incipient genital apertures and held on paper tissues under ambient conditions until emergence.

Mating Observations. Observations were conducted in the laboratory from dusk till dawn under ambient (moonlit) conditions. Six pairs of virgin moths were placed in a 10-liter plastic observation cage and observed at hourly intervals to establish the time of mating, after which continuous observation commenced. Observations were made on three nights, with three groups of moths.

Field Trapping. Apart from an initial trial with Pherocon IC traps (Zöecon Corporation), metal "triangular" traps (Lewis and Macaulay, 1976) were used for field studies. The bottoms of the triangular traps were lined with 15 × 20-cm acetate sheets smeared with Tangletrap (Tanglefoot Co. Grand Rapids, Michigan).

The traps were baited with moths and/or a 5-mm sleeve-type rubber stopper (Arthur H. Thomas Co. Philadelphia, Cat. No. 1780-B10) loaded with synthetic material. Moth baits included 1–6 virgin females (1 day old), mated females, dead virgin females, a virgin male, or virgin females enclosed in a small plastic bag. Moths were housed in a 5 × 5 × 5-cm wire mesh cage. Control traps were baited with empty mesh cages.

The traps were held on stakes just above the plant canopy or suspended in the top third of the plant in plants more than 2 m high. The trap positions were assigned randomly and rerandomized each time the catch was retrieved; the adhesive liner was replaced each time. When a trap held both a female in a cage and a rubber stopper, the two items were randomized independently when the traps were replaced. Experiments with synthetic chemicals were conducted with stoppers identified solely with a code number.

Although the virgin female first used remained attractive for three weeks

(Figure 3A), in most cases each female was used for one week only. The trap was cleared of males twice a week so each female produced two catches. During the 1981–1982 season, each female was assigned a code letter so that the relative attractiveness of each female could be assessed on the basis of these two catches before the variable under test was examined statistically.

Three areas of poroporo (a total of 360 plants covering 170 m^2) at the Mt. Albert Research Centre, Auckland (MARC), and five areas at the Pukekohe Research Station (a total of 780 plants covering 1800 m^2) were used as trap sites. Either five traps (virgin females 1979–1980) or two traps (synthetic material 1981–1982) were operated at MARC, with up to 22 traps in use at Pukekohe. Apart from a single remote station, the traps at Pukekohe were separated by approximately 9 m. Because of the scarcity of other host plants nearby, it was unlikely that large populations existed outside these areas.

Statistical Analysis. Unless otherwise stated, all quoted significance levels are derived from analysis of contingency tables of counts as log-linear models (McCullagh and Nelder, 1983). Any t statistics used are derived from differences between the parameters of the fitted models.

Preparation of Pheromone Extract. Ovipositors, with attached pheromone glands, were clipped from female moths (2–6 days old) and placed in redistilled pentane or methylene chloride. Collections were made during daylight (1980–1981) or between 2 AM and 4 AM (1982).

Gas Chromatography (GC) and Selected-Ion Mass Spectrometry (SIM). Preparative gas chromatography was performed on a Varian 1700 instrument fitted with a variable-ratio effluent splitter (Scientific Glass Engineering Pty) housed in the detector oven. The eluate was split between a flame ionization detector (FID) (one part) and a heated collection port (nine parts). Samples were collected by attaching the tip of a Pasteur pipet (ca. 10 cm × 1.6 mm OD) to the collection port, using Teflon tubing. During the collection, a section of the Pasteur pipet was cooled in a tray containing powdered solid carbon dioxide. The GC column was stainless-steel tubing (2 m × 2.1 mm ID) packed with 3% OV-1 on Chromosorb W AW-DMCS (80–100 mesh). A nitrogen carrier-gas flow of ca. 15 ml/min and temperature programs of 100–200°C at 4°/min or 140–190°C at 2°/min were used.

Analytical GC was performed using either a OV-101 WCOT (25 m × 0.3 mm ID, fused silica, Hewlett Packard, Avondale, PA, USA) or a Carbowax 20 M SCOT (50 m × 0.5 mm ID, glass, Scientific Glass Engineering, Melbourne, Australia) capillary column. The carrier gas was helium at 1.5 ml/min. The first column was temperature programmed from 150°C to 190°C at 4°/min, while the second column was used isothermally at temperatures between 165°C and 190°C.

GC-MS was carried out on a GC interfaced to an AEI MS30 mass spectrometer via a silicone rubber membrane separator (at 180°C). The mass spectrometer used electron impact ionization (20 eV) and operated in the selective ion monitoring (SIM) mode.

Electroantennogram (EAG) Analysis. The EAG responses of adult male *S. cordalis* to standard samples of acetates and alcohols were determined and standardized according to the procedures of Roelofs and Comeau (1971).

Chemicals. Our original samples of (*E*)-11- and (*Z*)-11-hexadecenyl acetates (*E*11–16:Ac, *Z*11–16:Ac) were kindly provided by Dr. E. W. Underhill (N.R.C., Saskatoon, Saskatchewan, Canada), and Dr. J. Weatherston, Albany International, Needham Heights, Massachusetts). Subsequently we synthesized the (*E*)-11-acetate by conventional means and prepared the corresponding alcohol from it by the method of Schwarz and Waters (1972). Other reference chemicals for EAG tests were from our collection. All samples were >98% pure by GC analysis.

Wind-Tunnel Experiments. A wind tunel was constructed based on the design of Miller and Roelofs (1978). Our wind tunnel lacked their illumination system, observations being made under ambient moonlit conditions supplemented by an incandescent light in a distant room. The motor-driven orange and black belt was replaced by a hand-driven white and black belt. Baffles reduced the speed of the fan-driven air current to 20 cm/sec. Synthetic mixtures on filter paper clipped to a wire fork acted as a pheromone source. Rubber stoppers taken from field traps were also used. For each test two or three males were released from small cages pressed into openings in the downwind wire mesh screen. The experiments were carried out between 1 AM and 4 AM.

Inhibition of Mating with Elevated Background Levels of Normal Pheromone Components. The impact of the identified chemicals on mating was measured with laboratory reared adults in sealed containers. Five unmated 1-day-old moths of each sex were enclosed in 10-liter plastic bowls containing a square of filter paper loaded with either 0, 1, 10, or 100 μg of a 1:1 mixture of *E*11–16:Ac and *E*11–16:OH. The experiment was replicated five times. The bowls were held for three days in darkness in the laboratory at ambient temperatures. Each female was then dissected and scored as mated when a spermatophore was recovered.

RESULTS

Sexual Behavior of S. cordalis. Sexual activity was observed only during the hours preceding daybreak. Very little spontaneous flight was seen until 1 AM (New Zealand summer time) (4 hr into scotophase). The first mating occurred at 1:20 AM, sexual activity peaked at 2 AM, and ceased at 4 AM, approximately an hour before daybreak. The resting posture of both sexes superficially resembled the "calling" posture of other species (Figure 1). The abdomen was tightly curled up over the thorax, the wings were spread with the apices pressed against the ground, the forelegs were outstretched, and the antennae were pressed down on the inner edge of the wing. In this position, prom-

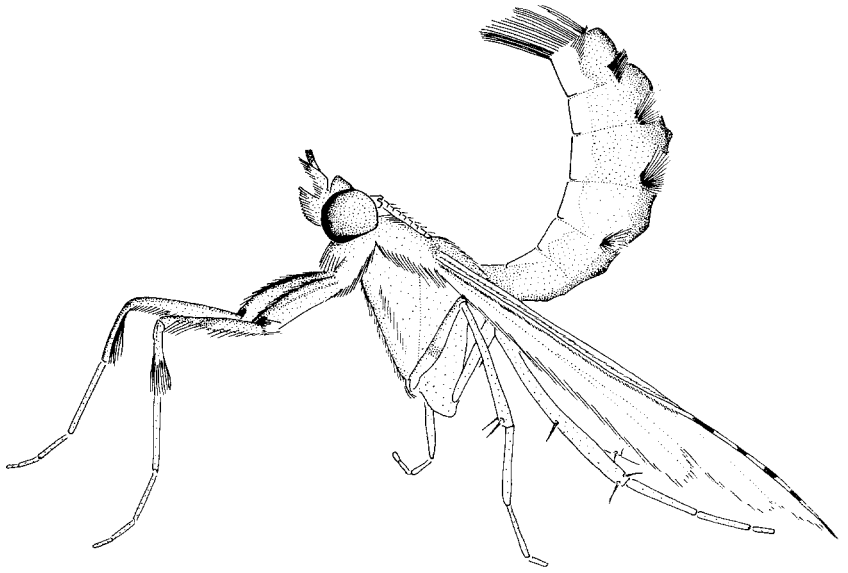

FIG 1. Male *Sceliodes cordalis* at rest (photophase and first half of scotophase), wingspan = 2 cm.

inent scales at the base of the abdomen were spread, exposing well-developed tympanic organs. With the onset of sexual activity, the moths lowered their abdomen. Females with the abdomen touching the substrate were sexually receptive and accordingly were thought to be "calling," although eversion of the sex pheromone gland on the dorsal surface of the ovipositor was not observed.

Low-amplitude antennal movements were the first signal of sexual arousal in the male. The wings were then lifted above the body and vibrated rapidly. The claspers were everted, and the legs were extended to full stretch (Figure 2) for several seconds before take-off. Males hovered below and to one side of the females and then made several short very rapid lunges with the claspers. As soon as coupling was achieved, the male hung head downwards.

Field Trapping with Live Moths. Sticky traps baited with *S. cordalis* females proved an effective means of capturing males. Five hundred sixty-four male *S. cordalis* were taken from five Pherocon IC traps, attracted by single, virgin females (Figure 3A). The traps were operated between December 5, 1979, and March 17, 1980, with the peak catch occurring in late February. Trapped moths were confirmed to be males by extrusion of the claspers with fine forceps. Most of the captured moths were found close to the female's cage and many had everted their claspers. Three individuals had produced a distinct, well-formed spermatophore.

There were no significant differences between the number of males caught

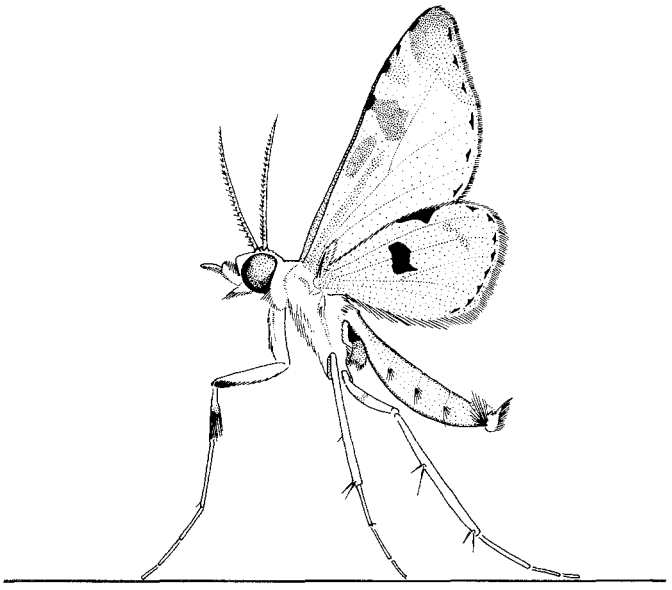

FIG. 2. Sexually stimulated male *s. cordalis* (last half of the scotophase in the presence of virgin females).

by traps containing one, two, or three females ($P > 0.50$), although cages with six females were less attractive, possibly due to physical interference during competition for calling positions on the wire mesh ($P < 0.005$) (Table 1). A single virgin female was used for all subsequent experiments.

Live, unrestricted virgin females were essential for trapping males. All other treatments, namely mated females, dead virgin females, live virgin males, and virgin females inside a small plastic bag, failed to catch a single male. No moths were captured in control traps.

Small numbers (57) of another pyralid moth, *Mnesictena flavidalis* (Doubleday) (Pyralidae; Pyraustinae) were also captured during the 15-week period (Figure 3A). All were males. This species was not captured by the control traps. All other moth species were captured in very small numbers, were of both sexes, and were also captured in the control trap.

Identification of Major Component of Sexual Pheromone of S. cordalis. The electroantennogram offered the first information on the identity of the major component of the pheromone. The strongest response of isolated antennae of male *S. cordalis* presented with a series of monounsaturated hexadecenyl, tetradecenyl, and dodencenyl acetates was observed with *Z*11- and *E*11–16 : Ac followed by the homologous *Z*11- and *E*11–14 : Ac (Figure 4).

The EAG results were supported by chromatographic analysis of the ex-

TABLE 1. *Sceliodes cordalis* MALES ATTRACTED BY ONE, TWO, THREE, OR SIX VIRGIN *Sceliodes cordalis* FEMALES

Trap No.[a]	No. of females	Catch/week	Trap No.[b]	No. of females	Catch/week
I	1	0.9	I	1	20
II	1	0.9	II	2	11
III	1	2.2	III	2	18
IV	3	1.3	IV	3	20
V	3	2.6	V	6	6[c]

[a] Traps with one or three females were compared between December 5 and 20, 1979 at MARC. The traps were sampled on 11 occasions in the course of 16 nights. Eighteen males were caught.
[b] Traps with one, two, three, or six females were compared between January 8 and 14 1980 at MARC. The traps were sampled on five occasions in the course of seven nights. Seventy five males were caught.
[c] Traps with 6 females caught significantly fewer males than traps with one, two, or three females ($P < 0.005$ χ^2 test).

tract from the female pheromone gland. Initially, a crude female abdominal tip extract (from about 10 moths) was fractionated on the 3% OV-1 packed column. The FID trace showed a number of peaks, the major ones being at 18.5 and 20.13 min. Timed, 1-min collections were made of the effluent. The individual collections were then assayed for EAG activity which revealed a distinct maximum in the 17- to 18-min and 18- to 19-min collections. Under the operating conditions (100–200°C at 4°/min), the retention time (R_t) of 12:Ac was 9.65 min, of 14:Ac = 14.45 min, and 16:Ac = 18.08 min. This suggested to us that a C_{16} acetate was a major component of the pheromone. Another extract (from 20 moths) was then fractionated with the 3% OV-1 packed column programmed from 140 to 190°C at 2°/min. Under these conditions, the R_t of 12:Ac was 6.00 min, of 14:Ac = 10.13 min, and 16:Ac = 15.75 min. Three collections were made at 3–7 min (A), at 7–12 min (B), and at 12–17 min (C). Of these fractions, only C was strongly active in the EAG assay.

The Pasteur pipet in which fraction C was collected was rinsed out with ca. 300 μl of methylene chloride, and the washings concentrated to ca. 5 μl under a nitrogen jet. After adding 14:Ac as an internal reference, a 1-μl aliquot of this extract was then analyzed by GC-MS on the Carbowax 20 M SCOT column, at 165°C. The mass spectrometer was operated in the SIM mode (*m/z* 192, 194, 196, and 222). Apart from the 14:Ac reference, R_t = 18.32 min, seen on the *m/z* 194 trace, the only signal seen was an intense one on the *m/z* 222 trace, R_t = 38.20 min. Under the same conditions *Z*7–16:Ac had R_t = 36.89 min. These results suggested that the tip extracts indeed contained a hexadecenyl acetate. Tetradecenyl acetates and alcohols and tetradecadienyl acetates and alcohols were either absent or below detection limits.

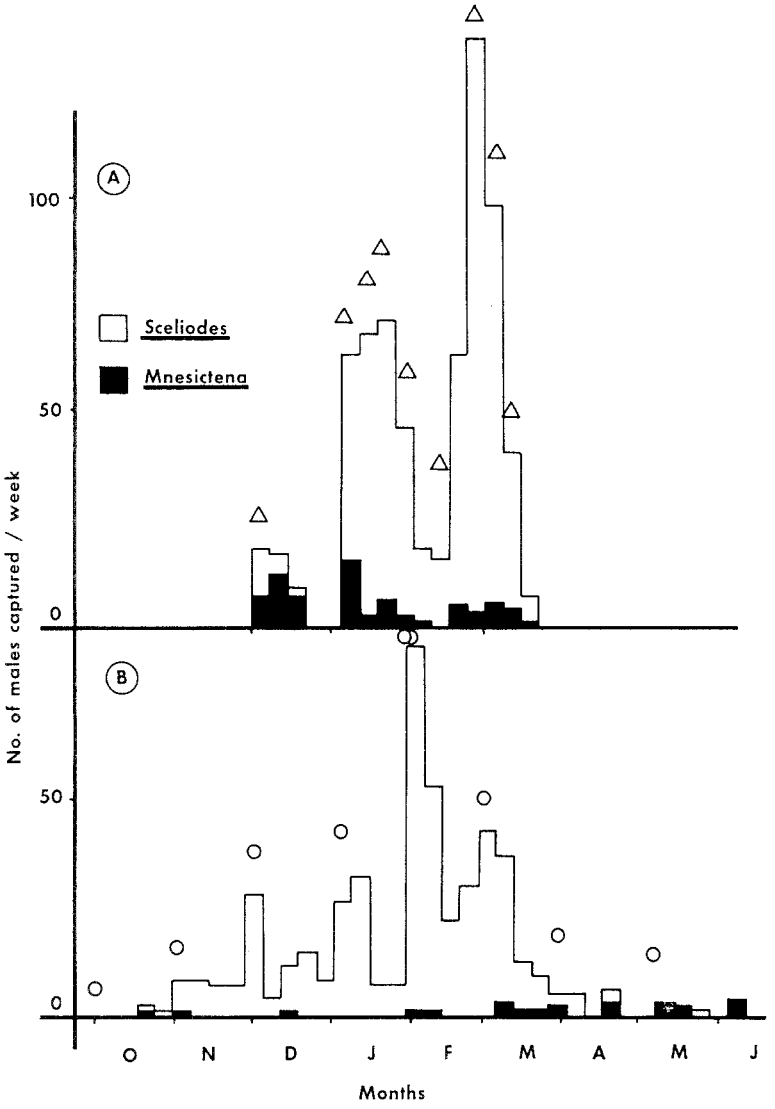

FIG. 3. Male *Sceliodes cordalis* and *Mnesictena flavidalis* captured in sex attractant traps (one-week intervals). (A) Males captured by five Pherocon IC traps baited with virgin females usually replaced (△) every one or two weeks (December 5, 1979, to March 17, 1980). (B) Males captured by two triangular traps baited with synthetic pheromone replaced (○) monthly (October 3, 1981, to June 11, 1982). The new cap replaced on February 1, 1982, was blown away by strong winds and replaced the next day.

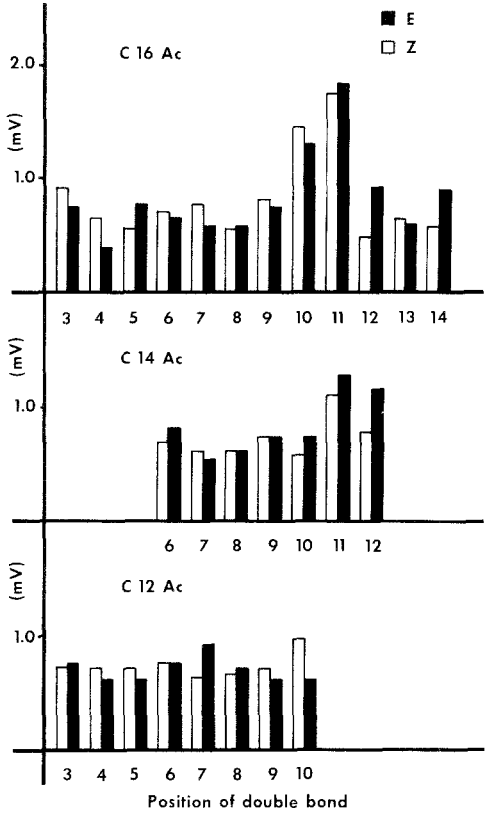

FIG. 4. Electroantennogram responses by antennae of male *Sceliodes cordalis* when stimulated with a series of C_{12}, C_{14}, C_{16} monounsaturated acetates. One microgram of each standard was used, and each point was replicated two or three times.

The presence of the hexadecenyl acetate was indicated by another experiment in which an aliquot of fraction C, to which 16:Ac was added as an internal reference, was analyzed on the Carbowax 20 M column, at 170°C, using SIM (*m/z* 192, 220, 222, and 224 detection). As before, apart from the reference, 16:Ac (R_t = 32.02 min), the only peak seen up to 45 min was on the *m/z* 222 trace (R_t = 35.50 min). The relative retention time (R_t^{rel}) of this component (16:Ac R_t^{rel} = 1.0) was 1.109. Under the same conditions *E*11–16:Ac had the same R_t^{rel} of 1.110 (within experimental error, ±0.004), while the R_t^{rel} of other hexadecenyl acetates were significantly different (e.g., *Z*11–16:Ac R_t^{rel} = 1.123). Hexadecadienyl acetates were not detected [no peaks on the *m/z* 220 trace, (*Z*,*Z*)-7,11-hexadecadienyl acetate had a relative retention time of 1.194]. At this point we considered that *E*11–16:Ac formed a major part of the sex pheromone.

Field Trapping with Synthetic Mixtures. Initial field trials at MARC with *E*11–16:Ac were disappointing. No *S. cordalis* were caught, although 22 *M. flavidalis* were taken. A virgin female caught 13 *S. cordalis* and two *M. flavidalis* during the same period (December 24, 1980, to January 14, 1981). Addition of varying amounts of the *Z* isomer caught more *M. flavidalis* and suggested that a 10:1 mixture of the two acetates might resemble the natural pheromone of this species (Table 2). The *Z* isomer alone or in combination with other chemicals captured large numbers of male *Eudonia* sp. nr *linealis* (Walker) (Pyralidae; Scopariinae). No *S. cordalis* males were captured. Although no additional components had been detected by EAG assay or by initial GC analysis of female pheromone glands, it was clear that at least one further component was required to lure male moths of this species. Three chemicals structurally related to *E*11–16:Ac were selected and tested in the field. (*Z*,*E*)-7,11-Hexadecadien-1-yl acetate and (*E*)-11-hexadecenal were each tested singly or in combination with *E*11–16:Ac and were as unsuccessful as *E*11–16:Ac presented alone. (*E*)-11-Hexadecen-1-ol appeared to be the missing component because traps baited with *E*11–16:Ac supplemented by the free alcohol caught 31 males in four nights (March 5–9, 1981).

A wide range of ratios of these two chemicals attracted males. The first series of traps at MARC caught a diminishing number of males as the proportion of the alcohol was lowered (Figure 5B,C). A third series run at Pukekohe confirmed the 1:1 ratio as the most attractive (Figure 5A). While all stoppers with both components attracted many males, the 1:1 ratio was significantly more attractive and the 9:1 ratio significantly less attractive than the 1:3 and 3:1 ratios ($P < 0.001$ χ^2 test). Three traps, each baited with a virgin female caught an average of 1.46 males/trap/night and were statistically indistinguishable from the 1:3 and 3:1 ratios of synthetic material. Each component offered by itself failed to capture a single male. Unless otherwise specified, a standard pheromone mix of 50 μg *E*11–16:Ac plus 50 μg *E*11–16:OH on a rubber stopper was used for all subsequent experiments.

If the two components were placed on separate stoppers, the catch dropped dramatically. Traps with a stopper loaded with 50 μg of *E*11–16:Ac touching

TABLE 2. *Mnesictena flavidalis* MALES ATTRACTED BY STOPPERS LOADED WITH *E*- AND Z11–16Ac (μg *E*:μg *Z*)[a]

	50:0	50:1	50:5	50:20	0:50
Catch/trap/week	0.4[a]	2.3[b]	4.6[b]	2.7[b]	0[a]

[a] A single example of each of the stoppers was sampled on five occasions in the course of 18 nights (January 14 to February 1, 1980 at MARC). Numbers followed by different letters are significantly different ($P < 0.0001$, χ^2 test). Twenty-six males were captured.

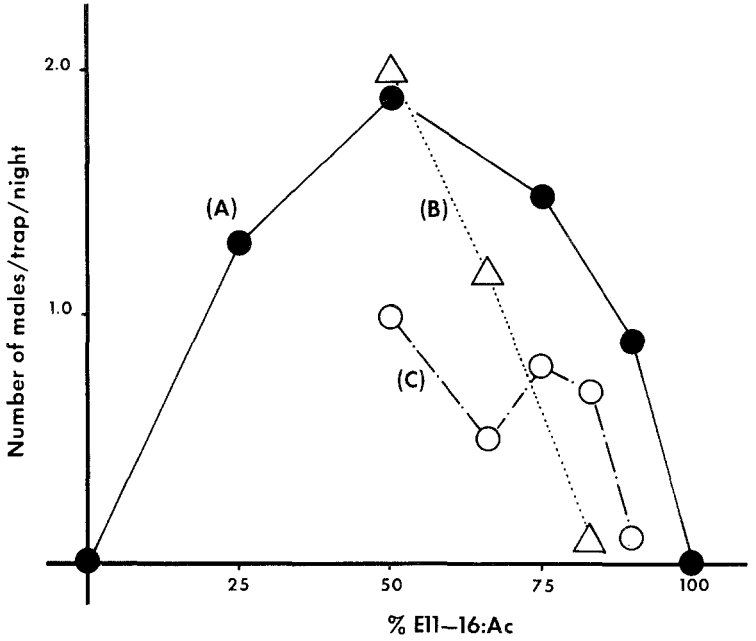

FIG. 5. Comparison between the males captured with stoppers loaded with an increasing percentage of E11-16:Ac. (A) Traps operated at Pukekohe between March 11 and April 1, 1981. Three replicates of each of six types of stopper were sampled on five occasions in the course of 21 nights; 352 males were captured. (B) Traps operated at MARC between March 11 and 21, 1981. A trap of each of three types of stopper was sampled on nine occasions in the course of eleven nights; 36 males were captured. (C) Traps operated at MARC between March 5 and 9, 1981. Two replicates of each of five types of stopper were sampled on three occasions in the course of four nights; 31 males were caught. Stoppers used contained a total of 100 μg synthetic material, in the following ratios. *E*11-16:Ac-*E*11-16:OH 0:1 (0% *E*11-16:Ac), 1:3 (25% *E*11-16:Ac), 1:1 (50% *E*11-16:Ac), 2:1 (66% *E*11-16:Ac), 3:1 (75% *E*11-16:Ac), 5:1 (83% *E*11-16:Ac), 9:1 (90% *E*11-16:Ac), 1:0 (100% *E*11-16:Ac)

a stopper loaded with 50 μg of *E*11-16:OH caught less ($P < 0.001$) than half of the catch of traps with the same amount absorbed on a single stopper (Table 3).

Less than 10% of the expected catch was taken from traps with widely separated stoppers. An acetate-laden stopper was placed near one trap opening and the alcohol-laden stopper placed at the opposite opening (14 cm separation) so that the airflow passing through the trap would produce a well-mixed odor plume. Separate odor plumes were produced by tucking similar stoppers in the sides of the trap (10 cm separation) perpendicular to the airflow. Wide separation of the two components severely reduces the catch ($P < 0.001$), but the

TABLE 3. REDUCTION IN CAPTURE OF *Sceliodes cordalis* MALES WITH INCREASING SEPARATION OF TWO COMPONENTS[a]

	Single stopper	Two stoppers		
		Adjacent	10 cm separation perpendicular to air flow	14 cm separation parallel to air flow
Catch/trapweek	20.8[a]	7.5[b]	0.5[c]	1.8[c]

[a]Single stoppers carried 50 μg *E*11-16:Ac plus 50 μg *E*11-16:OH. Other traps carried two stoppers, one with 50 μg *E*11-16:Ac alone, the second with 50 μg *E*11-16:OH alone. Two replicates of each of four dispositions of stopper were sampled on four occasions in the course of 14 nights (March 1-15, 1982, at Pukekohe). One hundred twenty-two males were caught. Catches followed by different letters are significantly different ($P < 0.001$, t statistic).

distribution pattern of the stoppers in the airflow appears to have had little effect (Table 3).

Four males found in synthetic baited traps had extruded a fully formed spermatophore while held by the adhesive layer.

Confirmation of E11-16:OH as Authentic Component of S. cordalis *Pheromone.* As we had found that this species mated in the hours before dawn, we made our next extracts between 2 and 4 AM. These extracts were analyzed first on the OV-101 capillary column, at 175°C, using SIM (*m/z* 222). Peaks were seen at 12.77 and 19.58 min in the positions expected for hexadecenols and hexadecenyl acetates, respectively. Under the same operating conditions *E*11-16:OH had R_t = 12.67 min, and *E*11-16:Ac 19.58 min. To provide better discrimination among the hexadecenols, the analyses were then repeated on the Carbowax 20-M capillary column at 190°C after adding 16:Ac as an internal standard. The SIM trace (*m/z* 222) showed peaks at 12.62 min, and 15.40 min (16:Ac, SIM *m/z* 224 appeared at 11.68 min). The R_t^{rel} of the two components, 1.080 and 1.318, respectively, corresponded well with those of *E*11-16:Ac (1.081) and *E*11-16:OH (1.324). Other hexadecenyl alcohols tested had different R_t^{rel}. Our successful detection of the hexadecenol only from the early morning extracts suggests that this component is synthesized immediately before release during calling.

The isolated male antennae also responded to this alcohol. There was no significant difference between the EAG response to *E*11-16:Ac and *E*11-16:OH (Wilcoxon's rank-sum test, $P < 0.10$) (Table 4). The saturated hexadecenyl acetate was less stimulatory (Kruskal-Wallis tests for equality of three populations, $P < 0.01$). We therefore concluded that the sex pheromone of *S. cordalis* contains as principal components *E*11-16:Ac and *E*11-16:OH. The relative abundance of the two ions suggested a 2:1 ratio of the two components.

TABLE 4. EAG RESPONSES TO *E*11-16:Ac, *E*11-16:OH, and 16:Ac[a]

	Response (mV)	
	10 μg	1 μg
*E*11-16:Ac	1.07	0.58[a]
*E*11-16:OH	0.87	0.63[a]
16:Ac		0.33[b]

[a] Average of seven replicates. Responses followed by different letters are significantly different ($P < 0.01$, Kruskal-Wallis test for equality of three populations).

Use of Pheromone Traps to Define Seasonal Distribution of S. cordalis. During the third season, two traps at MARC were baited with the standard pheromone loading and emptied daily from October 3, 1981, to June 11, 1982. Four hundred sixty-two male *S. cordalis* and 15 male *M. flavidalis* were captured. No females of either of these species were caught. The first male *S. cordalis* was captured on October 21 with the first peak in early November. These individuals had probably developed from overwintering prepupae (N. Martin, personal communication). The seasonal peak was reached in early February and male activity had largely ceased by mid-April (Figure 3B). The temporal difference in the seasonal peak populations in 1979–1980 and 1981–1982 (Figure 3A, B) may have been due to climatic differences, while the lower catches of *M. flavidalis* in 1981–1982 were possibly due to the different attractants used. This species is extremely common (Gaskin, 1966), so lack of available males in 1981–1982 was unlikely.

Pheromone Loadings and Male Capture. Trap catches were highly correlated with log pheromone concentration ($P < 0.0001$, $\chi^2 = 166$ with 3 *df*) (Figure 6). Lines A and B intersect the *x* axis between 7 and 9 μg, establishing a minimum effective concentration of pheromone in rubber stoppers in field traps. Only one insect was caught with 1 μg, and then only in a trap placed at the end of the line of poroporo at a distance of 37 m from the other traps. If we assume that the evaporation rates of *Z/E* isomers are similar and use the constants and equations of Butler and McDonough (1979, 1981), a minimally effective stopper would initially release *E*11-16:OH at a rate of 7.4×10^{-8} μg/sec ($t^{1/2} = 432$ days) and *E*11-16:Ac at a rate of 5.0×10^{-8} μg/sec ($t^{1/2} = 635$ days).

Interference between traps was observed. Significantly smaller catches ($P < 0.005$) were taken by all traps neighboring those baited with 1000 μg of pheromone (analysis of data shown in Figure 6A). As traps were only separated by 9 m, interference between traps was not unexpected. When increased loadings of 1000 μg and 10,000 μg were offered, the males exhibited a preference

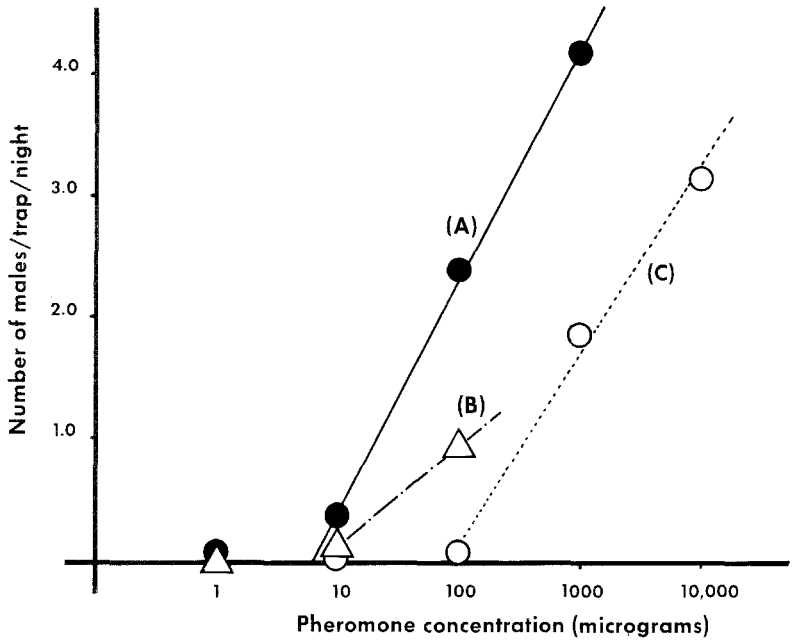

FIG. 6. Relationship between males captured and pheromone concentration in the rubber stopper (Ac to OH ratio = 1:1) (A) Concentrations of 1, 10, 100, and 1000 μg compared between February 8 and March 1, 1982, at Pukekohe. Two replicates of each of four types of stopper were sampled on six occasions in the course of 21 nights; 292 males were captured. (B) Concentrations of 1, 10, and 100 μg compared between April 1 and 15, 1981, at Pukekohe. Three replicates of each of three types of stopper were sampled on three occasions in the course of 14 nights; 43 males were captured. (C) Concentrations of 10, 100, 1000, and 10,000 μg compared between March 15 and 22, 1982, at Pukekohe. Two replicates of each of four types of stopper were sampled on two occasions in the course of seven nights; 72 males were caught.

for the highest loading, almost completely ignoring the usually attractive 100-μg bait (Figure 6C).

Impact of Trapping on Population. The deployment of traps baited with physiologically potent materials and removal of adult males may have a significant effect on the wild population within the catchment area of the traps. At both the MARC and Pukekohe field sites, the first catch markedly exceeded subsequent catches (Figure 7, Table 5). At the MARC site, the 24 moths caught on the first night included older battered individuals as well as apparently freshly emerged males. Later, smaller catches were made up of predominantly newly emerged (i.e., undamaged) insects. At Pukekohe, the first catch of 247 was followed by catches of 64 or less. Both the synthetic attractant and the natural attractant (virgin females) showed initial high catches (Figure 7). The drop in

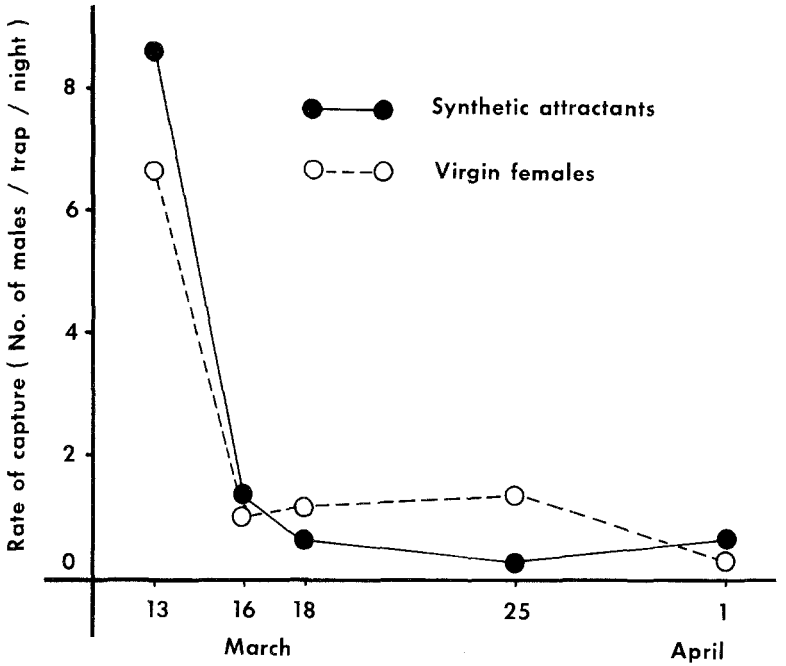

FIG. 7. Catches of male moths at Pukekohe between March 11 and April 1, 1982. Three traps baited with virgin females and 12 traps with stoppers loaded with synthetic material werer sampled on five occasions in the course of 21 nights. Synthetic baited traps caught 352 males and virgin females caught 92 males.

TABLE 5. COMPARISON OF FIRST NIGHTS' CATCH WITH AVERAGE SUBSEQUENT CATCH[a]

	Catch males/trap/night				
	Test A	Test B	Test C	Test D	Test E
First night[b]	2.4	8.6	6.7	8.7	4.5
Av. subsequent nights	0.4	0.6	0.9	1.0	2.5

[a]Test A: ten traps baited with synthetic attractant were smapled on three occasions in the course of five nights (March 5–9, 1981 at MARC). Thirty-one males were captured. Test B: twelve traps baited with synthetic attractant were sampled on five occasions in the course of 21 nights (March 11–April 1, 1981, at Pukekohe). Three hundred fifty-two males were captured. Test C: three traps baited with virgin females were sampled on five occasions in the course of 21 nights (as B). Ninety-two males were captured. Test D: three traps baited with synthetic attractant were sampled on 14 occasions in the course of 14 nights (February 6–20, 1985, at MARC). Sixty-six males were captured. Test E: two traps baited with virgin females were sampled on 14 occasions in the course of 14 nights (as D). Seventy-five males were captured.

[b]The first catches were significantly greater than subsequent catches ($P < 0.05$, χ^2 test).

catch with time was not due to the end of the flight season, as the same result could be obtained during the peak February flight (Table 5, columns D and E).

Inhibition of Male Attraction with Pheromone Geometrical Isomers. Addition of (Z)-11-hexadecen-1-yl acetate (Z11–16:Ac) or (Z)-11-hexadecen-1-ol (Z11–16:OH) to attractive synthetic mixtures and to traps baited with virgin females diminished the daily catch of male moths. Stoppers loaded with 50 μg of E11–16:Ac plus 50 μg of E11–16:OH attracted 37 males to four traps between March 18 and April 15, 1981. No males were captured in four neighboring traps with stoppers loaded with 50 μg of Z11–16:Ac in place of E11–16:Ac. The final group of four traps baited with stoppers containing 50 μg of Z11–16:Ac, 50 μg of E11–16:Ac, and 50 μg of E11–16:OH caught a single male.

The Z isomers diminished the attractiveness of virgin females. A stopper loaded with 100 μg of Z11–16:Ac placed on the trap floor next to the wire cage housing a virgin female reduced catches to 10% of the expected numbers (Table 6). Lesser amounts of this chemical did not significantly reduce catches. A different inhibition pattern was observed with the alcohol, with 30–50% reductions in the expected catch observed across the concentration range tested (Table 6).

Inhibition of Mating with Elevated Background Levels of Normal Pheromone Components. The binary pheromone mixture disrupted normal mating of

TABLE 6. REDUCTION IN NUMBERS OF *Sceliodes cordalis* MALES ATTRACTED TO TRAPS BY VIRGIN FEMALES IN PRESENCE OF INCREASING CONCENTRATIONS OF Z ISOMERS

	Males (catch/trap/week)			
	Virgin female alone	Virgin female +1 μg Z isomer	Virgin female +10 μg Z isomer	Virgin female +100 μg Z isomer
Z11–16:Ac[a]	11.3[a]	12.4[a]	10.8[a]	1.1[b]
Z11–16:OH[b]	7.2[c]	5.0[d]	4.5[d]	3.5[d]

[a] The impact of 0, 1, 10, or 100 μg Z11–16:Ac on the attractiveness of virgin females was measured on (1) January 26–February 8, 1982, and (2) April 1–15, 1981 at Pukekohe. (1) Two replicates of virgin females with no stopper or with one of three types of stopper were sampled on three occasions in the course of 13 days. Two hundred ten males were captured. (2) Three replicates of virgin females with no stopper or with one of three types of stopper were sampled on two occasions in the course of 14 days. Eighty-four males were captured.

[b] The impact of 0, 1, 10, or 100 μg of Z11–16:OH on the attractiveness of virgin females was measured from March 22 to April 8, 1982, at Pukekohe. Two replicates of virgin females with no stopper or with one of three types of stopper were sampled on five occasions in the course of 17 days. Ninety-eight males were captured. In each line, weekly catches followed by different letters were significantly different ($P < 0.001$, ANOVA).

TABLE 7. PERCENTAGE OF *Sceliodes cordalis* FEMALES REMAINING UNMATED IN PRESENCE OF MALES INFLUENCED BY VARYING AMOUNTS OF SYNTHETIC PHEROMONE[a]

	Synthetic pheromone			
	0 μg	1 μg	10 μg	100 μg
Unmated females (%)	4[a]	20[a]	40[b]	44[b]

[a] Five groups of five males and five females held for three days in the presence of 0, 1, 10, or 100 μg pheromone. Percentages with different superscripts are significantly different ($P < 0.05$ ANOVA on arc-sine transformed data).

moths enclosed in a sealed plastic bowl (10 liter volume). After three nights, almost half the females remained unmated when 10 or 100 μg of *E*11–16:Ac plus *E*11–16:OH (1:1) on filter paper was placed in the bowl (Table 7). The possibility of visual mate recognition was eliminated by keeping the bowls in complete darkness for the period of the experiment.

All mated females ($N = 153$) dissected had a single spermatophore. Multiple mating was not encountered in the laboratory or field.

Wind-Tunnel Experiments. On four separate nights, laboratory-reared males flew from a release cage to a pheromone release point. Two or three males in a small mesh cage clipped into a hole in the downwind screen remained inactive until stimulated by the pheromone plume. Sexual arousal began 5–10 sec after the introduction of the pheromone-bearing paper (50 ng of 1:1 *E*11–16:Ac + *E*11–16:OH) into the airstream. The heightened arousal stage lasted a further 5 sec before take-off and flight toward the source. Rubber stoppers from field traps were also adequate stimuli. Males flew at a high angle of incidence (>30°) with antennae raised and claspers partially extruded. The ground speed was low, about 1–3 cm/sec. The line of flight was not completely straight, the moth making short (1 to 2-cm) zig-zags as it flew upwind. When the moth drew near the pheromone paper, it hovered 5–20 cm away from the paper for 4–180 sec ($N = 10$ observations) occasionally darting close ($N = 4$), or immediately landing ($N = 7$). Considerable effort was necessary to dislodge some of the moths from the paper after landing.

When the floor stripes were moved downwind, increasing the apparent ground speed to a moth in midflight, the moth reduced its airspeed and was carried downwind, still facing the plume and zig-zagging slightly ($N = 11$). The moth could be made to retrace its flight path and land again on the downwind screen near to its original point of take-off. Removal of the pheromone source while the moth was still in flight elicited the "casting" behavior observed by Kennedy and Marsh (1974) in *Ephestia* (= *Cadra*) *cautella* (Walker). One to two seconds after removal of the paper, the steady upwind flight pattern

changed to wild swings across the tunnel as the male turned into a rapid crosswind flight with sharp reversals between left and right.

In the middle of one experimental period, the pheromone source was removed, and a group of four males observed for 10 min. During this period, no flight activity of any kind was observed. The moths had been actively following pheromone trails before the break. When the pheromone paper was reintroduced, standard upwind orientation toward the pheromone source was resumed. The two components of the pheromone failed to stimulate upwind flight when presented singly. *E*11–16 : Ac (50 ng) elicited slight antennal movements only ($N = 4$). Strong antennal and wing movements but no upwind flight followed exposure to a similar concentration of *E*11–16 : OH ($N = 4$). Antennal and wing movements and brief undirected flights were stimulated by *Z*11–16 : Ac ($N = 4$). This isomer, presented simultaneously with pheromone-loaded filter papers, inhibited the upwind flight response. A further series of three filter papers were prepared: 25 ng *E*11–16 : Ac + 25 ng *E*11–16 : OH (A), 25 ng *Z*11–16 : Ac (B), and 2.5 ng *Z*11–16 : Ac (C). These papers were presented in the sequence A, A + B, A, A + C. Upwind flight was stimulated by papers A, but addition of either concentration of the *Z* isomer completely inhibited the response. Of the 30 males used in these tests, 22 showed the state of high sexual arousal and 16 individuals flew in reproducible upwind flight patterns. These results confirmed the conclusions based on the field experiments that *E*11–16 : Ac and *E*11–16 : OH are the major, functional components of the pheromone, that both are essential for the full male response, and that the *Z* isomers inhibit the male response.

DISCUSSION

Although the stereoisomers *Z*11–16:Ac and *Z*11–16:OH have been frequently identified as pheromone components, the only species presently known to use *E*11–16:Ac are *Brachmia macroscopa* Meyrick Gelechiidae (Hirano et al., 1976) and *Mamestra brassicae* (L.) Noctuidae (Struble et al., 1980). Six other species respond to empirical mixtures containing this substance (Inscoe, 1982). *E*11–16:OH has not previously been extracted from insect pheromone glands but has been reported as an attractant synergist to two species (Inscoe, 1982).

The male *S. cordalis* does not appear to discriminate greatly between different ratios of the two pheromone components. This may be explained by the fact that, although the two components have a common carbon chain, the different functional groups result in significant differences in vapor pressure. This difference would make it difficult to maintain a sharply defined ratio of the two components at all distances from the emitting female over a range of temperatures (Roelofs & Cardé, 1977). Although the ratio of the two components may vary, complete superposition of the two plumes is important. Reducing the pro-

portion of *E*11-16:OH in the lure to 10–20% of the standard has the same impact on male catch as separating the source of the two components by less than a centimeter (Figure 5, Table 3). Separate sources might adequately attract males from a distance, but confuse short-range orientation.

Roelofs and Comeau (1968) first reported that the geometrical isomer of a pheromone was very effective in inhibiting the approach of males. *S. cordalis* males are deterred to the greatest extent by the opposite geometrical isomer of the major component, in agreement with the observations of Hill et al. (1974, 1975) on *Argyrotaenia citrana* (Fernald), Tortricidae, and *Platynota idaeusalis* (Walker), Tortricidae. The moderate inhibition of male approaches by all tested levels of *Z*11-16:OH contrasts with the almost complete avoidance of traps with 100 μg of *Z*11-16:Ac, suggesting that different behaviors are affected.

Control of *S. cordalis* by mating disruption appears possible. In enclosed bowls 1–10 μg/liter of the natural pheromone in air inhibits mating among half a laboratory population of moths for three nights. Shorey et al. (1967) demonstrated that 50 μg/liter decreased matings of *T. ni* to 10% of the control for one night while 500 μg/liter abolished mating completely. They then went on to demonstrate disruption of male orientation in the field. Cardé (1981), in a discussion of mechanisms by which elevated levels of pheromone disrupt mate finding by male *Grapholita molesta* (Busck), suggested that males remain responsive to pheromone and search actively but fail to find the females because a disruptant pheromone concentration of 2.5×10^{-2} g/hectare/day is well above the maximum production of the female. Unlike most moth species, which respond only to a narrow range of pheromone concentrations (Roelofs and Cardé, 1977), *S. cordalis* can locate traps baited with very high loadings (up to 10,000 μg in our field experiments). In the presence of the latter concentration, the normally effective 100-μg stopper (statistically more attractive than a virgin female) failed to capture males. These results suggest that sexually active male *S. cordalis* would continue to search in the presence of relatively high pheromone levels that effectively camouflage the normal output of their females.

The highest catches of *S. cordalis* are taken when the traps are deployed for the first time of each season. Howell and Quist (1980) ascribed high initial catches of codling moths to the removal of an accumulated pool of older males. Later, smaller catches then reflected the daily recruitment rate into the adult population. An alternative hypothesis suggests that the pattern of daily catches (Figure 7) results from the physical characteristics of the pheromone-laden stopper. Many pheromone formulations rapidly release attractant for the first one to five days before maintaining a steady low release rate (Maitlen et al., 1976; Caro et al., 1981). Because both the synthetic attractant and the virgin females showed initial high catches (Figure 7, Table 5), the differences in release rate with time are likely to make only a small contribution to this effect.

These initial high catches also suggest that the traps are removing a high proportion of the sexually active males. The first nights' trapping of 1981 (March

5, MARC) removed 24 males, leaving a population of approximately 32 males (estimated by mark–release–recapture studies: 28.7 $\times/\div$ 1.87 males on March 9 and 35.9 $\times/\div$ 1.54 males on March 23; Clearwater, unpublished results). Further trapping removed an average of 4.32 males per night (13.4% of the population). Freshly emerged males, with crisp wing patterns, made up most of the later captures. Halving the population initially and preventing population increases by removing newly emerged males suggests that population control by male removal might be possible with *S. cordalis*.

Males captured by pheromone traps (baited with synthetic material or virgin females) were occasionally found with a spermatophore stuck to the adhesive liner close to their claspers. Spermatophores are normally found only within the females reproductive tract, and this is the first report of spermatophore production following flight to a synthetic pheromone source. Preliminary electron microscopy (Clearwater and Hallet, in preparation) confirmed that the ejected capsules are spermatophores. Many elongated bodies inside the capsule were ultrastructurally similar to spermatozoa (Smith, 1968).

The capture of significant numbers of *M. flavidalis* was an unexpected result of our study. Males clearly responded to the acetate component of the *S. cordalis* pheromone. Many species sharing pheromone components are temporally separated by differing circadian rhythms of responsiveness or different seasons of emergence (Roelofs and Cardé, 1974), but our demonstration of attraction of *M. flavidalis* males by *S. cordalis* females indicates overlapping times of responsiveness. We suspect that attraction of *S. cordalis* males to *M. flavidalis* females is unlikely as Z11–16:Ac attracts *M. flavidalis* (Table 2) but inhibits *S. cordalis* (Table 6). Careful dissections failed to reveal any male scent system that might allow close range species recognition (Cardé et al., 1975; Clearwater, 1975).

M. flavidalis is a very variable entity. Color ranges from lemon yellow to light and dark browns. The wingspan and pattern differ greatly within local populations (Dugdale, personal communication). Our traps catch the small light to dark brown segment of the Auckland population. This data might be valuable in future taxonomic investigations of this species.

Our experiments, carried out over four seasons, captured 2573 wild *S. cordalis*. Only three were female, and control traps were consistently empty. The pheromone is highly sex specific, and the trapping of only three females in three years suggests that random captures are extremely low. If populations of *S. cordalis* cause economic losses as plantings of pepinos expand, the female-produced pheromone offers a potentially potent method of control.

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Underhill, Prairie Regional Laboratory, NRC, Saskatoon, Canada; and Dr. I. Weatherston, Albany International, Controlled Release Division, Needham Heights, Massachusetts, provided us with authentic samples of (*E*)11- and (*Z*)11-hexadecenyl acetates.

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NESTMATE RECOGNITION CUES IN LABORATORY AND FIELD COLONIES OF *Solenopsis invicta* BUREN (HYMENOPTERA: FORMICIDAE)
Effect of Environment and Role of Cuticular Hydrocarbons

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Abstract—Laboratory-reared *Solenopsis invicta* workers were tested for the ability to discriminate nestmates from nonnestmate conspecifics. Postcontact aggressive response to workers from local field colonies was significantly greater than the response to lab-reared workers, even when the latter were selected from colonies originating hundreds of miles away. Behavioral observations support the conclusion that lab-reared ants were less distinctive than field-collected ants with respect to recognition cues detectable on the cuticle. Potential environmental factors affecting colony odor are discussed. In addition, gas–liquid chromatographic and statistical analyses of the major *S. invicta* cuticular hydrocarbons indicate that cuticular hydrocarbon pattern was a poor predictor of laboratory colony response to field colony workers.

Key Words—Ants, Hymenoptera, Myrmicinae, *Solenopsis invicta*, nestmate recognition, colony odor, cuticular hydrocarbons, environmental effects, gas–liquid chromatography, multivariate analysis, Formicidae.

INTRODUCTION

Hamilton's theory of kin selection (1964) argues that individuals act altruistically toward others with whom they share alleles by common descent. One prediction of the theory is that mechanisms should exist that enable individuals to discriminate close relatives from nonrelatives, especially in the social insects (Alexander, 1974; Pfennig et al., 1983). The ability to discriminate colony members or nestmates from nonnestmates (i.e., "nestmate recognition") has been documented in a number of social insects (Wilson, 1971; Hölldobler and

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Michener, 1980; Carlin and Hölldobler, 1983; Breed, 1983; Jaffe and Marcuse, 1983). Typically, recognition in social insects is based upon olfactory cues that constitute a "colony odor" (Wilson, 1971) common to all colony members (Bradshaw and Howse, 1984). Elements of an individual's odor profile determine to a large extent whether it is fed, tolerated, or attacked in or near a colony. Chemical recognition cues can be genetically based (Kukuk et al., 1977; Greenberg, 1979; Jutsum et al., 1979; Hölldobler and Michener, 1980; Breed, 1981, 1983; Mintzer and Vinson, 1985), as well as environmentally determined or acquired (Lubbock, 1894; Kalmus and Ribband, 1952; Lange, 1960; Renner, 1960; Free, 1961; Jutsum et al., 1979; Hölldobler and Michener, 1980; Breed, 1983). As a consequence, recognition systems can be complex and dynamic, rendering chemical elucidation difficult.

This paper reports behavioral and chemical data used to investigate nestmate recognition at the colony level in the imported fire ant *Solenopsis invicta* Buren (Myrmicinae). *S. invicta* workers aggressively defend their nest against intruders, including conspecific neighbors. As in other myrmicine ants studied (Jutsum, 1979; Vilela and Howse, 1986), attack is released by close-range or contact chemoreception of surface compounds (see also Wilson, 1971; Bradshaw and Howse, 1984). By rearing colonies under controlled, laboratory conditions, it was possible to assess the effects of environment on nestmate recognition cues. It was also possible to test whether the cuticular hydrocarbon pattern was consistent with aggressive behavior between colonies, as might be predicted if cuticular hydrocarbons provide nestmate recognition cues in this species (Howard and Blomquist, 1982).

METHODS AND MATERIALS

Ant Rearing. Monogynous *S. invicta* colonies were established from newly mated queens collected near Gulfport, Mississippi, and Gainesville, Florida, and established at the USDA/ARS Insects Affecting Man and Animals Research Laboratory, Gainesville, Florida. For details of collection and laboratory rearing, see Banks et al. (1981). Colonies were maintained in plastic Petri dish cells (diameter = 14.0 cm) with Castone® floors at 26–27°C on a diet of honey-water, fly pupae, roaches, and hard-boiled egg. Illumination was provided by fluorescent lamps, and the light–dark cycle was variable. The Petri dish cells were placed in plastic trays (52.0 × 39.0 × 7.5 cm) that served as foraging arenas. The sides of the tray were Fluon®-coated.

Nestmate Recognition Bioassay. Eighteen months after establishment in the laboratory, six of these colonies were tested for their ability to discriminate nonnestmates introduced from (1) each of the five other lab-reared colonies and from (2) *S. invicta* field colonies ($N = 6$) situated in a 24.0 × 55.0-m section of lawn adjacent to the USDA/ARS Fire Ant Project laboratories. Ants intro-

duced into foreign colonies (i.e., "intruders") were collected in groups of 12–15 from either the mound perimeter of field colonies or from the foraging tray of laboratory colonies. Medias, i.e., ants of the intermediate size caste, were selected by visual inspection, and only these were introduced into foreign colonies. Collected ants were maintained in small, glass vials and then introduced individually on the end of forceps into the foraging arena of a laboratory colony. Only ants that walked undisturbed from the forceps into the "resident" colony tray were tested. Intruders were positioned on the tray floor so as to maximize both the initial distance (5–20 cm) between the site of introduction and resident ants as well as the distance from any previous introduction. The forceps were rinsed with acetone and thoroughly dried after each introduction. Controls consisted of lab-reared ants that were removed and subsequently introduced back into their colony of origin (as above).

Tests were conducted on two consecutive days. On day one, control introductions were conducted first, followed by tests of lab-reared intruders and finally by tests of field intruders. This sequence was reversed on the second day. A 90-min period was observed between the three series of introductions. Within each series (other than controls), replicates were randomized with respect to intruder origin and resident colony, and no resident colony was retested before all resident colonies had been equally replicated. Except when they entered the brood chamber, introduced ants (including at times those residents clinging to them) were removed after each introduction.

The recognition bioassay consisted of five behaviors observed following intruder–resident contact (Table 1). The two most aggressive, "hold/sting" and "hold," always resulted in intruder death, whereas the three least aggressive responses, "hold/release," "antennate/follow," and "no follow," resulted in continued intruder mobility throughout the foraging tray. An introduction was

TABLE 1. BEHAVIORS SCORED IN NESTMATE RECOGNITION BIOASSAY

Behavior	Description
Hold/sting	Resident grabs intruder with forelegs and mandibles, curls abdomen and attempts vigorous stinging during first minute of interaction
Hold	Resident holds intruder (usually by the petiole), but does not attempt stinging; intruder is not released, but eventually dismembered by other residents
Hold/release	Resident releases intruder after initial holding (as above); residents may repeatedly hold, release and follow intruder
Antennate/follow	Resident antennates (>5 sec), follows, but does not hold intruder
No follow	Resident antennates intruder, but does not follow

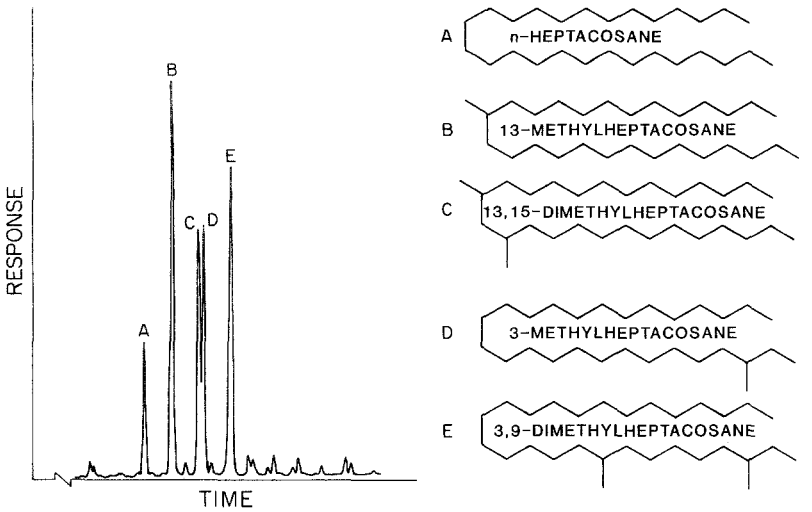

FIG. 1. Chromatographic pattern of *S. invicta* worker cuticular extracts (see Methods and Materials). Structures of the five major hydrocarbons (A–E) are included (after Nelson et al., 1980).

considered completed either when "hold/sting" was observed, or after five resident ants had contacted the intruder. Behavioral data were analyzed according to statistical methods described in Sokal and Rohlf (1981).

Chemical and Statistical Analysis of Cuticular Hydrocarbons. Samples ($N = 3$) consisting of three media workers were collected (as above) from each of the lab and field colonies on the second day of tests. Samples were extracted for 7 min in 150 μl of hexane. Previous studies (Vander Meer, unpublished data) indicate that hydrocarbons extracted by this method are exclusively of cuticular origin. Solvent was removed, reduced under a nitrogen stream to approximately 25 μl and analyzed by capillary gas chromatography: 30 m $\times$ 0.33 mm DB-1 column (J. and W. Scientific, Inc.), splitless; Varian 3700, FID, temperature program = 150 (1 min hold), then 4°C/min to 285°C (Figure 1). Normalized and autoscaled areas (Luc Massart and Kaufman, 1983) of the five major *S. invicta* cuticular hydrocarbons were obtained. These peaks have been chemically identified (Nelson et al., 1980) and are qualitatively invariant in this species. In addition, gas chromatographic comparisons of *S. invicta* cuticular extracts (containing internal standard) with aliquots eluted with hexane from Florisil (60–200 mesh, Applied Science) confirm that these peaks contain only hydrocarbons (Vander Meer, unpublished data). Statistical analysis included *t* tests of individual peak areas in lab and field colonies as well as discriminant function and nearest-neighbor discriminant analyses (SAS, 1982) of cuticular hydrocarbon patterns in the two types of colonies.

RESULTS

Bioassay. Most introduced ants moved immediately about the tray, although some groomed momentarily before doing so. Since the foraging trays contained a minimum of several hundred workers, intruder–resident contact (antennation) occurred within 1 min of introduction in all cases. No introduction took more than 3 min to complete. Forty-eight "control," 120 lab-reared, and 96 field intruders were tested. Eight lab-reared and 14 field intruder replicates were excluded from analysis as a result of either experimenter confusion as to ant identity ($N = 2$), trophallactic appeasement (Bhatkar, 1979) of resident ants by an intruder ($N = 2$), initiation of aggressive behavior by the intruder ($N = 7$), or rapid, erratic movement (escape attempts) following introduction ($N = 11$). Intruders from field colonies (5 of 96 introductions = 5.2%) were no more likely to exhibit initial aggressive responses than were intruders from laboratory colonies (2 of 120 introductions = 1.7%) ($\chi^2 = 2.15$, $DF = 1$, $P = 0.14$), but field intruders were more likely to attempt escape by moving rapidly to one side of the test arena and attempting to climb out (9 of 96 = 9.4% vs. 2 of 120 = 1.7%; $\chi^2 = 6.56$, $DF = 1$, $P = 0.01$).

Data (Table 2) clearly demonstrate that intruders introduced from either lab-reared or field colonies elicit more aggression from resident ants than control intruders. No control ants were killed, whereas 18.8% (21 of 112) of the laboratory intruders were dispatched (test of percentages; $t = 5.17$, $DF = 158$, $P < 0.001$). In addition only 10.4% (5 of 48) of controls were physically detained by residents (i.e., elicited "hold/release," "hold," or "hold/sting"), compared with 54.5% (61 of 112) of lab-reared intruders ($\chi^2 = 26.90$, $DF = 1$, $P < 0.001$). Resident ants responded more aggressively to introductions of field ants than to introductions of lab-reared ants. Both the proportion of intruders killed (61 of 82 = 74.4% vs. 21 of 112 = 18.8%; $\chi^2 = 60.06$, $DF =$

TABLE 2. *S. invicta* NESTMATE RECOGNITION BIOASSAY SUMMARY[a]

Behavior	Observed frequency of resident behaviors		
	Control	Lab/Lab	Field/Lab
Hold/sting	0 (0.0)	1 (0.9)	21 (25.6)
Hold	0 (0.0)	20 (17.9)	40 (48.8)
Hold/release	5 (10.4)	40 (35.7)	15 (18.3)
Antennate/follow	18 (37.5)	28 (25.0)	6 (7.3)
No follow	25 (52.1)	23 (20.5)	0 (0.0)
Total	48 (100)	112 (100)	82 (100)

[a] Scores are frequencies of resident ant responses to (1) nestmates (control), (2) nonnestmates from other lab-reared colonies (lab/lab), and (3) nonnestmates from field colonies (field/lab). Percent total response is included in parentheses.

TABLE 3. RESIDENT RESPONSE (NO. INTRUDERS KILLED AND NO. INTRUDERS RELEASED) WHEN INTRUDER AND RESIDENT COLONIES ORIGINATE FROM SAME POPULATION OR DIFFERENT POPULATIONS (i.e., ALACHUA COUNTY, FLORIDA, VS. GULFPORT, MISSISSIPPI).[a]

Intruder origin	From same population		From different population		χ^2, $1DF$[b]
	Killed	Released	Killed	Released	
Lab	10	41	11	50	0.45
Field	27	11	34	10	0.41

[a] Chi-square values are the result of separate contingency table analyses of resident response to lab-reared intruders and field-collected intruders.
[b] Chi-square$_{(0.05)}$ = 3.84

1, $P < 0.001$) and the proportion of intruders physically detained (76 of 82 = 92.7% vs. 61 of 112 = 54.5%; $\chi^2 = 5.44$, $DF = 1$, $P = 0.02$) were significantly greater among ants introduced from field colonies. These data are homogeneous with respect to the sequence of trials (i.e., day one vs. day two) for both the proportion of intruders killed ($\chi^2 = 3.83$ $DF = 2$, $P = 0.15$) and the proportion detained ($\chi^2 = 2.34$, $DF = 2$, $P = 0.31$). Finally, not significantly different proportions of intruders were killed when intruders and residents were from the same population (i.e., Florida or Mississippi) or from different populations (Table 3).

Analysis of Cuticular Hydrocarbons. A total of 36 samples were analyzed by gas chromatography. Hydrocarbon patterns gave no evidence of hybridization events involving *S. richteri* (Vander Meer et al., 1986), and all colonies tested were therefore included in the analysis. The five major hydrocarbons (Figure 1) constituted 72–77% of the total cuticular extract. Univariate analysis revealed significant differences between laboratory and field colonies in the mean, normalized peak areas of peaks A, B, and E (Table 4). (Variances associated with peak means were not significantly different in the two types of

TABLE 4. MEAN (±SEM) NORMALIZED PEAK AREAS OF FIVE MAJOR *S. invicta* CUTICLE HYDROCARBONS IN LABORATORY ($N = 18$) AND FIELD ($N = 18$) COLONIES[a]

Peak	Lab	Field	T, $DF = 34$	ASL
A	0.209 (0.009)	0.165 (0.012)	2.81	0.008
B	0.268 (0.007)	0.301 (0.009)	2.80	0.008
C	0.170 (0.008)	0.183 (0.008)	1.21	0.235
D	0.194 (0.010)	0.166 (0.011)	1.84	0.074
E	0.164 (0.008)	0.203 (0.011)	2.80	0.008

[a] Univariate test statistics and attained significance levels (ASL) are also presented.

colonies.) When particular laboratory vs. field colony comparisons were made, individual areas of peaks A, B, and E were not always significantly different. However, the absence of a significant univariate peak area difference between individual field and laboratory colonies could not be statistically correlated with significantly reduced resident aggression in the recognition bioassay. For example, it was determined (Newman-Keuls' test; $\alpha = 0.05$) that 19 of the 36 field vs. laboratory bioassay combinations were between colonies with not significantly different normalized, mean peak areas for peak A. Yet, the proportion of intruders killed (36 of 45) was not significantly different from the proportion killed when intruder and resident colonies exhibited significantly different peak A areas (25 of 37 killed; $\chi^2 = 1.65$, $DF = 1$, $P = 0.20$). Similarly, not significantly different proportions of intruders were detained when colony pairs with significant peak B differences were compared with those exhibiting no significant differences (34 of 37 vs. 43 of 45 detained; $\chi^2 = 0.48$, $DF = 1$, $P = 0.49$).

Both parametric (discriminant function) and nonparametric (nearest-neighbor) analysis generated classifications in which the colony "blends" of the five major hydrocarbons were maximally correlated with colony class (i.e., field or lab origin). In the former analysis, a classification criterion based upon the pooled covariance matrix resulted in nine "misclassified" field colony samples, i.e., samples from three colonies that, based upon cuticular hydrocarbon pattern, were more appropriately classified as originating from laboratory-reared colonies. The nearest neighbor discriminant analysis produced identical results utilizing both Euclidean and Mahalanobis distances. In summary, the classification rules developed from the five chemical descriptors (peaks A–E) accurately classified only 50% of the samples. More importantly, of the 21 field intruders not killed by residents (Table 1) only nine originated from these three misclassified ("lab-like") colonies ($\chi^2 = 0.43$, $DF = 1$, $P = 0.51$).

DISCUSSION

Effect of Environment on Nestmate Recognition Cues. S. invicta workers reared under controlled, laboratory conditions respond less aggressively to nonnestmate conspecifics reared under the same conditions than they do to field-collected conspecifics. This phenomenon has been observed in other laboratories as well (Les Greenberg, personal communication). That this effect is not due to reduced aggressiveness overall is indicated by the almost total recognition capability observed when intruders were selected from field colonies (76 of 82 intruders detained). Behavioral differences between lab-reared and field intruders (see above) do not account for these data, although the bioassay is admittedly insensitive to subtle, social interactions such as alarm and recruitment. Overall, however, the data suggest that nestmate recognition cues of laboratory-reared *S. invicta* colonies were less distinctive than those of field col-

onies. These recognition factors or other colony-specific cues may be transferred to the substrate. This is suggested by the significantly greater preencounter escape response of field intruders, although an alternative explanation may be that lab-reared ants had learned that escape was impossible (John Sivinski, personal communication).

The effect of laboratory rearing on detection of nonnestmates supports the hypothesis that nestmate recognition in *S. invicta* is subject to environmental modification. Environmental factors known to affect nestmate recognition in social insects include diet (Lange, 1960; Jutsum, 1979), ambient odors (Renner, 1960; Free, 1961), and nest material (Lange, 1960). The lack of nest soil in lab-reared colonies used in this study removed potentially distinctive odor cues that could conceivably be transferred to the ants' cuticle. In addition, absence of nest soil exposed workers on a continuous basis to a homogeneous rearing-room bouquet that could have become incorporated into the cuticle (see also Shellman-Reeve and Gamboa, 1985). Without knowledge of field colony response to lab-reared intruders, one cannot determine to what extent laboratory rearing homogenizes environmentally determined recognition cues among laboratory colonies or precludes their full expression.

It should be noted that "innate" cues (i.e., those not affected by environment) also contribute to distinctive colony odor in *S. invicta*. Resident response to lab-reared intruders was significantly greater than resident response to controls (Table 1), indicating that detectable differences persisted despite 18 months of laboratory rearing. That locale of intruder origin had no measurable effect on resident aggressive response (Table 3) suggests that variability in genetically based recognition cues is minimal among North American populations. Whether this is a consequence of limited introduction events and genetic drift particular to *S. invicta* (Tschinkel and Nierenberg, 1983), or a more general phenomenon in ants is unclear.

The effect of environment on nestmate recognition in *S. invicta* suggests that colony odor in this species may be, in part, a dynamic phenomenon. If so, it is likely that some form of learning is an underlying mechanism of recognition (Fielde, 1903; Soulié, 1960; Kukuk et al., 1977; Breed, 1981; Carlin and Hölldobler, 1983; Morel, 1983). There are both costs and benefits to a flexible recognition system based on learning. While such a system may increase colony susceptibility to slave-making (i.e., dulotic) species (Wilson, 1971), it may facilitate efficient colony functioning when queens are multiply-inseminated or when, as is sometimes the case for *S. invicta* in North America (Glancey et al., 1973), colonies are polygynous. Dulosis involving *S. invicta* is undocumented.

Role of Cuticular Hydrocarbons. Cuticular hydrocarbons were of interest to this study for several reasons. To begin with, the experiments of Howard et al. (1980, 1982) argued strongly in support of cuticular hydrocarbons as interspecific recognition cues in the termite genus *Reticulitermes*. Second, Howard and Blomquist (1982) demonstrated that cuticular hydrocarbons were both spe-

cies- and caste-specific in *S. invicta*. In addition, Vander Meer and Wojcik (1982) observed that myrmecophilous beetles inquilinous in the nests of *S. invicta*, *S. richteri*, and *S. xyloni* acquired the species-specific hydrocarbon pattern of their host. They suggested that the predaceous beetles reduced the probability of detection by chemically mimicking the host (see also Howard et al., 1980). Finally, hydrocarbons represent 70–75% of all *S. invicta* cuticular lipids (Lok et al., 1975).

However, although differences between the cuticular hydrocarbon patterns of laboratory and field colonies were observed, these differences were inadequate predictors of resident ant response to nonnestmates in the recognition bioassay presented here. Specifically, a negative correlation between similarity of cuticular hydrocarbon pattern and intercolony aggression was not obtained. Rather, intruders from "misclassified" field colonies (i.e., those with hydrocarbon patterns statistically not unlike residents') elicited as much aggression as did intruders from correctly classified field colonies. The same conclusion resulted when individual hydrocarbon peaks were considered separately.

Possible explanations for these results include: (1) Cuticular hydrocarbons are, in fact, not nestmate recognition cues in *S. invicta*. (2) Cuticular hydrocarbons other than or in addition to those addressed in this study provide intraspecific nestmate recognition cues. (3) The major cuticular hydrocarbons do provide nestmate recognition cues, but other, environmentally influenced or acquired surface compounds (perhaps in concert with cuticular hydrocarbons) permit discrimination when hydrocarbons alone do not. Hypotheses 2 and 3 are not mutually exclusive, and each could explain the high levels of aggression directed at intruders from "misclassified" field colonies. It is possible that the major cuticular hydrocarbons represent a part of the more genetically controlled recognition cue profile. If so, it is not surprising that hydrocarbon pattern was such a poor predictor of environmentally based behavioral differences in this study.

Conclusion. Both innate and environmentally based chemical cues appear to affect nestmate recognition in *Solenopsis invicta*. This conclusion supports findings for other Hymenoptera (Wilson, 1971; Jutsum et al., 1979; Hölldobler and Michener, 1980; Discussion in Breed, 1983). It should be possible with the bioassay presented here to elucidate the environmental factors affecting recognition chemistry in this species. In addition, it has been demonstrated that the major *S. invicta* cuticular hydrocarbons cannot account for the dramatically different response of laboratory-reared colonies to field-collected and laboratory-reared workers, respectively. However, it cannot be concluded at this time that these compounds do not constitute a portion of the recognition profile mediating intraspecific interactions.

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Journal of Chemical Ecology, Vol. 12, No. 9, 1986

Errata

SCALE ESTERASE: A Pheromone-Degrading Enzyme from Scales of Silk Moth *Antheraea polyphemus*

RICHARD G. VOGT and LYNN M. RIDDIFORD

Readers should note the following correction to this article which appeared in *J. Chem. Ecol.* 12:469–482 (1986).

On p. 482 the reference to Vogt and Riddiford (1986) should read:

VOGT, R.G., and RIDDIFORD, L.M. 1986. Pheromone reception: A kinetic equilibrium, pp. 201–208, in T.L. Payne, M.C. Birch, and C.E.J. Kennedy (eds.). Mechanisms in Insect Olfaction. Clarendon Press, Oxford.

Lespedeza PHENOLICS AND *Penstemon* ALKALOIDS: Effects on Digestion Efficiencies and Growth of Voles

RICHARD L. LINDROTH, GEORGE O. BATZLI, and SUSAN I. AVILDSEN

Several errors were incorporated into the final printed version of the above article's Table 3 J. Chem. Ecol. 12:713–728 (1986). The corrected version of this table is shown below.

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TABLE 3. MEAN (±1 SE) CONSUMPTION AND DIGESTIBILITY OF *Lespedeza*, *Penstemon*, AND CONTROL DIETS BY SUBADULT VOLES*

Species and diet	Ingested dry matter (mg/g $BW^{0.5}$/day)	Dry matter digestibility (%)	Digested dry matter (mg/g $BW^{0.5}$/day)	Apparent protein digestibility (%)	Protein digested (mg/g $BW^{0.5}$/day)
Lespedeza digestibility trials					
Prairie vole					
Control (10)	905 ± 43^{a}**	58.3 ± 0.9^{g}	528 ± 26^{i}	77.5 ± 1.2^{o}	67.2 ± 3.2^{s}
Lespedeza (10)	$1076 \pm 44^{a,b}$**	51.3 ± 0.9^{f}	555 ± 29^{i}	33.3 ± 2.1^{m}	34.0 ± 3.0^{r}
Meadow vole					
Control (10)	$974 \pm 47^{a,b}$	58.3 ± 1.2^{g}	567 ± 28^{i}	71.3 ± 1.3^{n}	66.6 ± 3.5^{s}
Lespedeza (10)	1124 ± 92^{b}	51.7 ± 0.8^{f}	576 ± 42^{i}	32.2 ± 1.8^{m}	34.8 ± 2.5^{r}
Penstemon digestibility trials					
Prairie vole					
Control (4)	851 ± 27^{c}	57.6 ± 0.5^{h}	490 ± 13^{j}	77.2 ± 1.9^{q}	62.9 ± 3.1^{t}
Penstemon (6)	982 ± 32^{d}	58.5 ± 0.9^{h}	573 ± 18^{k}	75.8 ± 1.4^{q}	71.2 ± 2.0^{t}
Meadow vole					
Control (6)	1125 ± 44^{e}	58.1 ± 0.5^{h}	654 ± 24^{l}	65.4 ± 3.8^{p}	71.0 ± 6.6^{t}
Penstemon (7)	$1099 \pm 45^{d,e}$	57.5 ± 0.5^{h}	632 ± 27^{l}	71.9 ± 2.9^{p}	76.0 ± 5.1^{t}

*Values within each digestibility trial-performance category that bear different superscripts are significantly different (LSD test, $P < 0.05$). Sample sizes are shown in parentheses.

**Ingestion rates for prairie voles in the *Lespedeza* trial are significantly different at $P < 0.055$.

Journal of Chemical Ecology, Vol. 12, No. 9, 1986

Errata

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DIETARY EFFECTS OF PHYTOECDYSONES IN THE LEEK-MOTH, *Acrolepiopsis assectella* ZELL. (LEPIDOPTERA: ACROLEPIIDAE)

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Abstract—Incorporation of certain phytoecdysones (ecdysterone, polypodine B, and ponasterone A) into a semisynthetic artificial diet induces pathophysiological effects in larvae of the leek-moth (*Acrolepiopsis assectella* Zell., Acrolepiidae). The effects include lethality of the newly hatched, first-instar larvae; special ecdysial failures associated with the appearance of larvae with two head capsules; and developmental anomalies during metamorphosis. The effective range of dietary ecdysteroid, as evaluated by larval mortality, varies from 25 to 250 ppm. The EC_{50} value is 100 ppm for polypodine B and 130 ppm for ecdysterone. The dietary effects of the phytoecdysones are similar to the previously observed effects caused by the dried flowers, but not leaves, of the leek plant. However, the active compound of the leek flowers is a saponin.

Key Words—*Allium porrum* L., Liliaceae; *Acrolepiopsis assectella* Zell., leek-moth, Lepidoptera, Acrolepiidae, ecdysone, ecdysteroids, 20-hydroxyecdysone, 5,20-dihydroxyecdysone, 20-hydroxy,25-deoxyecdysone, ecdysial failures

INTRODUCTION

The larvae of the leek-moth are specialist feeders of the leaves of leek (*Allium porrum*). In the laboratory, they can be successfully reared on semisynthetic diets containing small amounts of dried leek leaves. It has been observed that substitution of the dried leaves by an equal amount of dried flowers causes

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increased mortality of small larvae (Arnault, 1975). This action is occasionally associated with special symptoms of ecdysial failures, i.e., malformed larvae with two head capsules telescoped within the old exuviae (Arnault, 1975, 1979).

Although it is known that certain phytophagous insects became specialized to feed only on some parts of the host plant (Rosenthal and Janzen, 1979), the curious developmental anomaly characterized by larvae with two head capsules is rare. It has been induced experimentally in several other species of Lepidoptera, by rearing the larvae on the diets containing 50–100 ppm of various ecdysteroid compounds (Kubo et al., 1981, 1983; Kubo and Klocke, 1983). We have thus assumed that the flowers, but not the leaves, of the leek may contain some phytoecdysone that is responsible for these effects in larvae of the leek-moth. The assumption has been supported by phytochemical evidence, showing that the flowers of certain plants can contain more than 2% of phytoecdysones on a dry weight basis, while the leaves of the same plant contain only 0.2% or less (Achrem et al., 1973; Sláma, 1979). In this work, we have assayed in the diet of the leek-moth various concentrations of certain common phytoecdysones. The results have been compared with the previously described effects of the dried leek flower (Arnault, 1979).

METHODS AND MATERIALS

The methods of breeding *A. assectella* Zell. and the preparation of the artificial diets have been described previously by Arnault (1979). In the present experiments, we used glass test tubes 25 mm OD, 50 mm long. Each tube contained 5 ml of the artificial medium; the top was sealed by paper wool. After solidification and cooling, the diet in each tube was inoculated with 50–60 3-day-old eggs. The larvae maintained at 26–27°C hatched within 24 hr.

The ecdysteroid compounds tested were 20-hydroxyecdysone (ecdysterone), 5,20-dihydroxyecdysone (polypodine B) and 20-hydroxy,25-deoxyecdysone (ponasterone A). They are natural plant products (see Jizba et al., 1967). After addition of 4.5 ml of the freshly prepared, still-warm diet, the graded doses were supplied to the diet (0.5 ml) in 10% ethanolic solution. The content of each tube was then thoroughly mixed to give the final medium (Arnault, 1979).

Each concentration of ecdysteroid (except for ponasterone A) was assayed in duplicate series and the experiments were duplicated (4 × 50 larvae in each concentration). The results were evaluated at three-day intervals using the criteria of mortality and larval inability to ecdyse. The methanol extracts of leek flowers were assayed for ecdysteroid activity on ligated larvae of *Dermestes* according to the method of Sláma et al. (1974, p. 337).

RESULTS AND DISCUSSION

The addition of exogenous ecdysteroid into the diet of *A. assectella* resulted in an array of pathophysiological disorders. We have divided these syndromes into four categories: (1) death of the newly hatched first-instar larvae; (2) ecdysial failures in the young larval instars, i.e., larvae with two cuticles including two head capsules; (3) prothetelic effects in larvae that survived on ecdysteroid-containing diet until the 4th or 5th instar; and (4) developmental anomalies during metamorphosis.

The combined effects of ecdysteroid present in the diet can be expressed collectively by the rate of larval mortality. Figure 1 shows that the curves for larval mortality exhibit a common pharmacological dose–response pattern. The EC_{50} value is 100 ppm for 5,20-dihydroxyecdysone and 130 ppm for 20-hydroxyecdysone. Leek-moth larvae do not tolerate a dietary concentration of ecdysteroids higher than 250 ppm. With these high concentrations the newly emerged larvae of the first instar are unable to initiate feeding. In most cases these larvae perish without successful ecdysis. This response in the newly emerged larvae is similar to antifeeding or intoxication and apparently is related to the syndrome known as "hyperecdysonism" (see Williams, 1968).

The responses of the second category (ecdysial failures and appearance of larvae with additional head capsules) were responsible for most larval mortality

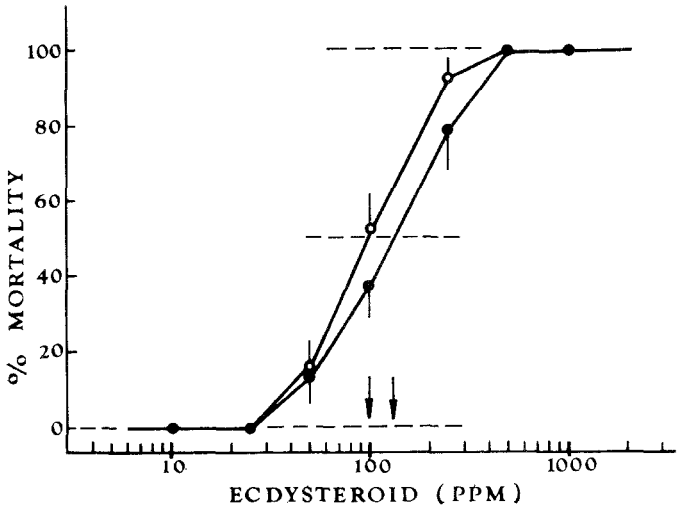

FIG. 1. Mortality of *A. assectella* larvae reared on artificial diet containing 20-hydroxyecdysone (solid circles) and 5,20-dihydroxyecdysone (open circles). Vertical bars are SEM, arrows indicate the respective EC_{50} values.

FIG. 2. Young leek-moth larvae exhibiting two head capsules and incompletely shed exuviae. These effects were induced with 100 ppm dietary 5,20-dihydroxyecdysone.

within a dose range of 100–250 ppm. The affected larvae remained enclosed within their old exuviae. In some cases, as shown in Figure 2, old exuvia was shed from some parts of the thorax or abdomen. However, these treated larvae were unable usually to withdraw the deformed mouth organs from the old head capsule. The latter remained attached permanently to the newly formed head and prevented feeding. The affected larvae had reduced or completely suppressed locomotory activity. The critical developmental stage for these effects to be elicited was the second larval instar. Larvae that succeeded for some unknown reason to ecdyse from a second to a third larval instar were also able to complete larval development.

The prothetelic effects (premature formation of pupal patterns in larvae) occurred occasionally among larvae that survived 50–100 ppm concentrations of ecdysterone or ponasterone A until the fifth larval instar. The characteristic features of prothetelic larvae were everted wing lobes covered with pupal cuticle (see Figure 3). Patches of brown, sclerotized pupal cuticle were also present commonly on the mouth organs, on the distal end of the larval abdomen, and on various parts of the thoracic segments. Prothetelies were not found in 5,20-dihydroxyecdysone-treated animals. By contrast, the largest incidence of the prothetelies (over 15%) occurred in ponasterone A-treated larvae.

The increased mortality of the pupae and pharate adults (effects of the fourth category) has not been included in calculating dose–response relation-

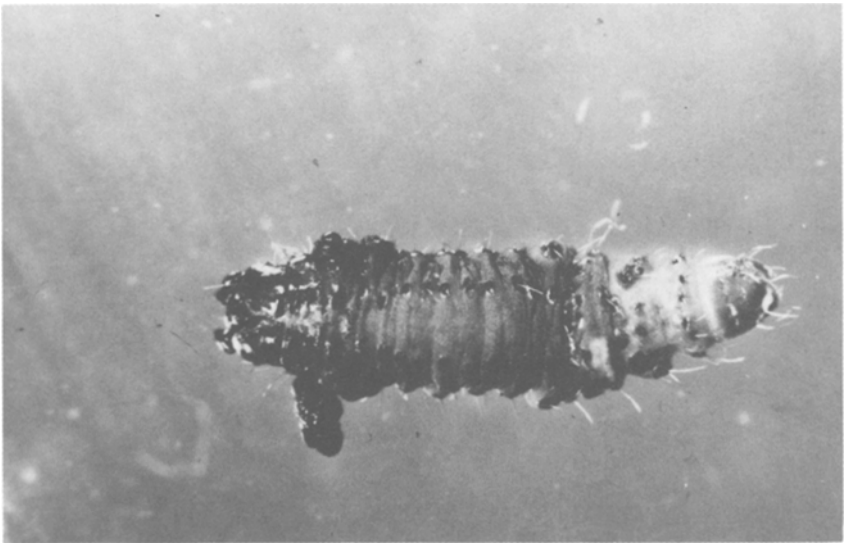

FIG. 3. Typical prothetely of the last-instar larva of the leek-moth. These effects were generated with 100 ppm dietary 20-hydroxy,25-deoxyecdysone. Note the everted and sclerotized wing lobes and deformed mouth parts.

ships in Figure 1. With a 100 ppm concentration of ecdysteroids, we observed that approximately half the pharate adults died enclosed within their pupal cuticular case. Since we have focused our attention mainly on larval effects, these results should be taken as preliminary.

Careful evaluation of our results obtained with ecdysteroids and comparison with the previously described effects of leek flowers (Arnault, 1979), led us to conclude that the dietary action of ecdysteroids in the leek-moth was indeed quite similar, and in certain respects actually identical, with the dietary action of the dried flowers of the leek. This prompted us to assay the ecdysone activity in the methanolic extracts of leek flowers. To this end, we used the assay on ligated terminal instar larvae of *Dermestes maculatus*, which are sensitive to less than 0.01 μg of ecdysterone equivalents and, in addition, respond positively to a number of ecdysone analogs (Sláma et al., 1974). However, the assays revealed an absence of ecdysteroid activity in the flower extracts of leek, although we used 200–300 μg of the dried methanolic extracts. This suggested that the effects of the leek flowers were not due to the presence of an unbound phytoecdysone.

Since the discovery of juvenile hormone activity in balsam fir (Sláma and Williams, 1966), this topic has been frequently discussed and reviewed (Sláma, 1969, 1979; Williams, 1970; Rees, 1971; Beck and Reese, 1976; Harborne,

1977; Bergamasco and Horn, 1984). Experimental evidence that ecdysteroids may cause adverse effects in insects when consumed has been provided by Robbins et al. (1970). The wide distribution of phytoecdysones (ecdysteroids) in plants is now generally known (Bergamasco and Horn, 1984), although some authors still expressed doubts about their real ecological importance (cf. Beck and Reese, 1976).

Our results in *A. assectella* are fully consistent with the observations of Kubo et al. (1981, 1983) on the dietary action of ecdysteroids in larvae of other lepidopteran insects, *Spodoptera*, *Pectinophora*, and *Bombyx*. Our observations are in accord with the reported dietary effects of ecdysteroids in larvae of various dipteran species (Singh and Russell, 1980; Singh et al., 1982; DeClerck and DeLoof, 1983). In all cases, we find the effective dietary range of ecdysteroid between 25 and 100 ppm. This range is comparable to the reported plant ecdysteroid concentration (0.001–0.1% on a dry weight basis; see Sláma, 1979). This supports the supposition that the ecological importance of ecdysteroids in insect–plant interactions cannot be ignored, especially when we consider that dry flowers of certain plants may contain up to 2.9% ecdysteroid (Achrem et al., 1973). These dietary interactions would be likely realized in minute first- and second-instar larvae. However, since these stages are rarely investigated, these effects can be overlooked.

The second larval instar evidently represents the stage most susceptible to the dietary effects of ecdysteroids. The second larval instar has certain unique properties, including the largest relative size increment, shortest duration of the interecdysial period, and the most active metabolic rate (Morohoshi, 1957; Sláma et al., 1974). In *Galleria*, ecdysteroids almost immediately cause cessation of feeding, arrest somatic growth, and reduce drastically respiratory metabolism of young larvae (Sláma, 1982).

Certain developmental effects of insect hormones can be nonspecifically mimicked by various hormonomimetic chemicals. This is mainly true for a number of structurally unrelated, mostly synthetic, bioanalogs of juvenile hormone (cf., Henrick, 1982; Sláma, 1985). By contrast, the action of ecdysone in insects can be simulated only by a limited group of structurally related ecdysteroids, all of which possess the essential steroid nucleus (Bergamasco and Horn, 1984). Our results show, however, that the dietary effects of ecdysteroids can be elicited by a compound from leek flowers that has no ecdysteroid activity in direct bioassays. Recent chemical analysis of leek floral natural products has revealed the active component to be a previously known saponin, aginosid (Harmatha et al., 1986). Experiments performed in the leek-moth with a common saponin, digitonin (Arnault and Mauchamp, 1985) show effects similar to those obtained with leek flowers or ecdysteroids. These data indicate that the described larval effects, such as reduced feeding, inability to ecdyse, or partial ecdysis in small larvae, which result in lethality (formation of larvae with two head capsules), cannot be regarded as the specific effects of the ecdysteroids.

By contrast, induction of the prothetely, produced in our experiments with some ecdysteroids but never with the saponins or flower extracts, could be regarded as a hormonal imbalance specifically caused by the ecdysteroids.

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FOOD SEARCH BEHAVIOR IN ARCTIC CHARR, *Salvelinus alpinus* (L.), INDUCED BY FOOD EXTRACTS AND AMINO ACIDS

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Abstract—Food search behavior in Arctic charr (*Salvelinus alpinus*) to extracts of granulated food was quantified and compared with the response to solutions of amino acid mixtures. The concentration of each of 18 amino acids was analyzed in the food extract and similar pure amino acid solutions were prepared. Earlier published electrophysiological data and the data of concentrations in the test area were used to order the amino acids with respect to their presumed contribution to the stimulatory effect of the food extract. The 18 amino acids were tested collectively (18 aa) or were divided into two groups, one group containing 7 amino acids (7 aa) which was presumed to have a high stimulatory effect and the other group containing the remaining 11 amino acids (11 aa). The solutions of 7, 11, and 18 aa were stimulatory at concentrations between 2 and 5×10^{-6} M, but no significant responses were observed at $2\text{–}5 \times 10^{-9}$ M. This can be compared with the food extract which elicited response at all concentrations tested, i.e., the total concentrations of 18 amino acids were 5×10^{-9}, 5×10^{-8}, 5×10^{-7}, and 5×10^{-6} M. The response to the 18 aa solution was compared with the food extract and was shown to be significantly lower at 5×10^{-9} M but not at 5×10^{-6} M. These results show that amino acids induce food search behavior in Arctic charr, but there are other substances which also contribute to the stimulatory effect of the food extract.

Key Words—Amino acids, Arctic charr, fish, food extract, food search behavior, *Salvelinus alpinus*.

INTRODUCTION

Chemical stimuli induce food search behavior in many species of fish (e.g., Bardach and Villars, 1974; Dempsey, 1978; Atema, 1980; Atema et al., 1980;

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Kamchen and Hara, 1980). Amino acids are important as stimuli, and they are detected both by olfaction and taste. The most important chemical sense in food search behavior varies from one species to the other. Taste is of major significance in food search and orientation in species of fish with taste buds on barbel and body flanks (Bardach et al., 1967; Atema, 1971, 1980; Holland, 1978). In yellow fin tuna (*Thunnus albacares*) food search behavior is induced by prey odor and amino acids with the aid of the olfactory sense. The tuna was most excited by odor from the species of sardine on which it was feeding at the time (Atema et al., 1980). Salmonids also respond with food search behavior to a supply of extract of their normal food (McBride et al., 1962; Kamchen and Hara, 1980) but hitherto no studies have been published which deal with the importance of free amino acids during the search for food.

Kamchen and Hara (1980) found that in white fish (*Coregonus clupeaformis*) food search behavior and attraction to water containing food extract is mediated by olfaction. The distribution of taste buds in salmonids is restricted to the oral cavity (De Kock, 1963; Sutterlin and Sutterlin, 1970; Meyer-Rochow, 1981; Ezeasor, 1982; Marui et al., 1983). Adron and Mackie (1978) found that L-amino acids are important as gustatory feeding stimulants in rainbow trout (*Salmo gairdneri*). The aim of this study was to quantify food search behavior in juvenile Arctic charr (*Salvelinus alpinus*) to extract of their normal food, i.e., granulated food pellets, and compare it with the response to mixtures of amino acids.

METHODS AND MATERIALS

One-year-old hatchery-raised Arctic charr (*Salvelinus alpinus*), deriving from wild fishes in Lake Torrön, Jämtland, Sweden, were used as test fish (2.5–6.0 g, $N = 40$). They were kept in a trough and fed daily at 4 PM with Ewos "Salmon Feed Extra" corresponding to 1% of body weight.

The chemical composition of tap water has been given by Pärt and Svanberg (1981). The water is rich in calcium, 2.8 mM Ca^{2+}. The water temperature in the test area and trough was measured daily. It increased during the experimental period, March 18, 1982, to June 9, 1982, from 7.5 to 9.8° C. The pH in the test area, measured weekly, was 8.0–8.1.

Test Apparatus. In order to quantify food search behavior in Arctic charr, we observed the upstream movement of specimens from their territory while supplied food extracts and amino acid mixtures. Tests were performed in a modified fluviarium with a group of five fish at a time (Höglund, 1961) (Figure 1). Screens of black plastic sheeting were placed in the downstream end of the test area to function as shelters and to decrease the risk that a dominant fish might interact with the others and affect their behavior and therefore their response during the tests. A few tests were omitted as the dominant fish was

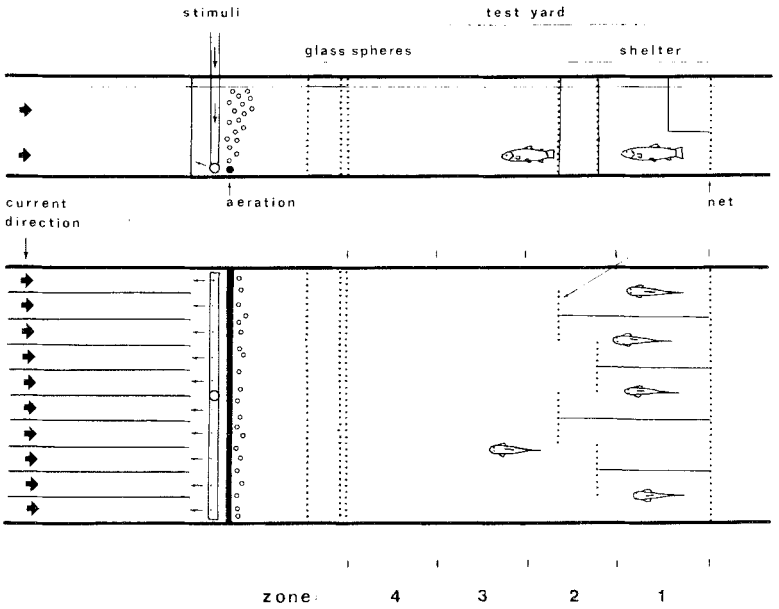

FIG. 1. The modified test area of the fluviarium. The positions of fish were usually noted as shown in the figure except when there was a supply of stimuli inducing the charr to swim upstream. The dominant individual was commonly located in front of screens which functioned as shelters to subordinate fish. Four zones used for analyzing upstream movement of fish were defined by lines on the bottom transverse to the direction of flow.

aggressive and chasing subordinate fish. During chemical stimulation, the test fishes were often observed to seize particles from the bottom. Changes in locomotor activity and direction of body axis were also studied, but these responses were not as distinct as upstream movement. We observed in a pilot test that food extract pumped into a holding tank with three-year-old Arctic charr gave a very strong response; i.e., increased locomotor activity and dispersion of the group.

The bottom of the test area was white. The flow rate of water was 0.8 cm/sec (17.1 liter/min). The light was turned on at 7:00 AM and off at 5:30 PM, both in the test area and in the trough. The light intensity in the test area just above the water surface was 135 $\mu W/cm^2$ (86 lux).

Control and test solutions were evenly distributed over the breadth of the fluviarium with two interchangeable T-tubes (diam. 10 mm) with 10 holes (diam. 0.5 mm) in each. To improve the mixing of solutions in tap water, the water stream passed a curtain of air bubbles and cage of stainless steel containing glass spheres (diam. 5–7 mm) before entering the test area. The admixture of control and test solutions in the test area was analyzed in tests with dye solutions. Samples of 1 ml each were taken every 10 sec during a 5-min period

at six different places both at the up- and downstream ends of the test area. The difference in dye concentration in different parts of the area was smaller than 7% according to absorbance measurements.

Five fishes (size difference < 1 g) were placed in the test area 60 hr before the initial test. After this adaptation period, tests were performed with the fish-group during five days. The fish were fed once a day (1% of the body weight) and food was supplied through a tube after the last test. The tube was located close to the front net of the test area just above the water surface. Food extract was supplied through the T-tubes at the same time as feeding the test fish. The concentration of food extract varied from day to day but corresponded to one of the tested concentrations.

Experiments. Four tests were performed each day, at 9 AM, 11 AM, 1 PM, and 3 PM. Each test lasted for 20 min and was divided into the following four periods of 5 min: (1) Adaptation period (A). No solutions were supplied. (2) Control period (C1). Control solution was supplied (pure water) through a T-tube placed in the fluviarium in the beginning of the period. (3) Test period (T). Control or test solution was supplied through a T-tube in the same way as in C1 but with another T-tube. (4) Control period (C2). Control solution (pure water) was supplied through the same T-tube as in C1.

Each test solution was tested with at least two different groups of fish and the order of experiments with control and test solutions in T was determined by a pseudorandom scheme. The test fish were photographed every 10 sec (started in A) with a film camera connected to a flash filtered through Kodak Wratten gelatin filter No. 89B (no transmittance < 670 nm, cf. Kodak Filters publ. 1978) to reduce visual disturbances (Ali, 1975).

Analysis of Behavioral Response. The test area was divided into four equal zones by lines drawn on the bottom transverse to the stream direction (Figure 1), and numbered from 1 (furthest downstream) to 4 (furthest upstream). The snout position of each fish was recorded, and the behavioral response for the entire group during a test period was calculated by the following formula: Zone value $= Zv = [(n_1 \times 1) + (n_2 \times 2) + (n_3 \times 3) + (n_4 \times 4)]/(n_1 + n_2 + n_3 + n_4)$; where n_i = number of observations in zone "i" ($i = 1$–4) during a period. The initial four photo-recordings, which corresponded to the time (ca. 30 sec) for the frontal line of the solutions to reach the median part of the test area (tested with dye), were excluded from the calculations of the *Zv*s of each period. A mean zone value (*MZv*) was calculated on the basis of the *Zv*s for each of the four periods (A; C1; T; C2) for all tests with the same solutions. All *MZv*s are given in Figure 2.

Statistical treatment was performed in four different ways: (1) *Zv* for C1 was compared with the corresponding value for period T in the same test (*Zv*T − *Zv*C1). The difference between *Zv* for T and C1 is positive if the fish respond to the solution by swimming upstream. (2) *Zv* for C1 was compared with the corresponding value for A in the same test (*Zv*C1 − *Zv*A). All tests performed

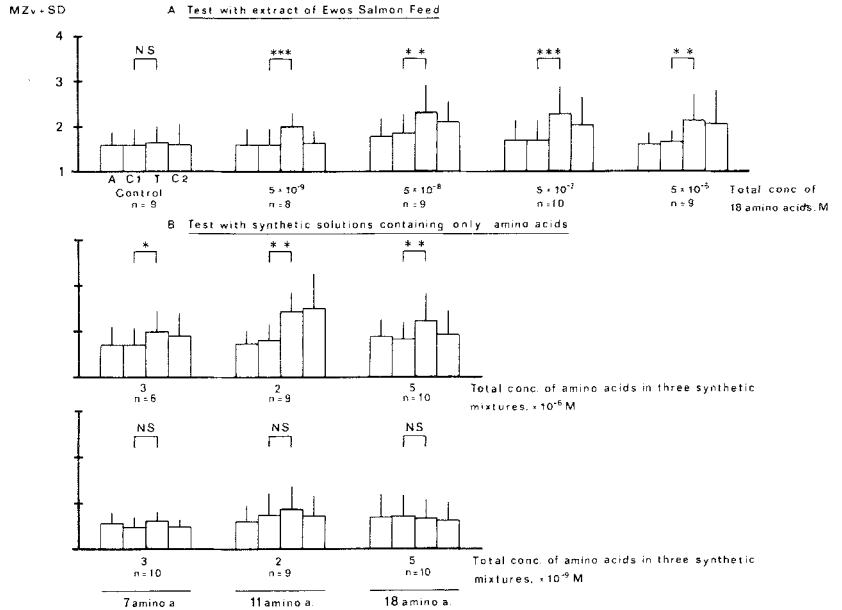

FIG. 2. Mean zone values ($MZv \pm SD$) for separate 5 min. periods in tests with different solutions supplied during the T period. (A) Control tests (pure water) and tests with food extract. The total concentration of 18 amino acids is noted. (B) Experiments with solutions of 7, 11, or 18 amino acids acting as stimuli (see Table 1). A paired t test (one tailed) was used in order to find out whether fish respond to the test solution by swimming upstream. NS = $P > 0.05$; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

were included in the same analysis ($N = 99$). (3) Zv for C1 and C2 in the same test were compared in order to examine whether the response induced in period T was still significant in C2 ($ZvC2 - ZvC1$). A paired t test (Colquhoun, 1971) was used in the comparisons for 1–3. (4) A one-way ANOVA was performed to examine differences in responses to test solutions (Sokal and Rohlf, 1969). The ($ZvT - ZvC1$) value of each test was used as an observation.

Test Stimuli. The normal fish food, Ewos Salmon Feed Extra, was ground and dried at 40°C. The pulverized food was suspended in distilled water and homogenized (Ultra-Turrax). The suspension was centrifugated at 20,000g during 10 min, and the supernatant was used as crude food extract solution. The solution was divided in small aliquots and kept in a freezer (−20°C). The crude extract was prepared in a total quantity, which was sufficient for all tests performed. The free amino acid content in this extract was analyzed using an amino acid analyzer, Durrum 500 (Eaker, 1970). The mixtures of pure amino acids in distilled water were prepared on the basis of this analysis.

The method used for amino acid analysis cannot discriminate between

serine, asparagine, and glutamine. Instead it resulted in a total concentration for these three amino acids which was divided into three equal parts (69.1×10^{-6} M each) in the preparation of the amino acid solution. The 18 dissolved amino acids were divided into two groups according to their expected significance for inducing food search behavior. The expected significance was based on threshold concentrations of the olfactory sense in Arctic charr for each amino acid observed in an electrophysiological study (Belghaug and Døving, 1977) and the amino acid concentrations in the test area (quotient: conc. in the test area/electrophysiol. threshold) during supply of the highest concentration of food extract, i.e., 5×10^{-6} M of 18 amino acids in the test area. Leucine and valine, due to their high concentration in the crude extract (only alanine had a higher concentration), were included in the group which was expected to have a great effect, although the quotient (conc. test area/electrophysiol. threshold) was less than one for both (0.3 and 0.7, respectively).

Threshold concentrations observed in a corresponding study with rainbow trout (Hara, 1982) were used when results with Arctic charr were lacking (Arg, Gly, Ile, Thr, Tyr, Val). No information on the threshold concentrations of aspartic acid (Asp) and phenylalanine (Phe) was available. These amino acids are relatively weak olfactory stimuli at 10^{-4} M in rainbow trout and, at this concentration, were ranked together with L-amino acids with thresholds between 10^{-6} and 10^{-5} M (Hara, 1973, 1982). The threshold concentration of Asp and Phe was assumed to be 10^{-6} M, the same as for glutamic acid in Arctic charr shown by Belghaug and Døving (1977).

Two different test solutions were prepared: one containing 7 amino acids (7 aa) which were presumed to contribute greatly to the stimulatory effect of the food extract (quotient range: 0.30–3.46; median: 1.50), and the other solution containing the remaining 11 acids (11 aa) (quotient range: 0.002–0.39; median: 0.05). A third solution contained all 18 amino acids (18 aa). The mixture ratios in the different solutions were the same as in the original extract. Amino acid solutions were kept in small aliquots in a freezer (−20°C). The three different amino acid solutions were tested in two different concentrations in the test area, namely, 3×10^{-6} and 3×10^{-9} M with 7 aa; 2×10^{-6} and 2×10^{-9} M with 11 aa; 5×10^{-6} and 5×10^{-9} M with 18 aa.

The food extract was tested at four concentrations, 5×10^{-6}, 5×10^{-7}, 5×10^{-8}, and 5×10^{-9} M, of the 18 amino acids. These concentrations were obtained by calculating the dilution of crude extract, i.e., in a 1000-ml glass beaker (75 ml crude extract + 925 ml pure tap water, 7.5 ml crude extr. + 67.5 ml distilled water + 925 ml tap water, and so forth) and then in the fluviarium (165 ml/min to 17.1 l/min), i.e., approximately 1400 times dilution of the highest crude extract concentration tested (Table 1). Control and amino acid solutions contained the corresponding amount of distilled and tap water as given for food extract.

Control, amino acid, and crude extract solutions were supplied from a

TABLE 1. FREE AMINO ACID CONTENT IN WATER SUSPENSION OF EWOS SALMON FEED EXTRA (CRUDE EXTRACT)[a]

Amino acid	Conc. in crude extract (10^{-6} M)	Conc. in test area (10^{-6} M)	Electrophysiol. threshold (10^{-6} M)	Source electrophysiol.[b]
Ala	1555.3	1.11	0.32	B & D
Leu	891.7	0.64	2.1	B & D
Val	686.6	0.49	0.7	H
Gly	631.3	0.45	0.3	H
Met	241.9	0.17	0.068	B & D
Asn[c]	69.1	0.05	0.025	B & D
Gln[c]	69.1	0.05	0.068	B & D
Glu	549.5	0.39	1.0	B & D
Ile	430.8	0.31	7.0	H
Pro	307.6	0.22	100	B & D
Lys	305.3	0.22	6.8	B & D
Thr	294.9	0.21	1.0	H
Phe	261.5	0.19	—	—
Arg	231.5	0.17	1.0	H
Ser[c]	69.1	0.05	0.96	B & D
Asp	205.0	0.15	—	—
Tyr	63.3	0.04	1.0	H
His	56.4	0.04	6.8	B & D

[a] The content of each aa in the test area is given for the highest concentration of crude extract tested. Electrophysiological thresholds are noted. The 18 aa analyzed were divided into two groups, 7 aa and 11 aa, presumed to have a major and a minor contribution, respectively, to the stimulatory effect of the crude extract. For further explanation see Materials and Methods.

[b] B & D = Belghaug and Døving (1977); H = Hara (1982).

[c] Ser + Asn + Gln = 207.3 × 10^{-6} M in crude extract.

1000-ml glass beaker in which the solutions were continuously mixed with a magnetic stirrer. Test and control (dist. water) solutions were pumped into the fluviarium at a rate of 165 ± 5 ml/min.

RESULTS

All concentrations of food extract tested induced upstream movement in the fish (Figure 2). Amino acid solutions with 7, 11, or 18 amino acids (7 aa, 11 aa, or 18 aa) induced a response at the highest concentration level, 10^{-6} M, but none of these solutions gave a significant response when the total amino acid content was reduced to a level of 10^{-9} M.

No significant difference in zone value (Zv) between period A and C1 ($t = 1.12$, $P > 0.05$, $df = 98$) was noted, which indicates that pure tap water

TABLE 2. ONE-WAY ANOVA TO EXAMINE DIFFERENCES IN FOOD SEARCH RESPONSE IN CHARR INDUCED BY DIFFERENT CONCENTRATIONS OF FOOD EXTRACT AND AMINO ACID SOLUTIONS[a]

Variance	*df*	*F* value	*P* value[b]
Test solutions	10	3.95	***
Food extract (4 concentrations)	3	0.43	NS
Food extract (5×10^{-6} M) vs. 18 aa (5×10^{-6} M)	1	0.97	NS
Food extract (5×10^{-9} M) vs. 18 aa (5×10^{-9} M)	1	5.84	*
All aa solutions, 10^{-6} M vs. 10^{-9} M	1	18.78	***
7 aa vs. 11 aa vs. 18 aa, 10^{-6} M	2	1.50	NS
7 aa vs. 11 aa, 10^{-6} M	1	3.43	NS
7 aa vs. 11 aa vs. 18 aa, 10^{-9} M	2	0.33	NS
Within test solutions	88		

[a] Tests with control solutions are included in the ANOVA. The difference in *Zv* between T and C1 in one test (*Zv*T - *Zv*C1) was used as an observation. The proportion of each amino acid was the same as in food extract, which is shown in Table 1.
[b] NS = $P > 0.05$; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

through the T-tube did not influence the fish. Comparisons between *Zv* for C1 and C2 reveal that fish did not return to the positions they occupied in period C1, after the supply of food extract at an amino acid concentration of 5×10^{-7} M in period T ($0.01 < P < 0.02$). Moreover, when testing food extract of 5×10^{-6} and 5×10^{-8} M, respectively, *Zv* for C2 was significantly higher than for C1 ($0.05 < P < 0.1$). *Zv* for C2 was significantly higher than for C1 when using a solution of 11 aa at 2×10^{-6} M ($0.02 < P < 0.05$).

No difference in response to varied concentrations of food extract was found (Table 2). The response to the food extract was significantly stronger than the response to the corresponding 18 aa solution at the lowest concentration (5×10^{-9} M) but not at the higher concentration (5×10^{-6} M). A significant difference in response was observed when comparing all three amino acid solutions at the high concentration level (10^{-6} M) with all amino acid solutions at the low level (10^{-9} M), but there was no difference between amino acid solutions (7aa vs. 11aa vs. 18aa) at the same concentration level. Neither was there any difference between 7aa and 11aa at the same concentration level.

DISCUSSION

The results of this study show that pure amino acid solutions do induce food search behavior in Arctic charr. There are, however, some other substances in addition to the 18 amino acids analyzed which are important in in-

ducing the behavior as the lowest concentration of the 18 aa solution (5×10^{-9} M) did not have any stimulatory effect when compared to food extract at the corresponding amino acid concentration. This is the first study which deals with the significance of mixtures of amino acids, in the same proportions as in food, for the induction of food search in a salmonid species.

Earlier studies with other fishes have shown that a great part of the stimulatory effect of food extract is due to free amino acids, but for many species a complete extract is a more potent stimulus than single or mixed amino acids (Steven, 1959; Hashimoto et al., 1968; Konosu et al., 1968; Carr and Chaney, 1976; Pawson, 1977; Harada and Ikeda, 1984). Ammonia, ornithine, L-cysteine, and L-cystine were not included in the synthetic solutions but may contribute to the strong stimulatory effect of the food extract (Steven, 1959; Hara, 1973, 1977; Harada and Ikeda, 1984). The crude extract contained ammonia (3190.1×10^{-6} M) and ornithine (76.0×10^{-6} M), which resulted in a concentration of 2×10^{-6} M and 5×10^{-8} M, respectively, in the test area when testing the highest extract concentration. L-Cysteine and L-cystine were not analyzed in the crude extract.

In addition, betaine is important as a feeding stimulant and as an attractant in some species of fish (Carr and Chaney, 1976; Mackie et al., 1980; Mackie, 1982). This substance is a weak olfactory stimulus in rainbow trout (Hara, 1976), and Adron and Mackie (1978) found that betaine is of minor importance as a feeding stimulus for rainbow trout.

We did not notice any "dose–response" relationship showing increased response to higher concentrations of food extract. This might be due to the fact that all concentrations tested induced maximum response. There is also the possibility of an "all or nothing" response which means that the fish respond to the stimuli by swimming upstream irrespective of concentration, provided that the threshold concentration is exceeded. It is likely that the specimen in the test group which has the keenest sense of smell and is motivated by hunger initiates the response which is transmitted to the others in the group (cf., Ringler, 1979). An "all or nothing" response to chemical stimuli has been observed in a freshwater catfish (*Ictalurus punctatus*) (Holland and Teeter, 1981).

The presumed low stimulatory solution containing 11 aa was as effective as the high stimulatory solution with 7 aa. The reason may be that the behavioral thresholds are much lower than electrophysiological thresholds and the total amino acid concentrations of the two solutions are the same. But even if each amino acid may not reach its threshold concentration in the 11 aa solution, the mixture may have a stimulatory effect due to additive and synergistic interaction between different amino acids. Studies with radioactively labeled amino acids and with electrophysiological methods have shown that different amino acids may bind to the same receptor site (Cagan and Zeiger, 1978; Brown and Hara, 1981; Caprio, 1982; Hara, 1982). Caprio (1982) studied the electrophysiological response of the olfactory epithelium to amino acid stimulation and distin-

guished by cross-adaptations only a few types of receptor sites, and each site had the highest affinity for a special group of amino acids.

Although salmonids depend a great deal on vision for detection and capture of prey (e.g., Ringler, 1979; Wankowski, 1981), chemical stimulants are of some importance (McBride et al., 1962; Kamchen and Hara, 1980). Their dependence on olfaction should increase in habitats with poor light conditions. Arctic charr populations in deep lakes are often present as shallow and deep living forms or genetically different stocks (Nilsson and Filipsson, 1971; Johnson, 1980; Hammar, 1984). In some Arctic charr populations, different ages are segregated into separate habitats (Klemetsen and Grotnes, 1980; Hammar, 1984). For example the S-charr in Lake Torrön, the charr studied in this experiment, have been shown to make habitat shifts during the life cycle with classes 1+ and 2+ living benthic in deep water (Hammar, 1984). The charr may detect enhanced ammonia and amino acid concentrations and other substances also emitted by their food organisms (Gardner and Miller, 1981; Bengtsson, 1982), giving rise to arousal followed by increased locomotor activity in connection with food search behavior.

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FLAVANONE GLYCOSIDES AS OVIPOSITION STIMULANTS IN A PAPILIONID BUTTERFLY, *Papilio protenor*

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Abstract—Identification of chemical compounds responsible for the oviposition behavior in a Rutaceae feeder, *Papilio protenor demetrius*, was undertaken with the epicarp of sour orange (*Citrus natsudaidai*) which exhibited potent stimulatory activity as did its leaves for egg-laying by the females. The stimulants were present in the hydrosoluble fraction, and the kairomonal activity displayed by the peel was regarded as originating from the synergistic effect of the total chemical complex. One of the active compounds was identified as a flavanone glycoside, naringin (naringenin-7β-neohesperidoside), which, although showing no appreciable effectiveness when bioassayed alone, elicited positive response at the concentration of 0.2% either when admixed with other unidentified components or provided the females had been conditioned with them in advance. Another flavanone glycoside, hesperidin (hesperetin-7β-rutinoside) that was contained in a trace amount in the peel also had a positive effect comparable to that of naringin under similar conditions, while their corresponding aglycones were less active or inactive. In contrast, a flavone glycoside, rhoifolin, coexisting in the peel, and some other flavones and flavonols tested as possible candidates for oviposition stimulants were all found entirely ineffective.

Key Words—Oviposition stimulant, *Papilio protenor*, Lepidoptera, Papilionidae, flavanone glycoside, naringin, hesperidin, synergy, *Citrus natsudaidai*.

INTRODUCTION

A survey of numerous investigations so far conducted on oviposition behavior in many butterfly species covering major families such as Pieridae, Papilionidae, Nymphalidae, and Danaidae reveals that the oviposition sequence in gen-

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eral consists of the following successive phases: (1) Searching for host plants guided by visual and/or olfactory cues, (2) semirandom or nonrandom (where search image is formed) alighting on plants, (3) drumming on the surface of foliage with foretarsi for the purpose of discrimination and recognition of the host plant, and (4) abdominal curling and probing appropriate ovipositional site with the ovipositor extruded, followed by egg deposition (Chew and Robbins, 1984).

Recent work has documented that in the incipient step of oviposition behavior certain butterflies primarily utilize leaf shape to visually distinguish potential larval food plants from manifold vegetation of which the majority would be unsuitable for larval growth and to enhance the frequency at which they encounter the host plants (Benson, 1978; Rausher, 1978; Stanton, 1982). In selecting the ovipositional sites, females of some species appear to be affected also by the color of leaves of host plants (Ilse, 1937; Vaidya, 1969; Saxena and Goyal, 1978) or by the presence of previously laid conspecific eggs or egg mimics on plants (Rothschild and Schoonhoven, 1977; Rausher, 1979; Shapiro, 1981; Williams and Gilbert, 1981).

Despite the profound involvement in host seeking of visual stimuli and, in some cases, of volatile chemicals emitted by host plants which attract preovipositional females (Saxena and Goyal, 1978; Feeny et al., 1983), it is well established in Pieridae and Papilionidae that dominant stimuli acting in the final stage of host selection at close range as the cues permitting the females to assess the suitability of the plant they landed on are secondary plant metabolites. These are liberated during drumming, are perceived by foretarsal chemoreceptors and induce oviposition (David and Gardiner, 1962; Nishida, 1977; Ichinosé and Honda, 1978; Saxena and Goyal, 1978; Rodman and Chew, 1980; Abe et al., 1981; Feeny et al., 1983). The importance in host recognition of tarsi, especially foretarsi, which are endowed with chemotactile sensory receptors, is fully substantiated in several butterfly families, and their external morphology has been examined microscopically (Myers, 1969; Ma and Schoonhoven, 1973; Calvert, 1974; Ichinosé and Honda, 1978; Stanton, 1979; Feeny et al., 1983).

However, insufficient knowledge of the chemical basis for differential response of ovipositing females to various plants has been accumulated (Nishida, 1977; Ichinosé and Honda, 1978; Abe et al., 1981; Feeny et al., 1983). Only a few compounds have been identified as a trigger for abdominal curling from which egg-laying ensues. Sinigrin (allylglucosinolate) has been reported to stimulate oviposition in *Pieris brassicae* (David and Gardiner, 1962) and *P. rapae* (Renwick and Radke, 1983). Recent study with *Papilio xuthus* (Ohsugi et al., 1985) has revealed that the ovipositional response is elicited by a flavone glycoside, vicenin-2 (6,8-di-C-β-D-glucopyranosylapigenin), which, however, was effective only when admixed with other, as yet unidentified, components.

The present paper reports on the chemical compounds that evoke egg-laying from an oligophagous papilionid butterfly, *Papilio protenor*, which in na-

ture exclusively feeds on specific plants in the limited range of genera, *Citrus* (main), *Poncirus*, *Fagara*, and *Zanthoxylum* belonging to the family Rutaceae.

METHODS AND MATERIALS

Insects. Female butterflies of *Papilio protenor demetrius* were obtained from stock cultures maintained in our laboratory, or collected in the fields in Kanagawa Prefecture. They are of the same population which infests principally *Citrus unshiu* and *C. natsudaidai* in nature.

Extraction and Fractionation of Active Compounds. The epicarp of *C. natsudaidai* was extracted with ca. threefold amount of methanol at room temperature for a month. Female butterflies exhibited positive response to the methanol extract, suggesting that the active substance(s) inducing oviposition is present in the extract. The methanolic extract was concentrated in vacuo below 50°C. The residue (13.3 g), dissolved in 100 ml of water, was extracted with three 50-ml portions of isobutanol. The aqueous layer (fraction 2, 11.7 g) was subjected to ultrafiltration at a level of mol wt 1000 with a membrane filter (UH-1, Tôyô Roshi Co., Ltd.) to give two fractions: one (0.7 g) containing compounds with a molecular weight of more than 1000 (fraction 3) and the other (11.0 g) composed of smaller molecules (fraction 4). An aliquot of fraction 4 (1.5 g) was further fractionated by reverse-phase chromatography on a ODS column (LRP-2, 45 μm, Whatman Inc.; 2 cm ID $\times$ 30 cm) yielding fractions 5 (1.36 g), 6 (0.03 g), 7 (trace), 8 (0.06 g), and 9 (trace). The column was eluted stepwise with methanol–H_2O (20:80), methanol, and acetone, successively. The schematic procedure for separation is shown in Figure 1.

Authentic Chemicals. Commercially available naringin and hesperidin (Tokyo Kasei Kogyo Co., Ltd.) were recrystallized from water and pyridine, respectively. Naringenin, rutin (Tokyo Kasei Kogyo Co., Ltd.), hesperetin, rhoifolin, apigenin, diosmin, and kaempferol-7-neohesperidoside (Sigma Chemical Co.) were all used without further purification.

Bioassay for Ovipositional Activity. Artificial green plastic leaves, shaped like a compound leaf (25 cm long) consisting of 15 fusiform "foliole" (16 mm long $\times$ 11 mm wide), were dipped into aqueous methanol solution of a test sample of a given concentration and air-dried at room temperature to remove methanol. The artificial leaves were remoistened by spraying distilled water on the surface and presented to 3- to 10-day-old fertile females, which, prior to the experiments, had been fed with 10% aqueous sucrose and kept in a transparent plastic cage (25 $\times$ 35 cm; height, 15 cm) that permits them free flight. Appraisal of positive response was based on the following criteria: release of the sequential oviposition behavior usually observed in nature that consists of drumming on a leaf with forelegs and subsequent abdominal curling followed by settling of the ovipositor in contact with the underside of the leaf. The fe-

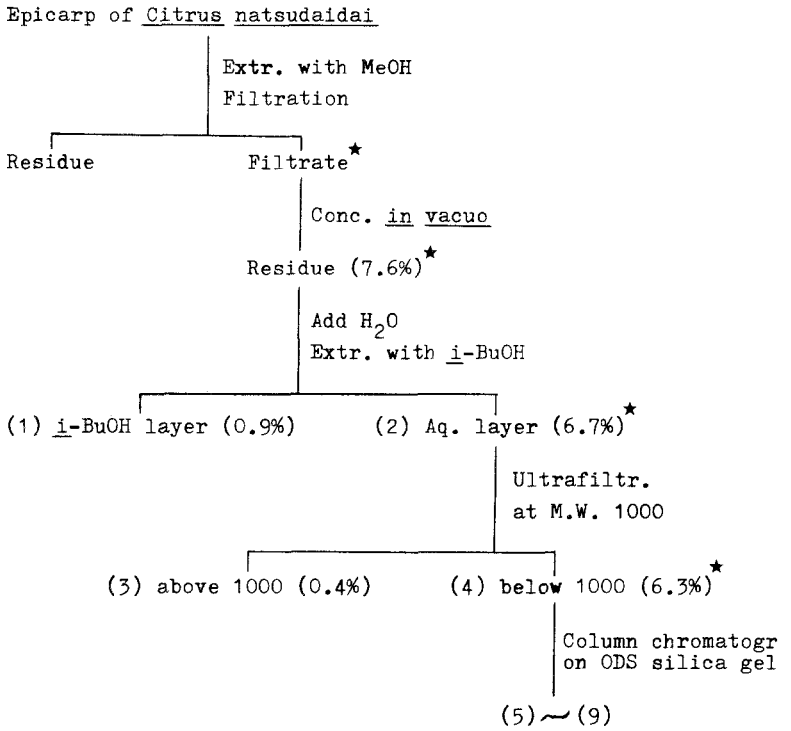


FIG. 1. Schematic procedure for the separation of oviposition stimulants from the epicarp of sour orange (*C. natsudaidai*). Fractions marked with asterisk were consistently active when bioassayed with 0.2–0.5% solution. Relative amount of each fraction to the original fresh weight of the epicarp is given in parentheses.

males screened as available for the behavioral test were only those that showed responses negative to distilled water alone and positive to the leaves of *C. natsudaidai*. Unresponsiveness of the females to the control (water) was carefully confirmed every three trials of sample presentation, for some females appeared to get "hypersensitive" during the experiment, positively responding without any chemical stimuli other than water. The rate of response was expressed by the number of positive responses/number of alighting on the leaves that was accompanied with drumming.

NMR Spectroscopy. [^{13}C]NMR spectra were recorded at 67.8 MHz in CD_3OD on a JEOL JNM-GX 270 FT-NMR spectrometer by proton-decoupled operation with tetramethylsilane as the internal standard. Temperature, flip angle, pulse repetition time, and the number of data points were 25°C, 45°, 5 sec and 64 K, respectively. Chemical shifts were represented by δ unit. Multiplicity of signals determined by means of INEPT technique was abbreviated as follows: singlet (s), doublet (d), triplet (t), quartet (q).

Mass Spectrometry. Fast-atom-bombardment mass spectra (FAB-MS) were measured with a JEOL JMS-DX 300 mass spectrometer. The target was bombarded with 6 KeV Xe atoms employing a mixture of sodium iodide and glycerine as a matrix.

High-Pressure Liquid Chromatography (HPLC). Reverse-phase HPLC analyses were carried out with a Hitachi 635S liquid chromatograph equipped with a wavelength-tunable spectrophotometer using a stainless column (4.6 mm ID $\times$ 15 cm) packed with Hitachi gel 3056 (ODS silica gel, 5 μm). Mixed solvent composed of MeOH–H_2O–AcOH (20 : 80 : 0.1) was used as an eluent, and the column eluate was monitored at 280 nm. The system was operated at an ambient temperature, and the flow rate was regulated at 1 ml/min.

RESULTS

As shown in Figure 1, major active compound(s) was present in the hydrosoluble fraction (fraction 2), and found to be a relatively small molecule whose molecular weight was less than 1000 (fraction 4). Further fractionation by reverse-phase column chromatography on ODS revealed that the active principle is composed of more than two components: the one being contained in fraction 5, and the other in fraction 8 (Figure 2). However, the potency of stimulatory effect on oviposition was quite different between these two fractions. Although 1% solution of fraction 8 was effective to a certain extent, its activity drastically diminished at lower concentrations, and eventually gave out

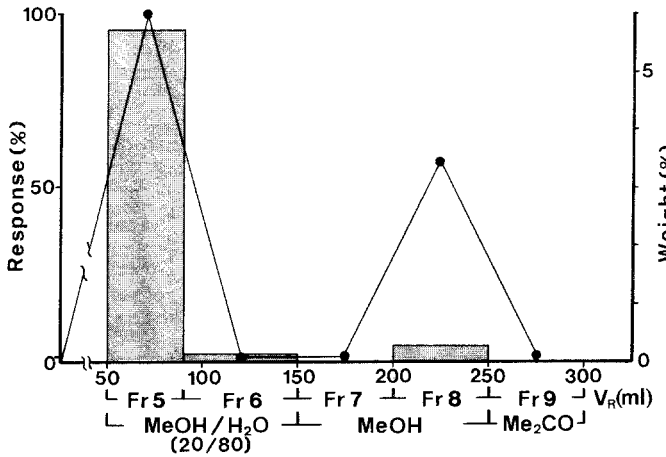


FIG. 2. Ovipositional response of *P. protenor* to fractions 5–9 separated from fraction 4 by reverse-phase (ODS) column chromatography. Three females were tested, and assay concentration of the sample was 1%. The relative weight of each fraction is indicated by a shaded bar, and the response by a closed circle.

almost completely at a level of 0.2%, whereas fraction 5 of the same concentration still provoked strong response at a rate as much as 100%. Therefore, the principal oviposition stimulant in the peel of *C. natsudaidai* was considered to be localized in fraction 5.

Upon further chemical analysis by thin-layer chromatography (TLC) on silicic acid with methanol, fraction 5 was found to be a mixture of a number of compounds, and each of the four fractions separated retained sufficient activity for inducing egg-laying at a level of 0.2%, indicating that at least several compounds combine to evoke oviposition behavior in this species. Since every fraction derived from fraction 5, however, seemed to gradually lose its activity during storage, fraction 5 was not pursued further in this study.

In contrast, fraction 8, although its stimulatory activity was not high, appeared far less labile, and gave a yellow precipitate when stored in a refrigerator for several days. The crude precipitate was recrystallized twice from hot water and dried at 150°C to afford pale yellow crystals (mp: 172–173°C), which, on HPLC analysis, was found to correspond to the major peak (A) in fraction 8 (Figure 3). Compound A was bioassayed for ovipositional response, but showed no appreciable activity at the concentration of 0.2% and was only slightly active at the level of 0.5%. However, the stimulatory effect of this substance, if only the females were "conditioned" beforehand with 0.2% solution of fraction 5, markedly increased to an extent adequate to release egg-laying (Table 1). Although a solid explanation for such remarkable enhancement of activity is hard to find at present (this will be discussed later), compound A should be regarded

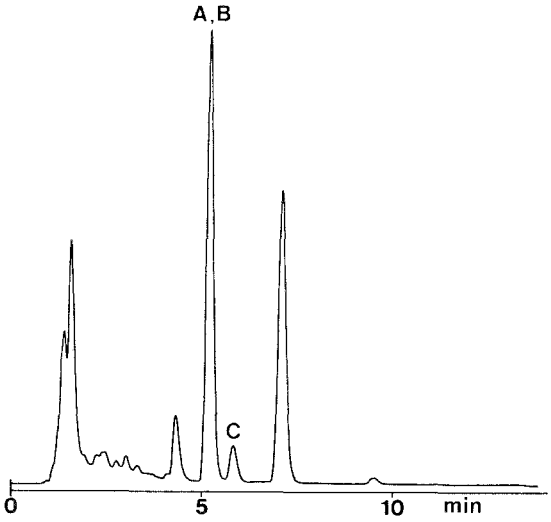


FIG. 3. HPLC chromatogram of fraction 8 on a ODS column. A,B: naringin and rhoifolin; C: hesperidin.

TABLE 1. OVIPOSITION-STIMULATORY ACTIVITY TO *Papilio protenor* OF SAMPLES DERIVED FROM EPICARP OF *Citrus natsudaidai*

Sample	Response (No. of trials)[a]		
	0.1%[b]	0.2%	0.5%
MeOH extract	0.23 (22)	1.00 (24)	1.00 (20)
Fraction 5	0.86 (21)	1.00 (21)	1.00 (22)
Compound A	0 (24)	0 (22)	0.12 (26)
Compound A[c]	0 (20)	0.81 (21)	0.92 (26)

[a] Twelve females were tested.
[b] Percent indicates concentration of sample.
[c] The females were conditioned with fraction 5 (0.2%) prior to the experiments. Unresponsiveness to the control (water alone) was carefully confirmed after conditioning to rule out the possibility of a direct effect of fraction 5.

as one of the oviposition stimulants. Therefore, an attempt was made at structural elucidation of compound A.

The aqueous solution of compound A turned reddish-purple when treated with Mg-HCl, thereby adducing the possibility of the compound being a sort of flavonoid. The result of elemental analysis, combined with FAB-MS information [$(M + H)^+$ at *m/z* 581 and $(M + Na)^+$ at *m/z* 603], determined the molecular formula of compound A to be $C_{27}H_{32}O_{14}$. More informative data were obtained by CMR measurement giving the following signals, δ: 197.8(s), 165.9(s), 164.2(s), 164.0(s), 158.4(s), 130.3(s), 130.2(s), 128.7(d), 128.6(d), 116.0(d), 104.5(s), 102.1(d), 99.0(d), 97.5(d), 96.4(d), 80.3(d), 78.9(d), 78.7(d), 78.5(d), 77.7(d), 73.6(d), 71.9(d), 70.9(d), 69.7(d), 62.0(t), 43.9(t), 43.6(t), 18.2(q), which were suggestive of the presence of one carbonyl carbon, no less than 10 aromatic carbons, and at least 10 carbons that may be assigned to the sugar moiety.

Compound A was thus assumed to be a flavonoid glycoside, possibly a flavanone glycoside among others. On the other hand, CMR spectrum of the acid hydrolysis (5% aq. HCl) product (pale yellow crystals soluble in ethyl acetate) of compound A, which was regarded as its aglycone, coincided well with that of authentic naringenin. Furthermore, the resonance at 18.2 ppm assignable to a methyl group was indicative of the presence of rhamnose in the sugar residue. This spectral evidence suggests that compound A is likely to be naringenin-7-rhamnoglucoside, probably either naringin or narirutin. Good agreement in melting point and spectral data of authentic naringin with those of compound A confirmed the identity of the two. Compound A was thus determined to be naringin (naringenin-7β-neohesperidoside, Figure 4).

HPLC analysis and CMR measurement of the crude crystals separated from fraction 8 revealed that fraction 8 also contains a small amount of rhoifolin

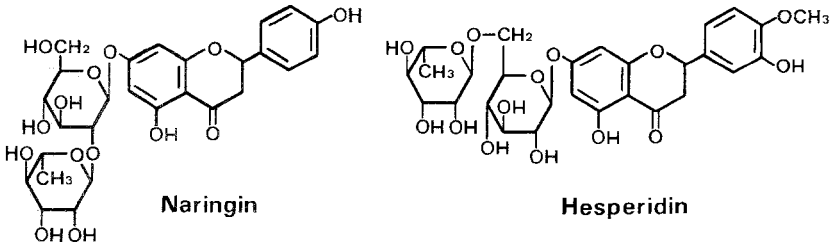


FIG. 4. Chemical structures of flavanone glycosides appraised as active for egg-laying by *P. protenor*. Naringin: naringenin-7β-neohesperidoside; Hesperidin: hesperetin-7β-rutinoside.

(apigenin-7β-neohesperidoside, peak B in Figure 3), which is superimposed on peak A. In addition, the existence of a trace of hesperidin (hesperetin-7β-rutinoside, Figure 4) in fraction 8 was also presumed by HPLC retention data (peak C in Figure 3).

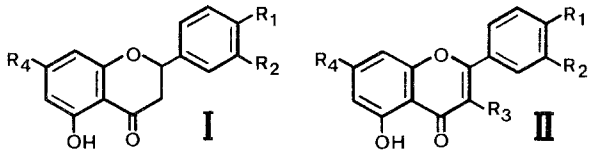
Naringin, hesperidin, rhoifolin (the former two being flavanone glycosides, and the latter, a flavone glycoside), and other several flavonoid compounds available as possible candidates for oviposition stimulants were then bioassayed. The results are summarized in Table 2.

Unless the females were conditioned with fraction 5 prior to the experiment, naringin was virtually inactive when assayed alone, while pretreatment with or coexistence of fraction 5 remarkably promoted its activity, and eventually naringin turned out active. As all the other substances tested exerted little or no stimulatory action by themselves, their activity was evaluated after similar conditioning. Of these compounds, only flavanones such as naringin, hesperidin, and hesperetin, to which females evidently displayed a positive response, were appraised as active. In contrast, flavones and flavonols were entirely inactive, although limited solubility of some compounds precluded examining their dose–response correlation. As for naringin and hesperidin, the minimal concentration necessary for eliciting ovipositional response by females was estimated equally at ca. 0.2%. The latter, however, appears a little more active since the effectiveness of 0.1% solution of hesperidin was somewhat higher as compared with that of naringin, and besides, its aglycone, hesperetin, exhibited unequivocal ovipositional activity despite the inertness of naringenin (aglycone of naringin).

DISCUSSION

The present results are congruent with previous work by Ichinosé and Honda (1978) in that egg-laying of *Papilio protenor* is stimulated by water-soluble substances in *Citrus* plants and further demonstrate definitely that the

TABLE 2. OVIPOSITIONAL RESPONSE OF *Papilio protenor* TO FLAVONOID COMPOUNDS



Compound	Type	R1	R2	R3[a]	R4[a]	Response (No. of trials)[b] 0.1%[c]	0.2%	0.5%
Naringin[d,e]						0 (22)	0.08 (26)	0.13 (24)
Naringin[d] and Fr.5 (0.05%)[f]	I	OH	H		Ne	0.05 (21)	0.88 (25)	0.93 (30)
Naringin						0.04 (24)	0.84 (25)	0.96 (27)
Naringenin	I	OH	H		OH	0 (24)	0 (24)	0.15 (20)
Hesperidin[e]	I	OMe	OH		Ru	0.19 (21)	0.80 (25)[g]	0.84 (25)[g]
Hesperetin	I	OMe	OH		OH	0 (20)	0.04 (23)	0.83 (23)
Rhoifolin[e]	II	OH	H	H	Ne	0.09 (22)	0.14 (29)	0.13 (24)[g]
Apigenin	II	OH	H	H	OH		0 (25)[g]	0.04 (25)[g]
Diosmin	II	OMe	OH	H	Ru		0.03 (31)[g]	0 (22)[g]
Kaempferol-7-neohesperidoside	II	OH	H	OH	Ne		0 (23)	0 (20)
Rutin	II	OH	OH	Ru	OH		0 (20)	0 (25)

[a] Ru: Rutinose, Ne: Neohesperidose.
[b] Recorded using 12 females that were preconditioned with fraction 5 (0.2%), unless otherwise noted.
[c] Percent indicates concentration of sample.
[d] Unconditioned.
[e] Compounds whose presence in the epicarp of *Citrus natsudaidai* was confirmed or presumed.
[f] Fraction 5 elicited no appreciable response at 0.05%.
[g] Because of the scanty solubility, some portions of crystals remained indissolved.

kairomonal activity for oviposition displayed by the peel of *C. natsudaidai* is the result of synergistic interaction among manifold chemical compounds. Similar synergy of chemicals involved in host selection has been reported for the cabbage butterfly, *Pieris rapae*, where sinigrin, although it stimulates oviposition, does not act alone to mediate discrimination among potential host plants, and combination with other water-soluble compounds evoked more potent ovipositional response (Renwick and Radke, 1983). A recent study with a swallowtail butterfly, *Papilio xuthus*, has also dealt with an analogous topic where vicenin-2 (flavone glycoside), which was inactive alone, proved active when admixed with another water-soluble fraction (Ohsugi et al., 1985).

The stimulatory action of naringin and hesperidin on egg deposition by the females appears to prevail only to a limited extent and to be subsidiary, because they neither exhibited any appreciable activity when assayed alone nor played an essential role in oviposition as evidenced by the fact that fraction 5, devoid of the two compounds, did suffice singly to elicit ovipositional response. Accordingly, such flavanone glycosides are not principal determinants of oviposition; nevertheless, either compound should be regarded as factors conducive to the synergy. Although it is difficult to put an explicit interpretation on the enhanced response by ovipositing females to flavanones caused by pretreatment with fraction 5, a possible reason is that this phenomenon was due to "memory effect" brought about by a trace of residual active substances adsorbed on the tarsal chemoreceptors of forelegs. In fact, repetitive washing of foretarsi with water resulted in much reduced response to flavanones.

Naringin and hesperidin are not specific to *Citrus* plants (although not being widespread in plants); however, most *Citrus* plants have been reported to be excellent producers of these compounds, which are contained not only in fruits but also in leaves (Hattori et al., 1952; Nakabayashi, 1961; Nishiura et al., 1969; Namba et al., 1985). In this respect, it is very likely that *Papilio protenor*, one of *Citrus* feeders, has evolutionarily come to rely not on ubiquitous flavonoids such as rutin (flavonol) but on certain specified flavanones in discriminating more precisely its host plants out of a diversity of unacceptable plants.

Some *Citrus* plants are also known to yield several flavones as minor flavonoid constituents. *P. protenor*, however, made little or no response to such flavone glycosides as rhoifolin and diosmin, the former being frequently encountered in *Citrus* plants (Hattori et al., 1952; Nakabayashi, 1961; Nishiura et al., 1969). Therefore, flavones in general appear essentially inactive to *P. protenor*, whereas a sort of flavone glycoside, vicenin-2, has been reported to be active to a related species, *P. xuthus* (Ohsugi et al., 1985). Feeny et al. (1983) have shown that for *P. polyxenes*, a umbellifer-feeding swallowtail, naringin and hesperidin were ineffective altogether, while chlorogenic acid and apigenin-7-glucoside (flavone) were slightly active. Such disparity may partly reflect interspecific difference in host preference.

In *P. protenor*, all of the components present in active fractions seem not indispensable for inducing oviposition; however, the whole complex of constituents should form the chemical basis for host recognition and preference. To possess a comprehensive response spectrum to or against a variety of compounds, not relying on any single stimulant, may serve to ensure correct and strict discrimination of host plants by mother butterflies.

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DYNAMICS OF 6-METHOXYBENZOXAZOLINONE IN WINTER WHEAT
Effects of Photoperiod and Temperature

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Abstract—6-Methoxybenzoxazolinone (6-MBOA), a compound derivable from some freshly growing plants, is known to stimulate reproduction in some mammals and birds. Winter wheat was studied under controlled laboratory conditions to determine the effects of photoperiod and temperature on derivable 6-MBOA content. Longer photoperiods decrease the amount of derivable 6-MBOA per gram of fresh material in 4-day-old wheat seedlings. Higher temperatures also decrease the amount of derivable 6-MBOA in 4-day-old wheat. 6-MBOA content decreases as the plant ages. Comparisons of only the first centimeter above the seed produced the same age-related result. 6-MBOA is concentrated in the meristematic region with decreasing amounts found in higher portions of the plant. Roots from 9-day-old plants contain 6-MBOA. Unsprouted wheat seeds contain negligible amounts of 6-MBOA. These results demonstrate that environmental variables have a significant effect on derivable 6-MBOA levels, but that under all the regimes studied, 6-MBOA is present in freshly sprouted wheat.

Key Words—6-Methoxybenzoxazolinone, hydroxamic acids, wheat, photoperiod, temperature.

INTRODUCTION

The timing of the onset of breeding of microtine rodents in natural populations can be highly variable. In field populations of *Microtus montanus*, the montane vole, the initiation of reproductive activity is correlated with the beginning of vegetative growth of their plant food resource in the spring, rather than at a

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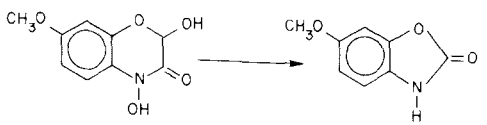
specific daylength (Negus et al., 1977). These herbivorous rodents eat primarily grasses and sedges, consuming fresh plant material when available during the growing season. Sanders et al. (1981) identified a factor derived from young winter wheat, 6-methoxybenzoxazolinone (6-MBOA), which increases uterine weight in female *M. montanus* and ovarian weight in *Mus musculus*. Using this compound in field studies, Berger et al. (1981) found that nonbreeding winter populations of *M. montanus* could be stimulated to breed several months before the onset of vegetative growth in the spring. Increases in reproductive function were observed in both sexes. In the laboratory, 6-MBOA increases litter size and frequency in this species (Berger et al., 1986a). This compound has been tested on other vertebrate species and has been found to have stimulatory effects on female reproductive function in rats (Butterstein et al., 1985), kangaroo rats (Rowsemitt, 1984), bobwhite quail (Berger et al., 1986b), rabbits, and mink (Berger and Negus, unpublished).

Prior to the work of Sanders et al. (1981), interest in 6-MBOA and related compounds centered on their role in the defense of young crop grasses against insect predators such as aphids (Argandona et al., 1980, 1981), corn borers (Klun and Brindley, 1966), and other pests. Although 6-MBOA is reported to be absent from intact plants, its precursor, 2,4-dihyroxy-7-methoxy-(2H)-1,4-benzoxazin-3(4H)-one (DIMBOA) has been reported in several species of the family Poaceae (Virtanen and Hietala, 1960; Loomis et al., 1957; Tang et al., 1975). This compound, present as a glucoside in intact tissue, is converted by enzymatic hydrolysis to the aglycone (DIMBOA) after tissue damage. The latter compound is unstable and decomposes to 6-MBOA (Figure 1) (see Virtanen and Hietala, 1960, for review). Therefore, 6-MBOA is formed when the plant's predator masticates the plant tissue. Argandona et al. (1981) examined the distribution of hydroxamic acids in wheat (*Triticum durum*) as an indicator of the major hydroxamic acid present, DIMBOA. On a per gram basis, maximum levels were reached in shoots by the fourth day after germination. Similar results were observed with *Triticum aestivum, Secale cereale,* and *Zea mays*. For *Triticum durum*, hydroxamic acid levels drop in old leaves, and new leaves on 30-day-old plants have lower levels than new leaves on younger plants. The authors did not report the environmental conditions under which the plants were grown.

Microtus montanus and some related species live in habitats with a high



DIMBOA 6-MBOA

FIG. 1. Chemical structure of 6-MBOA and its precursor DIMBOA.

degree of temperature and rainfall variability on a year-to-year basis. The freshly sprouted grasses which they appear to use to cue the onset of reproduction (Negus et al., 1977; Berger et al., 1981) are growing under different temperature and photoperiod conditions in different years. While laboratory studies examining the distribution of derivable 6-MBOA have been performed (Argandona et al., 1981; Klun and Robinson, 1969), no attempts have been made to examine variations in 6-MBOA in young grasses growing under the range of conditions typical of natural habitats. We are currently involved in field studies of available 6-MBOA in wild grasses which are food resources for *M. montanus* and other microtine rodents. In order to more closely examine the effects of environmental variables on 6-MBOA availability in grasses, we have chosen to use winter wheat as a model system to examine the concentrations of derivable 6-MBOA in grasses grown under various photoperiod and temperature regimes.

METHODS AND MATERIALS

General. Winter wheat seeds were soaked in tap water for 1 hr, then planted 24 hr later in Vermiculite and grown in an environmental chamber with high-pressure sodium and metal halide high-intensity discharge lamps. Light intensity was 0.5 mmol photosynthetically active radiation/m^2/sec. Four days after planting, triplicate 1.0-g samples of whole plants were extracted for derivable 6-MBOA. Samples were collected 3 hr after the onset of light. Entire shoots were utilized, cutting at the seed. Plant material was ground with sand to disrupt cell walls, incubated in distilled water for 1 hr at room temperature to allow enzymatic conversion to the aglycone, boiled for 30 min to allow conversion to 6-MBOA, then extracted three times with redistilled reagent-grade dichloromethane and dried under nitrogen. The dried samples redissolved in CH_3OH were analyzed for 6-MBOA by GC-MS. A Dupont model DP102 instrument equipped with an integrator and a SP2250 GC column isothermal at 200°C was used. The peak for the mass ion at *m/z* 165 was the most intense in the fragmentation spectrum and was integrated.

A standard curve was obtained using 0.06, 0.6, and 1.2 μg injections of a pure 6-MBOA in CH_3OH solution and was reproducible to $\pm 5\%$ at the low-weight end of the curve. Statistical comparisons were analyzed by linear regression unless otherwise specified.

Regimen I. 6-MBOA Concentrations under Various Photoperiods. Plants were grown under 8, 10, 14, and 16 hr of light per day with a temperature cycle of 12 hr at 25°C and 12 hr at 15°C such that the warm portion of the day occurred during the light and was symmetrical with the temperature regime.

Regimen II. 6-MBOA Concentrations under Various Temperatures. Plants were grown in 12 hr of light per day under the following temperature regimes

(°C): 35:15, 30:20, 20:10, 15:5, with the higher temperature given during the light portion of the day.

Regimen III. Age and Distribution Profile of 6-MBOA. Plants were grown under 12 hr of light per day with a daytime temperature of 30°C and a nighttime temperature of 20°C. Whole plant samples were taken at days 4, 9, and 16; 1.0-g samples of the first centimeter above the seed were also taken at days 4, 9, and 16. On day 9, the distribution of 6-MBOA within plants was examined by extracting 1.0-g samples of the first, fourth, and tenth centimeter above the seed.

Regimen IV. 6-MBOA Contents of Roots and Seeds. The 6-MBOA contents of roots and seeds were determined. Triplicate 1.0-g samples of roots were extracted from the 20°/10° sample in regimen II. Triplicate 1.0-g samples of unsprouted seeds were ground to a powder and extracted.

RESULTS

Regimen I. Longer daylengths decrease the amount of 6-MBOA present per gram of plant material ($r^2 = 0.852$, $P < 0.001$) (Figure 2). There was no relationship between photoperiod and plant height ($r^2 = 0.077$; $P = 0.68$). Plant height ranged from 2.0 cm in 10 hr of light per day to 5.0 cm in 14 hr of light per day.

Regimen II. Increases in day and night temperatures decrease 6-MBOA content per gram of plant material ($r^2 = 0.867$, $P < 0.001$) (Figure 3). Plant height decreased with increasing temperature ($r^2 = 0.996$; $P = 0.039$). Height ranged from 7 cm in the 35°/25° regime to 0.5 cm in the 15°/5° regime.

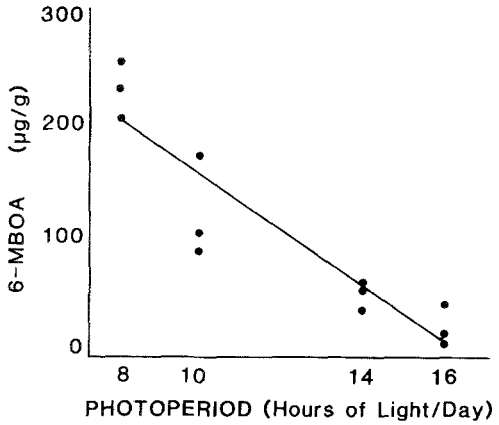


FIG. 2. Effect of photoperiod on derivable 6-MBOA in 4-day-old wheat plants. Regression: $y = -25.82x + 425.31$. $r^2 = 0.852$, $P < 0.001$.

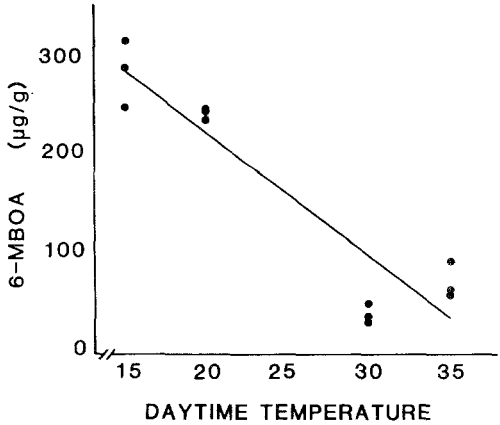


FIG. 3. Effect of temperature on derivable 6-MBOA in 4-day-old wheat plants. (Nighttime temperatures are 10° lower.) Regression: $y = -13.52x + 506.78$. $r^2 = 0.867$, $P < 0.001$.

Regimen III. Age has a highly significant effect on derivable 6-MBOA, with highest levels found in the 4-day sample ($r^2 = 0.650$, $P = 0.0156$) (Figure 4A). Comparisons of only the first centimeter above the seed yielded similar results with the 4-day sample having the highest concentration of derivable 6-MBOA ($r^2 = 0.944$, $P < 0.001$) (Figure 4B). Comparing the concentration of 6-MBOA in the first centimeter with that in the entire plant shows no significant difference at day 4, but significantly higher concentration in the first centimeter than in the whole plant at days 9 and 16 ($P = 0.001$, $P = 0.039$, respectively, Student's t test). This conclusion is supported by the results in Figure 5, showing concentrations in several portions of the plant ($r^2 = 0.976$, $P < 0.001$).

Figure 5 shows the distribution of derivable 6-MBOA within plants at 9 days of age grown under the conditions described above. The highest concentrations are found at the meristematic region at the base of the grass with decreasing concentrations in the higher portions of the plant.

Regimen IV. Root samples from 9-day-old plants as described above averaged 101.3 μg derivable 6-MBOA/g plant material (± 14.0 SD). Unsprouted seeds contained 3.3 μg 6-MBOA/g (± 5.8 SD).

DISCUSSION

To understand the availability of 6-MBOA in natural food resources of microtine rodents such as *Microtus montanus*, we have used one grass (wheat) as a model system. Under controlled environmental conditions in the laboratory, we have studied the effects of altering both photoperiod and temperature

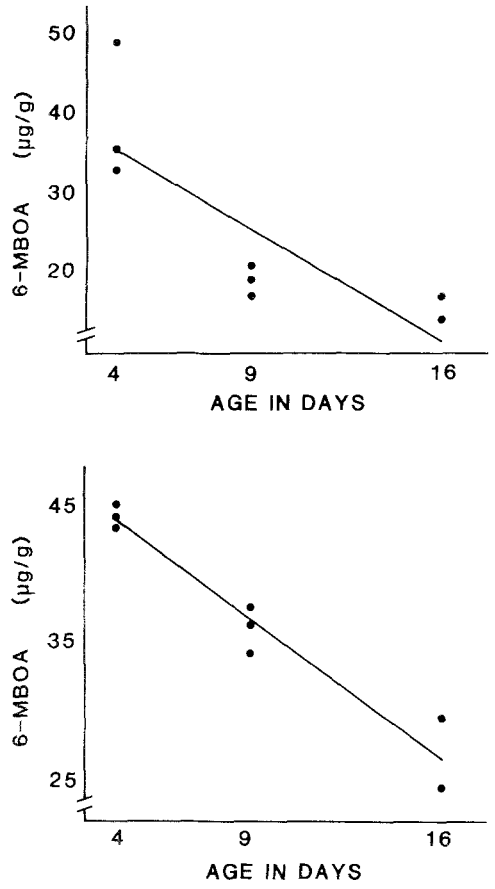


FIG. 4. Effect of age on derivable 6-MBOA in wheat. (A) Whole plant. Regression: $y = -2.05x + 44.78$. $r^2 = 0.650$, $P = 0.016$. (B) First centimeter. Regression: $y = -1.36x + 50.23$. $r^2 = 0.044$, $P < 0.001$.

on derivable 6-MBOA. While we recognize that the absolute values determined here for wheat are not likely to be equivalent to those found in the natural grasses which are food resources for microtine rodents, our purpose is to elucidate how the plants' investment in the production of the precursor to 6-MBOA varies under different growing conditions. Other work in progress examines 6-MBOA content of known food resources of *M. montanus* (Negus, Berger, and Epstein, in preparation).

Our results demonstrate that both photoperiod and temperature affect the concentration of derivable 6-MBOA in 4-day-old plants. Increasing the hours of light per day decreases the concentration of derivable 6-MBOA per gram of

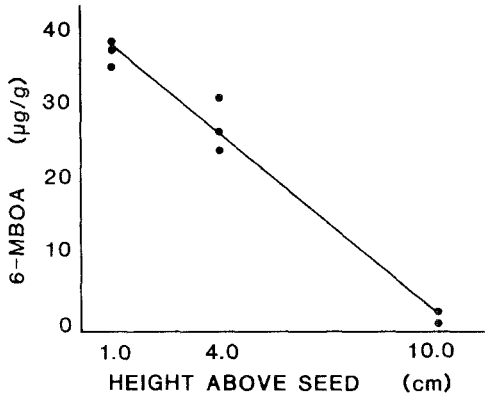


FIG. 5. Distribution of derivable 6-MBOA in the first, fourth, and tenth centimeter above the seed. Regression: $y = -3.73x + 41.86$. $r^2 = 0.976$, $P < 0.001$.

fresh plant material. Increasing the temperature also decreases the amount of derivable 6-MBOA. Since higher temperatures produce more biomass per plant, the temperature effect may be due, in part, to the fact that more individual plants are required to make a gram of material in the wheat grown at lower temperatures than at the higher temperatures. The result is that more meristematic material is present in the samples at lower temperatures. Since 6-MBOA occurs in higher concentration in the first centimeter, a 1.0-g sample of shorter plants will contain more individual plants and thus more first-centimeter portions (the meristematic region in grasses) than a 1.0-g sample of taller plants and will therefore contain more 6-MBOA if plant height is the important factor. The photoperiod effect on 6-MBOA concentrations cannot be explained in this manner because the plant height did not vary in a systematic fashion with photoperiod. Our data demonstrate that, regardless of photoperiod and temperature conditions, freshly sprouted wheat seedlings contain significant amounts of derivable 6-MBOA, with approximately a fivefold range in 6-MBOA on a per gram fresh weight basis under the different treatments.

The results of our examination of the distribution of 6-MBOA within the plant are consistent with those of other workers. Highest concentrations were found at the first centimeter above the seed, with decreasing concentration at greater heights. This is in agreement with the results of Klun and Robinson (1969) for several strains of corn and with those of Argandona et al. (1981) for wheat. Direct comparisons of our results with those of other workers are of limited value for several reasons. The intraspecific differences in 6-MBOA can be very dramatic in inbred strains of crop grasses. Examination of 11 strains of corn in one study revealed almost a 10-fold range in 6-MBOA concentrations (Klun and Brindley, 1966). Furthermore, the growing conditions varied substantially between studies. In spite of high variability in actual 6-MBOA content

in various grasses, interspecific generalities are emerging regarding the distribution of 6-MBOA in those grasses which contain it.

Our age profile, with samples taken at days 4, 9, and 16, showed decreasing levels from day 4, whether the entire grass was sampled or the first centimeter only was used. Other age profile work shows similar results. Argandona et al. (1981) show decreases in hydroxamic acids in wheat after four days of age. Argandona et al. (1980) took their first sample at 10 days of age. For wheat, decreases occurred after day 10; for rye (*Secale cereale*) days 10 and 16 produced similar levels of hydroxamic acids with decreases starting at day 22. Klun and Robinson (1969) reported 6-MBOA concentrations based on plant height in several strains of corn (*Zea mays*). Their first sample, at 6 in., was the highest value for every strain examined. Therefore, highest concentrations are found in the young plants.

The relevance of the root levels of 6-MBOA to microtine food resources is not clear. Behavioral studies of *Microtus breweri* have shown a seasonal shift in preference for various portions of their major food resource, the beach grass, *Ammophila breviligulata*. In late summer, the roots are the preferred portion of the plant (Rothstein and Tamarin, 1977). However, our own and other studies of 6-MBOA in roots have been from young actively growing plants; we know nothing about the availability of 6-MBOA in a perennial grass late in the growing season. Derivable 6-MBOA has been found in the roots of wheat, rye, and Job's tears (Tang et al., 1975). The availability of 6-MBOA in roots may be of importance to reproductive patterns of root-eating subterranean rodents such as pocket gophers (Geomyidae).

Our inability to detect significant amounts of derivable 6-MBOA in wheat seeds is consistent with the results of Argandona et al. (1981). They found no hydroxamic acids in wheat seeds (*Triticum durum*). This fact is of particular interest regarding the breeding patterns of some heteromyid rodents. Certain desert-adapted kangaroo rats, primarily seed-eaters, include fresh vegetation in their diet when it is available after rainfall (Reynolds, 1960; Reichman and Van De Graaff, 1975). Breeding correlates with the availability of the fresh vegetation. In one species of kangaroo rat, *Dipodomys ordii*, 6-MBOA causes uterine hypertrophy (Rowsemitt, 1984). If 6-MBOA is absent from the kangaroo rats' usual diet but is present in the fresh vegetation ingested after rainfall, it may provide a cue for breeding in these animals.

The exact results of this work are not expected to be quantitatively comparable to the values found in field populations of various grasses. The light intensity used was lower than full sunlight. Also the 6-MBOA content of different species of crop grasses, as well as our own unpublished work, show that interspecific differences are substantial. Argandona et al. (1980) demonstrated that hydroxamic acids are present in wheat and rye, but nondetectable in barley. Therefore, not all grasses contain these compounds. The main purpose here has been to examine the range of phenotypic plasticity of this parameter in one

system. The results presented here suggest that 6-MBOA concentrations may be higher per gram of plant tissue in grasses sprouting in the colder temperatures and shorter photoperiods of early spring than in those sprouting later in the season when temperatures are warmer and photoperiods are longer. Therefore, the animals may encounter higher concentrations of 6-MBOA in newly sprouted grass at the beginning of the breeding season than in grasses sprouting later. The dynamic aspects of 6-MBOA concentrations in plants may prove to be an important factor in breeding patterns of many herbivores.

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VARIATION OF SOME SECONDARY METABOLITES IN JUVENILE STAGES OF THREE PLANT SPECIES FROM TROPICAL RAIN FOREST[1]

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Abstract—Results from qualitative and quantitative evaluation of some chemical constituents, particularly phenols and volatile terpenes, from juvenile stages of three primary species belonging to the perennial rain forest are presented. The degree of infection and the time of the year were taken into account. TLC, GC, and simple chemical methods were used to estimate differences among components. Biological assays were conducted to evaluate fungicide potential of the extracts from studied plants. In general terms, the results show significant differences in the chemical composition of the species studied (*Nectandra ambigens, Omphalea oleifera*, and *Licaria alata*) related to the time of the year and the degree of infection. These differences can be related as well to their intrinsic resistance to infection and ability to grow to the adult stage.

Key Words—Secondary metabolites, juvenile stages,. phenols, volatile terpenes, degree of infection, *Nectandra ambigens*, *Omphalea oleifera*, *Licaria alata*.

INTRODUCTION

Seedlings and juvenile stages of primary species from perennial rain forests are kept in latency during long periods of time due primarily to light conditions prevailing in the lower layers of the forest (Richards, 1959; Whitmore, 1975; del Amo, 1978). During this period of growth suppression, plants are subject to constant pressures of many types, and these are a consequence of the inter-

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action between biotic and abiotic factors. One of the main factors of stress during the first stages of growth is the attack of pathogenic microorganisms.

The importance of chemical mechanisms of defense developed by some plants against pathogenic microorganisms, insects, and other herbivores, through formation and accumulation of secondary metabolites, has been noted by Levin (1976), Whittaker and Feeny (1971), and Seigler (1978), among others. Of the metabolites, those most frequently related to pathogen resistance have been the phenolic compounds and the terpenes (Cobb et al., 1968; Hunter et al., 1978). As for the terpenes, Cobb et al. have suggested that some monoterpenes such as myrcene, phellandrene, limonene, pinene, and carene, produced by *Pinus ponderosa*, play an important part in its resistance to infection by several microorganisms; they also mentioned that their activity can be related to that of phenolic compounds. Monoterpenes and sesquiterpenes are found in the so-called essential oils.

The contents and production of essential oils may vary according to climate, soil conditions, stage of development of the plants, and genetic factors (Swain, 1963; Rice et al. 1978).

The main goal of the present work was to evaluate the qualitative and quantitative variation of chemical components, mainly terpenes and phenolic compounds, in juvenile stages of three primary species from the perennial rain forest, taking into account the degree of infection and the time of the year. At the same time, extracts of each plant were evaluated against phytopathogenic fungi isolated from the same species studied in order to evaluate their fungicidal potential.

METHODS AND MATERIALS

Juvenile stages of plants of three primary species from Ebitrolotu (station for tropical biology at Los Tuxtlas, Veracruz, Mexico, belonging to the National University of Mexico) were used: *Nectandra ambigens*, *Licaria alata* (Lauraceae), and *Omphalea oleifera* (Euphorbiaceae). These species are common in the Tuxtla region, and their seedlings and young plants form a dense mass in the understory of this tropical rain forest (del Amo, 1978; Cordova, 1980).

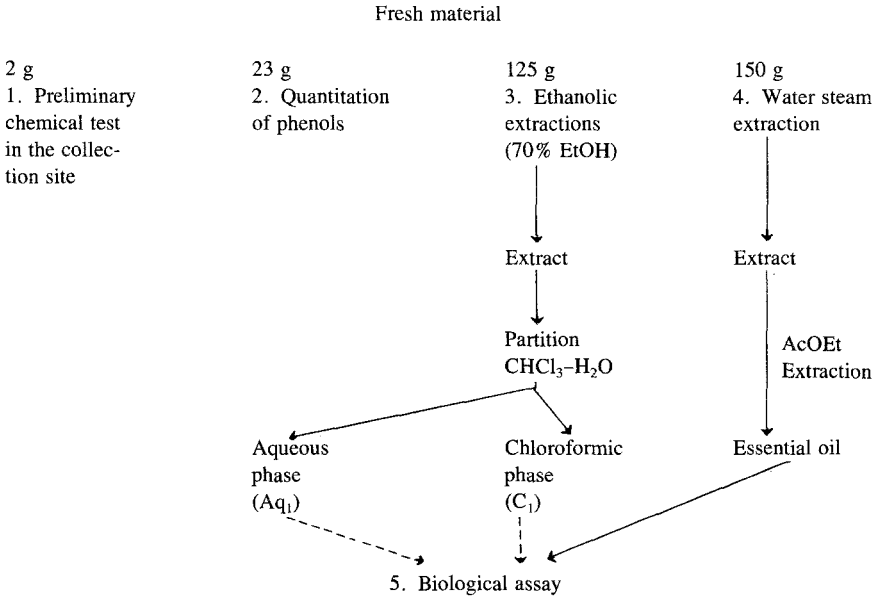
Samples were collected and classified as "damaged" and "healthy" plants, according to the degree of infection. There were no completely undamaged or healthy plants in the case of *Omphalea oleifera* specimens. Even so, the three species were classified in the same manner. It is important to note that the term "damaged" was given to plants with evident signs of infection or insect depredation.

Samples were collected twice during the year: during the dry season in March and the rainy season in July, with the purpose of detecting variations in secondary metabolites according to environmental conditions, specifically plu-

vial precipitation. The samples were processed as soon as possible in order to avoid metabolic transformation due to action of plant enzymes or microorganisms.

Laboratory Methods. Solvents were purified by distillation. Thin-layer chromatography (TLC) was done using silica gel GF 254-60 Merck and microcrystalline cellulose on flexible plates, "Bakerflex," and were made visible with iodine and/or anisaldehyde–H_2SO_4 followed by UV light.

Gas–liquid chromatography (GLC) was carried out in a Varian 3700 apparatus, using a 10% FFAP on Chromosorb WHP column. As an external standard, a mixture of pure terpenes selected according to chemotaxonomic criteria was used (Figure 1). Phenols were measured using a Varian 364 spectrophotometer. Fresh material, 300 g, was processed according to the Scheme 1.



SCHEME 1.

Preliminary Chemical Test (at Site of Collection). One gram of fresh material was triturated in a mortar, extracted with ca. 10 ml of EtOH and filtered; aliquots of this filtrate were used to search for phenols (reaction with ferric chloride), saponins (foam formation), alkaloids (reaction with Mayer, Dragendorff, Wagner, and phosphotungstic acid reagents); and essential oils (characteristic odor).

Evaluation of Phenols. Phenols were quantified by the Price and Butler (1977) method, but using 6 g of fresh material instead of the dry material used by these authors. The material was extracted with 60 ml of distilled water and the extracts filtered. An aliquot of 5 ml of filtrate was diluted to 50 ml with

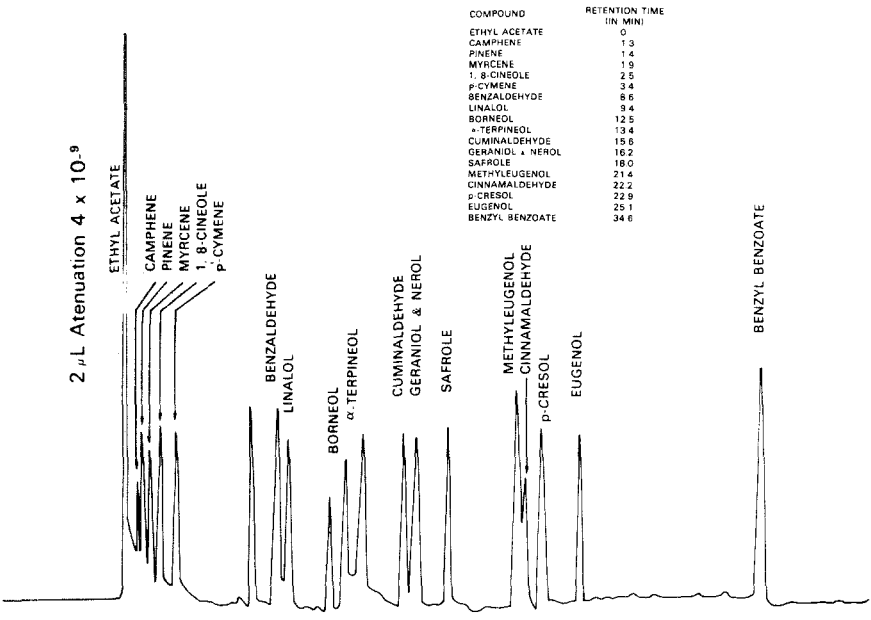


FIG. 1. Standards for GLC (retention time) of essential oils of juvenile plants with different degrees of infection.

water, 0.3 ml of ferric chloride solution was added, and the two were mixed. Afterwards 0.3 ml of potassium ferricyanide was added and the solution stirred. The mixture was allowed to stand for exactly 10 min and read at 720 nm using distilled water as a blank; it is important to read exactly at that time because afterwards absorbance varies and a precipitate may be formed. The test was done in triplicate using a standard of tannic acid, with a curve of concentrations from 1 to 6 μg/ml. Concentrations in micrograms of phenols (as tannic acid) per milliliter of extract are obtained and results are given in milligrams per gram of dry material and in percent of dry material.

Ethanol Extract. Fresh material (125 g) was extracted three times with 70% EtOH at room temperature for 24 h. Extracts were homogenized, filtered and concentrated in a rotary evaporator at a temperature below 60°. The oily aqueous residue was extracted with 100-ml portions of chloroform. Two fractions were obtained: chloroformic (C_1) and aqueous (Aq_1). The chloroformic fraction was evaporated to dryness to obtain a brown powder which was chromatographed by TLC using $CHCl_3$–MeOH (9 : 1) as eluent, made visible with anisaldehyde–H_2SO_4 in UV light. The aqueous phase was lyophilized and the resulting solid treated in the same way and also chromatographed on microcrystalline cellulose with butanol–acetic acid–water (8 : 2 : 3) as eluent.

Essential Oils. Fresh material (150 g) was water-steam extracted for 8 hr.

The suspended oils were further extracted with ethyl acetate and the latter eliminated by distillation. TLC of essential oils was carried out on silica gel using hexane–ethyl acetate (1 : 1) as eluent. As visualizing agents, iodine and anisaldehyde–sulfuric acid were used.

GLC was done under the following conditions: spiral glass column 200 cm in length, 2 mm OD and 0.85 mm ID, filled with 10% FFAP on Chromosorb WHP. Temperature programming: initial 80°C, final 220°C; velocity 4°/min; carrier gas, nitrogen; detector temperature, 250°; injector temperature, 250°.

A mixture of pure terpenes (Figure 1) was used as an external standard. When the retention time of some of the components of the essential oils coincided with one of those of pure compounds, the latter was used as the internal standard, measuring the increment of the corresponding area. Quantification of each component was carried out measuring the area under the curve by the triangle method.

Biological Assay. Pathogenic fungi were isolated from *L. alata* and *N. ambigens*. It was not possible to isolate fungi from *O. oleifera*. To isolate the microorganisms, pieces of infected leaves were scattered in a system of potato–dextrose–agar (PDA) and kept in a humid chamber (Sharvelle, 1969). Depression microscope slides were used, placing one drop of standard suspension of spores (50,000/ml) and one drop of the studied extract in five different dilutions: 25.5, 12.25, 6.12, 3.06, and 1.53 mg/ml.

Extracts of each plant were tested against pathogenic microorganisms isolated from the same plant, but in the case of *O. oleifera*, from which fungi could not be isolated, its extracts were tested against fungi isolated from the other two species of plants.

The extracts were dissolved in an acetone–water (1 : 10) system, adding 0.1% of PDA to facilitate germination. Only extracts available in sufficient amounts to make several dilutions were tested.

Preliminary tests of fungal tolerance to osmotic pressure were conducted, using mannitol in different concentrations: 0, 0.04, 0.08, 0.1, and 1 M.

Incubation time was 20 hr at 20°, after which the number of germinated and nongerminated spores in each scope of the microscope were counted using six scopes for every one of the three repetitions. Spores were considered as germinated whenever the length of the mycelium exceeded the spore diameter.

RESULTS

Preliminary Chemical Test

All the species studied contain, in different amounts, phenolic compounds and essential oils. There were no saponins present, and the amount of alkaloids was negligible. It is important to note that *O. oleifera* contains a lesser amount

of phenolic compounds than essential oils when compared to the other two species studied.

Quantification of Phenolic Compounds

The presence of phenolics is shown in Table 1, expressed as milligrams of tannic acid per gram of dried sample and in percent in dry material. The standard curve used showed a correlation of 0.999; and the standard deviation (SD) of measurements (each was done three times) was always less than 0.1. It can be noted that in the case of *L. alata* there was no significant difference in the phenolic content (rather small) between damaged and healthy plants. In the case of *O. oleifera*, and *N. ambigens*, there were appreciable differences between damaged and healthy populations, specially in the case of *O. oleifera*, where the amount of phenols was always greater in the healthy samples.

Ethanol Extract

Thin-layer chromatography (TLC) of the chloroformic (C_1) and aqueous (Aq_1) fractions of ethanol extracts showed a great number of components (an average of eight for *L. alata* and *O. oleifera* and of 15 for *N. ambigens*). Some of those of phenolic nature were made evident by the characteristic color of the spots after spraying with anisaldehyde–H_2SO_4.

No separation was intended, due to the small amounts of material available; however, it was discerned that the number of components was always greater in the damaged populations than in the healthy ones. For example (Table 2), the chloroform fraction (C_1) of *L. alata* extract shows the presence of three more components (R_f 0.4, 0.43, 0.55) in damaged plants. Similar results were

TABLE 1. QUANTITATIVE DIFFERENCES IN PHENOLIC CONTENT (PC) IN SEEDLINGS WITH DIFFERENT DEGREES OF INFECTION (EXPRESSED AS TANNIC ACID)

Sample	Humidity	PC (mg/g dry sample)	Dry weight (%)
Omphalea oleifera			
Healthy plants	82	0.388	0.038
Damaged plants	82	0.866	0.086
Licaria alata			
Healthy plants	68.6	0.216	0.0216
Damaged plants	68.6	0.207	0.0207
Nectandra ambigens			
Healthy plants	49.8	0.199	0.0199
Damaged plants	49.8	0.344	0.034

TABLE 2. R_f VALUES (TLC) OF ETHANOL EXTRACT FROM *Licaria alata*

Aqueous fraction (Aq_1)		Ethanol fraction (C_1)	
Healthy	Damaged	Healthy	Damaged
0.30	0.30	0.02	0.02
	0.38	0.1	0.1
0.46	0.46	0.24	0.24
0.58	0.58	0.35	0.35
0.64	0.64		0.40
0.74	0.74		0.43
	0.81	0.46	
0.94	0.94		0.55
		0.65	0.65
		0.83	0.83

found in the aqueous fractions (Aq_1) which, in the case of *N. ambigens*, differed in three components (R_f 0.4, 0.56, and 0.83); there was a difference of two in *L. alata* and of only one in *O. oleifera* which, as shown before, was the least damaged species.

Essential Oils

As in ethanol extracts, thin-layer chromatography (TLC) shows qualitative differences between populations from a single species differing in degree of infection; however, due to the nature of the compounds of this extract, the resolution that can be attained is not very good, and better results can be obtained by GLC. For example, the average number of components found by TLC is 9–10, whereas by GLC a minimum of 22 and a maximum of 48 (depending on the extract) is obtained.

Utilizing GLC, differences in composition of essential oils from the three species studied, in damaged and healthy populations and at two different times of the year, are shown in Figures 2 and 3. Main qualitative and quantitative differences in composition of extracts are in Tables 3 and 4. A short account on the average results, shown in Figures 2 and 3, follows.

O. oleifera

Differences Regarding Degree of Infection. In the first crop, there were no substantial differences, either between damaged and undamaged or healthy plants, or between components of their essential oils. The main component was identified as benzyl benzoate, the amount of this component being slightly greater in the healthy plants (Figure 2A).

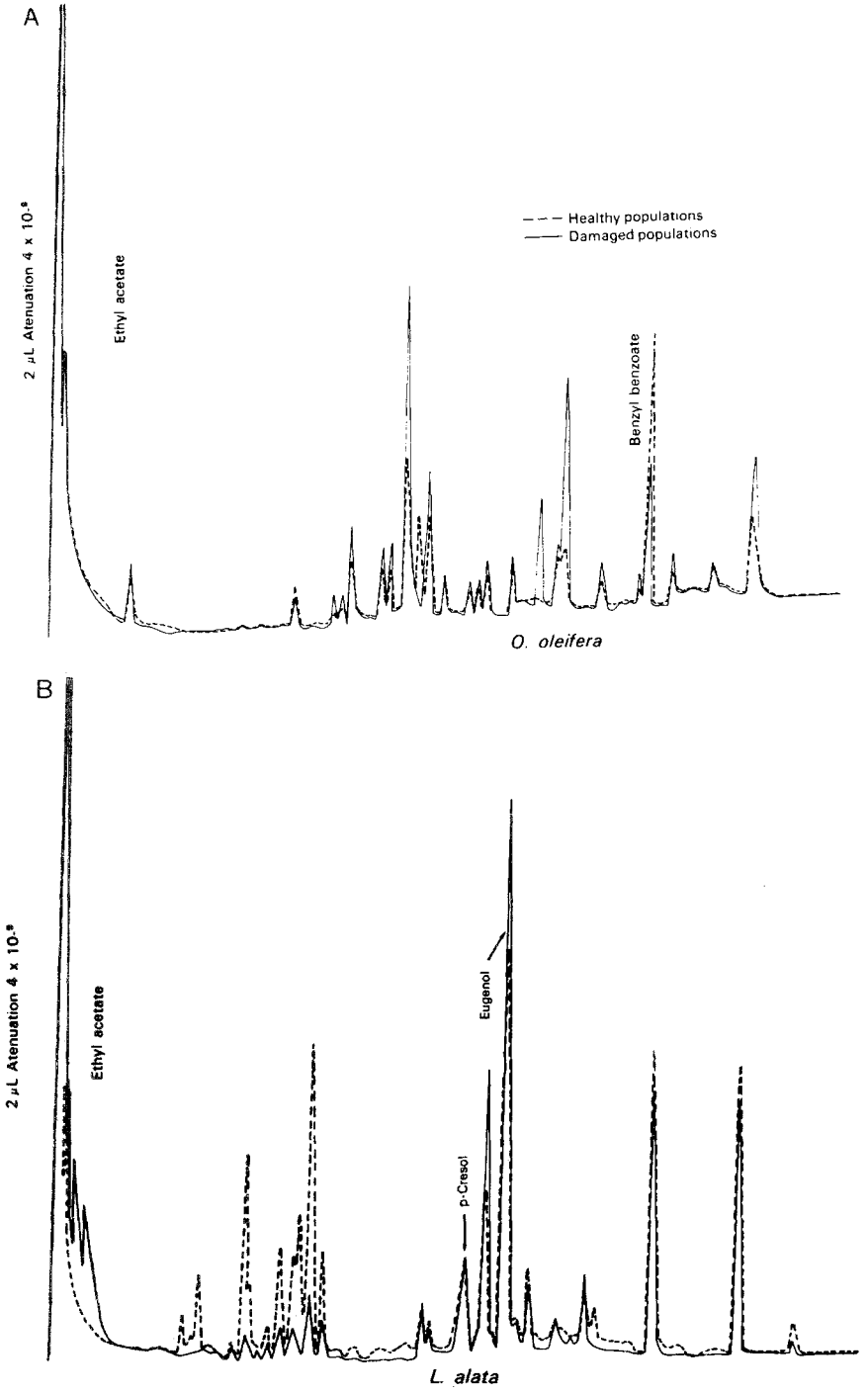


FIG. 2. Gas–liquid chromatography of the essential oils from *Omphalea oleifera* (A), *Licaria alata* (B), and *Nectandra ambigens* (C) in populations with different degrees of infection (first crop, dry season).

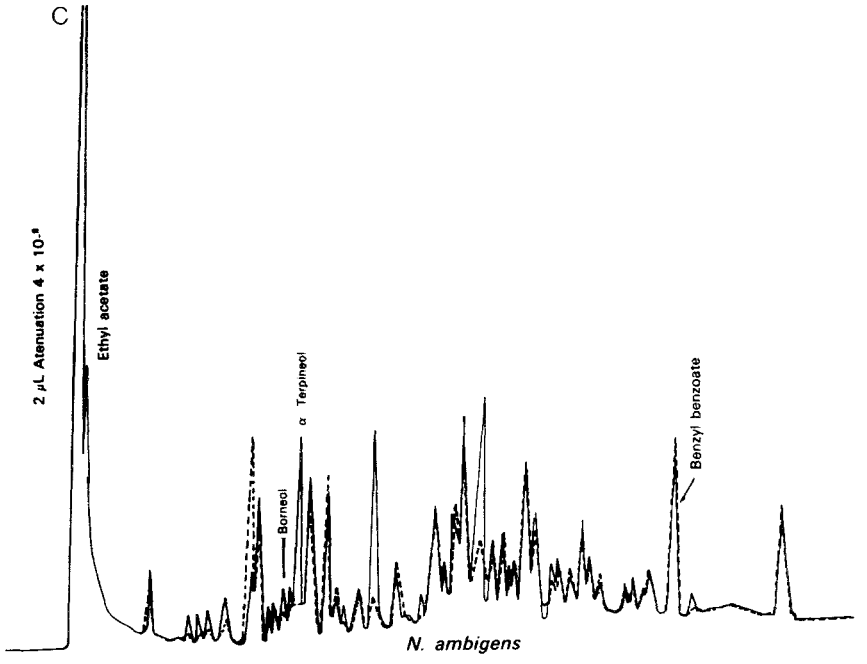


FIG. 2. (Continued)

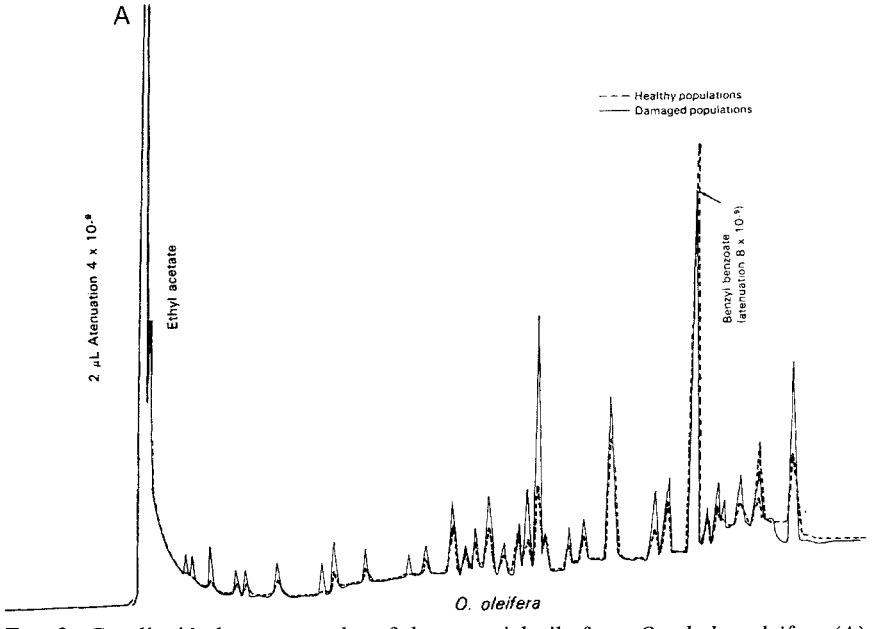


FIG. 3. Gas–liquid chromatography of the essential oils from *Omphalea oleifera* (A), *Licara alata* (B), and *Nectandra ambigens* (C) in populations with different degrees of infection (second crop, rainy season).

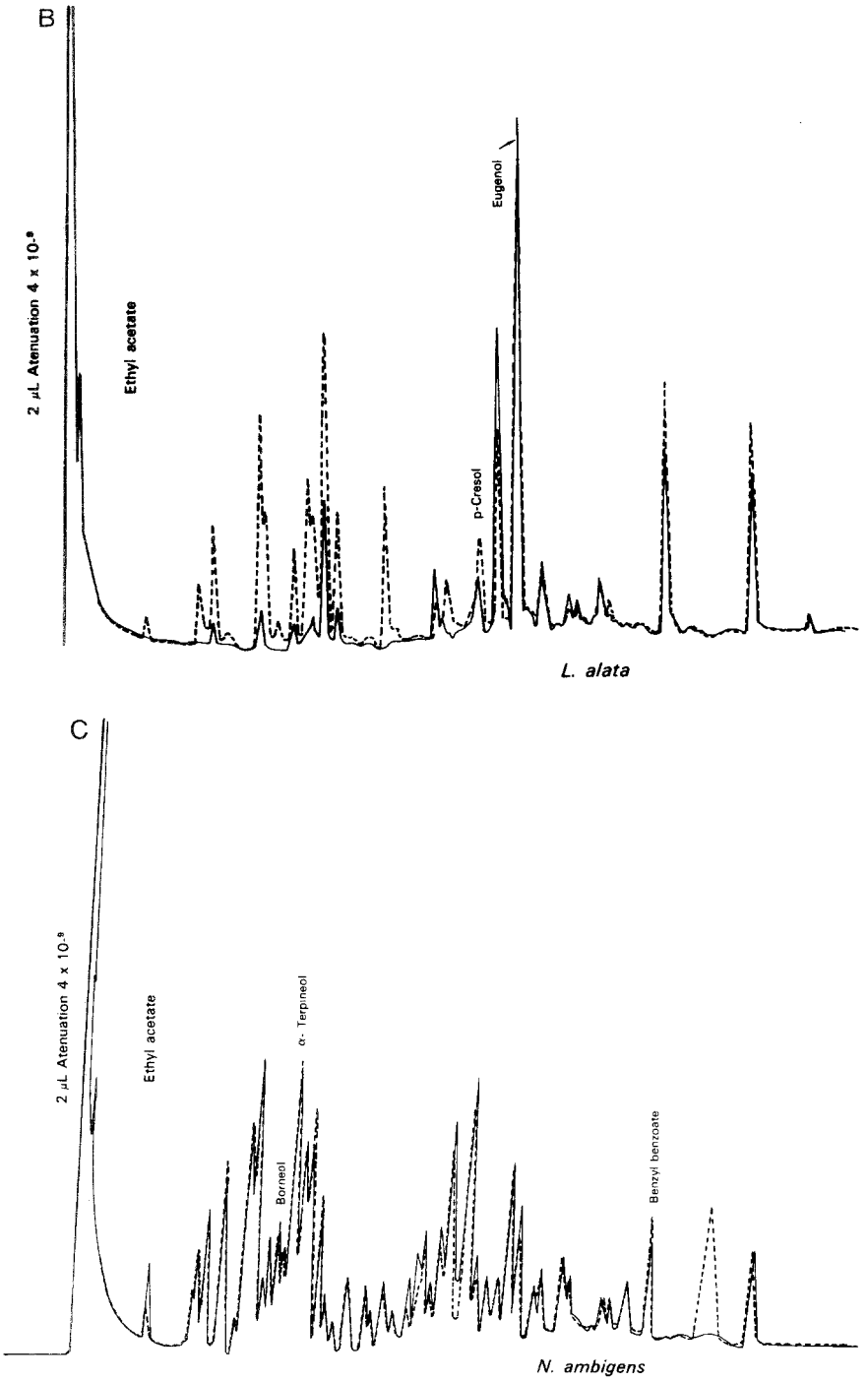


FIG. 3. (Continued)

TABLE 3. QUALITATIVE AND QUANTITATIVE DIFFERENCES IN COMPOSITION OF ESSENTIAL OILS FROM POPULATIONS WITH DIFFERENT DEGREES OF INFECTION (FIRST CROP, DRY SEASON)[a]

Plant	Degree of infection	Component (Retention time, min)	%	Differences in percent between populations (Only differences greater than 3%)
Omphalea oleifera	Damaged	24.8*	12.01	−8.52
		34.6* Benzyl benzoate	18.01	+4.21
		38.5	9.0	+6.23
		40.6	10.51	−5.44
	Healthy	24.8*	3.49	
		34.6* Benzyl benzoate	22.2	
		38.5*	15.2	
		40.6	5.0	
Licaria alata	Damaged	11.0	0.84	+4.87
		13.8	0	+5.66
		14.6*	2.72	+7.69
		14.0	14.71	−9.27
		25.1* Eugenol	31.36	−11.49
	Healthy	11.0	5.71	
		13.8	5.66	
		14.6	10.41	
		24.0	5.44	
		25.1 Eugenol	19.87	
Nectandra ambigens	Damaged	10.6*	1.53	+3.1
		13.4 Terpineol	6.44	−3.09
		17.4*	5.53	−4.72
		22.6	7.88	−3.47
		23.8	8.65	−5.8
	Healthy	10.6*	4.63	
		13.4 Terpineol	3.55	
		17.4	0.81	

[a] (+) Indicates greater amount in healthy populations; (−) indicates lesser amount in healthy populations; (*) component varying also in accordance to crops; (__) component present in both crops.

TABLE 4. QUALITATIVE AND QUANTITATIVE DIFFERENCES IN COMPOSITION OF ESSENTIAL OILS FROM POPULATIONS WITH DIFFERENT DEGREES OF INFECTION (SECOND CROP, RAINY SEASON)[a]

Plant	Degree of infection	Component (retention time, min)	%	Differences in percent between populations (Only differences greater than 3%)
Omphalea oleifera	Damaged	19.8	1.9	+9.7
		20.6*	24.2	−17.2
		21.2*	0	+8.0
		22.2*	8.8	−6.2
		28.3*	6.4	−6.4
		29.4*	16.9	−13.74
		34.6* Benzyl benzoate	5.4	+12.5
		40.6	12.2	−6.4
	Healthy	19.8*	11.6	
		20.6*	7	
		21.2*	8	
		22.0	2.6	
		28.3*	0	
		29.4*	3.2	
		34.6	18.9	
		40.6	5.8	
Licaria alata	Damaged	11.0	1.6	+5.5
		11.2	0	+4.2
		13.6*	0	+6.8
		17.6*	0	+4.0
		24.0	17.1	−10.9
		25.1 Eugenol	23.9	−8.8
	Healthy	11.0	7.0	
		11.2	4.2	
		13.6*	6.8	
		17.6*	4.0	
		24.0	6.2	
		25.1 Eugenol	15.1	
Nectandra ambigens	Damaged	38.2	0	+4.7
	healthy	38.2	4.7	

[a]Symbols as in Table 3.

In the second crop, there were slight differences between damaged and healthy plants; in the latter benzyl benzoate was augmented by ca. 12.5%, and there were two unidentified components (ret. R_t 20.6 and 29.4) that were diminished by 17.2% and 13.7% respectively (Figure 3A).

Differences between Crops. The groups of substances whose retention time indicates they belong to terpenes, such as safrol, cinnamaldehyde, and terpineol, were significantly augmented in extracts belonging to the second crop (rainy season); they were present in both healthy plants as well as in damaged ones.

L. alata

In the first crop, there were marked differences between damaged and healthy or undamaged populations. In the latter, the amount of substances with retention times similar to those of borneol and terpineol increased. Eugenol was identified as one of main constituents (31% in damaged plants and 19.9% in the healthy ones) (Figure 2B).

In the second crop, as happened in the first crop, terpenes found in the first part of the chromatogram were substantially augmented in the case of healthy plants; this fact might be related to plant resistance to disease (Figure 3B).

Differences between Crops. There were few differences in the composition of essential oils from the two crops. Among them, eugenol was found in smaller quantities in plants from the second crop (Tables 3 and 4).

N. ambigens

In the first crop, very slight differences between damaged and healthy populations were found. One of the appreciable differences was the presence of terpineol in the damaged plants and its absence in the healthy ones (Figure 2C).

In the second crop, there were almost no differences between components in the extracts of plants with different degrees of infection, with the important exception of one component which had a retention time (38.5) longer than that of benzoyl benzoate which exists only in undamaged plants (Figure 3C).

As this substance could be responsible for a protective effect in healthy plants, we found it interesting to undertake a further study of such a possibility. Isolation from the original extract was not possible because of the small amount available and because collection of substantial amounts of seedlings would have resulted in ecologic damage. For these reasons, the search of this component in the essential oil from leaves of mature plants was attempted. Unfortunately, composition of the essential oil was somehow different, and the compound in question was not present in the latter material.

Biological Assays

As indicated earlier, no pathogenic fungi were isolated from *O. oleifera*. Antifungal tests were done for species of *Phomopsis*, *Alternaria*, and *Colletotrichum* isolated from *L. alata* and *N. ambigens*. Partial results of these experiments were reported separately (Nava and del Amo, 1985; Nava et al., 1985). Only the inhibitory activity of essential oils studied is reported here; stimulatory activity, when found, was not taken into account.

Following is a brief account of the results obtained, (also shown in Figure 4):

Essential Oil from O. oleifera. Only the essential oil of undamaged plants during the rainy season was tested. This is not a highly inhibitory material for *Phomopsis* sp., isolated from *L. alata*, and for *Alternaria* sp. from *N. ambigens*; only higher concentrations were inhibitory. *Colletotrichum* sp. isolated from *N. ambigens* was more easily affected and was inhibited by the five dilutions used.

Essential Oil from L. alata. Among the three species studied, the essential oil from this plant showed the highest inhibitory activity against all the fungal

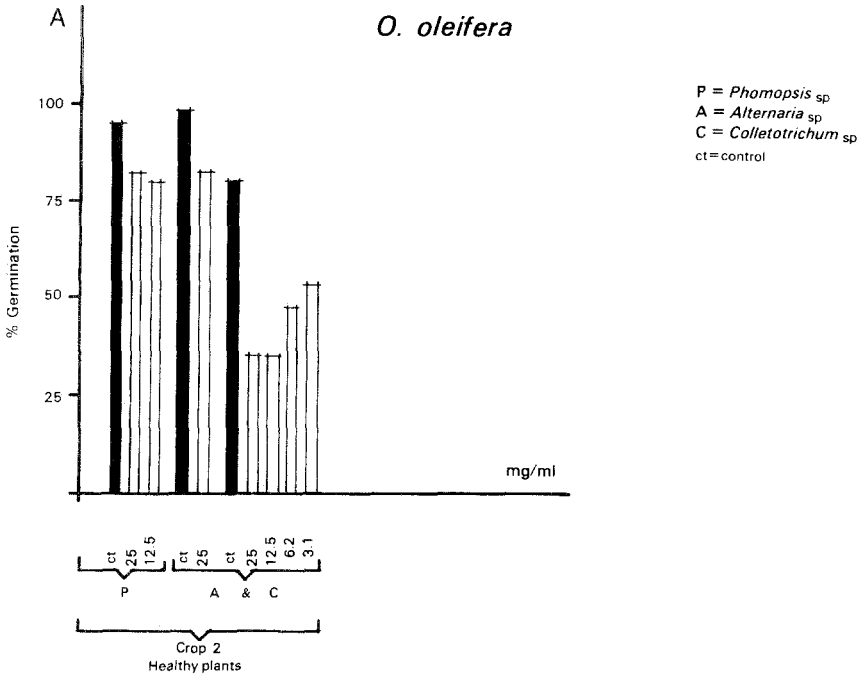
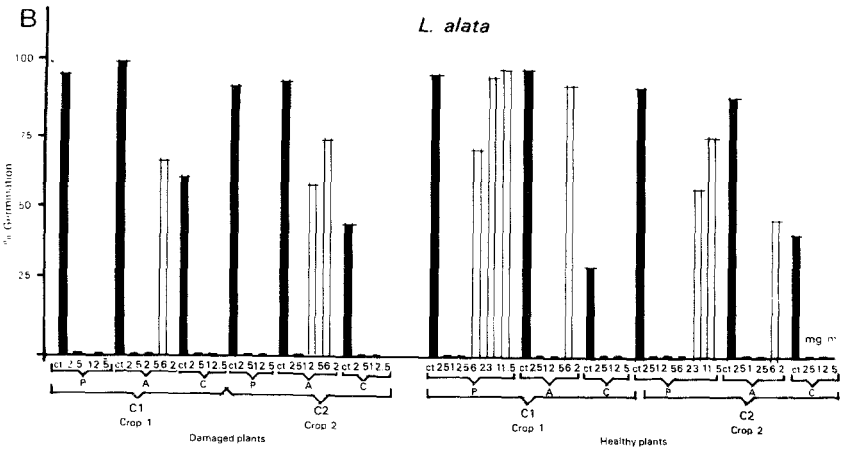


FIG. 4. Effect of the essential oils of *Omphalea oleifera* (A), *Licaria alata* (B), and *Nectandra ambigens* (C) on germination of fungal species from the genera *Phomopsis*, *Alternaria* and *Colletotrichum*.



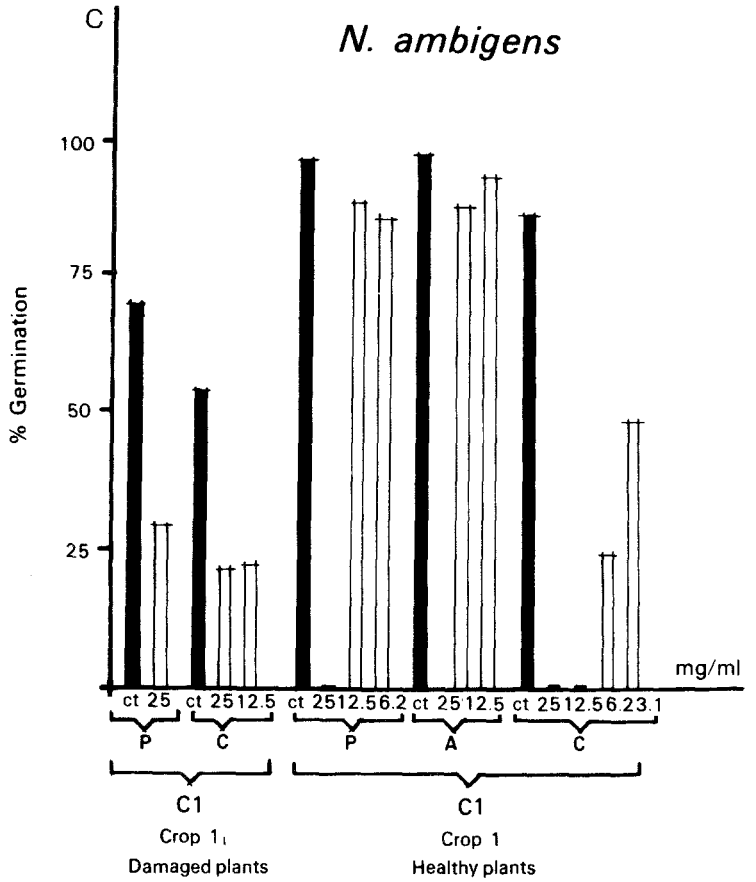


FIG. 4. (Continued)

species used. The minimum inhibitory doses were found to be among the lower concentrations of extract (Figure 4), for both types of populations, damaged and healthy. When higher concentrations were used, they completely inhibited the growth of fungi, with *Phomopsis* being the more affected species, especially by the extracts of healthy plants from the rainy season crop. This material has among its components eugenol and *p*-cresol, the latter being well known for its germicidal activity.

Essential Oil from N. ambigens. Only the extracts from the dry season (damaged and healthy) were tested. Essential oil from undamaged plants showed the highest inhibitory activity, especially against *Colletotrichum* sp. It is interesting to note that the latter differs only in one component from the corresponding extract of the damaged plants. Among its components, borneol, terpineol, and benzyl benzoate were identified.

DISCUSSION

In general terms, experimental results showed significant differences in chemical composition related to the time of year and degree of infection. These differences were also reflected in differences in biological activity against phytopathogenic fungi.

Qualitative and quantitative differences related to the degree of infection in plants corroborated the statement of Stoesl (1970) and Callow (1977). There are compounds that remain constant which would belong to the so-called constitutive components and others that are found present only in damaged populations, a fact that would place them within the groups of phytoalexins or induced metabolites. As evidence, we can note the greater amount of components present in extracts of damaged plants (Table 2).

Regarding the total evaluation of each of the plant species studied, the following can be said: In *O. oleifera* there are quantitative differences between crops, but there are no significant differences related to the degree of infection. The essential oil belonging to healthy plants from the rainy season showed a marked inhibitory effect. It is important to note that quantitative determination of phenolic compounds showed this species to be the one with the greatest amount of these compounds. This could be related to the greater resistance to infection that this species shows, given the known antifungal activity of phenols (Farkas and Kiraly, 1962; Wakimoto and Yoshii, 1958; Piankas and Chorin, 1969).

In *L. alata* the differences in chemical composition due to seasonal effects were less evident than those due to degree of infection. This was reflected in the biological activity of the extracts tested. Inhibition produced by different concentrations of extracts from healthy plants was especially noticeable in the case of *Phomopsis* sp. (Figure 4). This activity could be related to some of the

compounds in the first part of the chromatogram (GLC) that increased in the undamaged populations.

From the other Lauraceae studied, in *N. ambigens* some differences were found, both between crops and between plants with different degrees of infection. The essential oil of this species is scarce, and only the material from the dry season crop was biologically tested. Interestingly, the material from the undamaged plants was more active, especially against *Colletotrichum* sp., and it differed only in one component (R_t 38.2) from the material from damaged populations. Thus, the indicated activity could be related to that single component. Unfortunately, its isolation and identification, although attempted, have not been achieved to date.

In summary, of the plant species studied, the greatest fungicidal potential was found in *L. alata*. The fact that the genera of fungi used to test the plant extracts were some of the more important pathogens for cultivated plants makes it of great interest to extend the bioassays to some crop species.

Seedlings and juvenile stages which are important parts in the life cycle of the primary species are subject to suppression of growth and to attack by phytopathogenic microorganisms for extended periods of time. Thus, the development of chemical mechanisms of defense, such as the ones mentioned in the present study, represents an advantage in adaptation that will allow them to survive until adequate conditions make it possible for them to grow and reach adult stages.

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ULTRASTRUCTURE OF EXOCRINE PROTHORACIC GLAND OF *Datana ministra* (DRURY) (LEPIDOPTERA: NOTODONTIDAE) AND THE NATURE OF ITS SECRETION[1]

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Abstract—*Datana ministra* larvae possess a gland situated in the ventral anterior portion of the prothorax. The gland consists of a single sac and is only charged with secretion in the last instar. Its orifice, a simple slit, opens into a transverse invagination of the integument in the cervical margin of the prosternite. The components of the gland have been identified by GC and GCMS and have been shown to be dodecanol, dodecyl acetate, and dodecyl formate. The morphology of the gland and the chemistry of the secretion are discussed in relation to other notodontid larvae which have been investigated. It is conjectured that the secretion is not defensive in nature but may function as a dispersal pheromone.

Key Words—*Datana ministra*, Lepidoptera, Notodontidae, exocrine prothoracic gland, ultrastructure, dispersal pheromone, dodecanol, dodecyl acetate, dodecyl formate.

INTRODUCTION

That larvae of the family Notodontidae possess exocrine prothoracic glands at the cervical margin of the prosternite has been known for more than 200 years.

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The first report was probably that of De Geer (1750) who, working with a species referred to as "la chenile à double queue du Saule" (possibly *Cerura vinula*), accurately described the gland and the irritating secretion produced and ejected when the caterpillar was disturbed. He described the secretion as possessing an "odeure très forte et pénétrante entièrement semblable à celle de liqueur que jettent les grands Fourmis de bois." Among the other early reports are those of Saunders (1881), Poulton (1887), Denham (1888), Packard (1895), and Latter (1897).

Poulton (1887) reported that species of the genus *Cerura*, when disturbed, emitted a fine spray which could be directed to the source of irritation and which was composed of 40% formic acid. Packard (1895) indicated that all *Cerura* species, *Macrurocampa marthesia*, and *Schizura concinna* possess such glands. Although the function of these glands is thought of principally for defense, Latter (1897) presented evidence that the formic acid, in the case of *Dicranura* (*Cerura*) *vinula* is also used for treating the silk of the cocoon, making it very hard, "waterproof and completely closed against aggressors." Studies by Saunders (1881) on *S. concinna* indicated that this species produced a secretion which was incorrectly identified as hydrochloric acid by Denham (1885). Detwiler (1922) noted both the range and direction of the spray, the location of the gland, and correctly identified the active principle of the secretion as formic acid, although he believed there to be other components.

This was confirmed by Weatherston et al. (1979) who showed that the gland consists of two sacs, the anterior and posterior glands joined by an interglandular neck. Both glandular sacs contained formic acid while the anterior sac also contained four minor components, three of which were identified as decyl acetate, dodecyl acetate, and 2-tridecanone, occurring in the ratio 92:9:2, and accounting for about 3.5% of the secretion.

A prothoracic gland complex very similar to that reported for *S. concinna* (Weatherston et al., 1979) was previously described by Eisner et al. (1972) for *Heterocampa manteo*. In this species, the gland contents were identified as formic acid (20–37%) with 2-undecanone and 2-tridecanone in the ratio of 3:1, and accounting for 1.4% of the secretion. *C. vinula* and *Notodonta anceps* have been shown to produce 40% formic acid (Hintze, 1969), the former species also containing DL-threonine, DL-aspartic acid, and L-lysine (Schildknecht and Schmidt, 1963). *Schizura leptinoides* secretes formic acid with no minor ancillary components (Eisner et al., 1961). According to Pavan and Valcurone-Dazzini (1976), nine species of notodontid larvae have been shown to secrete formic acid.

Within the genus *Datana*, Detwiler (1922) found that *D. ministra*, *D. contracta*, *D. intergerrima*, and *D. angusii* all "possessed glands somewhat similar to that of *S. concinna* but evidently very much simpler in structure." He also reported that the gland contents of *D. intergerrima* were acidic. In the present

study, the morphology and ultrastructure of the prothoracic gland of *D. ministra* are described and components of the secretion identified.

METHODS AND MATERIALS

The insects used in this study were collected from service-berry (*Amelanchier saguinea*) as larvae at Big Basswood Lake, Thessalon, Ontario, and maintained on this host in the laboratory.

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

For ultrastructural studies, insects were injected with cold 5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.3, containing 2% sucrose and 0.01 M $CaCl_2$. The glands were dissected out of the insects, and placed in fresh glutaraldehyde. A small slit was made in each sac to allow proper fixation and infiltration of the embedding medium. Further processing of tissue was carried out as previously described (Percy and MacDonald, 1979).

For chemical studies insects were killed by freezing, decapitated while frozen, and the glands dissected from the thoracic cavity. The glands were stored at room temperature in N sodium hydroxide or methylene chloride until analyzed.

Gas–liquid chromatography (GLC) analyses were carried out on a Hewlett-Packard 5890 gas chromatograph fitted with flame ionization detectors, a 15-m × 0.25-mm fused silica column coated with Carbowax 20 M, and interfaced to a Hewlett-Packard 3392A integrator; and a Hewlett-Packard 5792 instrument fitted with flame ionization detectors, a 11.5-m × 0.20-mm fused silica column coated with methyl silcone, and interfaced to a Hewlett-Packard 3390A integrator.

The temperature program used in both cases was an initial temperature of 60°C (1 min) rising at 30°/min to 120°C, then at 10°/min to a final temperature of 210°C held for 4 min. With the Carbowax 20 M column, the operating conditions were: injector temperature 160°C, detector temperature 250°C, and a carrier gas (H_2) flow of 30 cm/sec. The operating conditions for the methyl silcone column were: injector temperature 240°C, detector temperature 310°C, and a carrier gas (H_2) flow of 42 cm/sec.

Gas chromatography–mass spectrometry (GC-MS) was carried out on a Kratos MS80 system operated at 70 eV and fitted with a 30-m × 0.32-mm fused silica column coated with DB-1 and programmed from 60°C (3 min) at 20°/min to 140°C, then at 10°/min to 250°C (3 min), then to 270° at 20°/min.

Decyl, lauryl, myristyl, palmityl, and stearyl acetates were obtained from Sigma Chemical Co., St. Louis, Missouri, and 2,4′-dibromoacetophenone from Aldrich Chemical Co. Inc., Milwaukee, Wisconsin. Dodecanol was prepared

by base methanolysis of lauryl acetate, and dodecyl formate by formylation of dodecanol according to standard procedures.

RESULTS

Ultrastructural Studies. When disturbed, *D. ministra* adopts a position typical of notodontid larvae with both the thorax and posterior abdominal segments elevated (Figure 1A). Despite this apparently overt defensive behavior, all attempts to induce the larvae to spray or exude the contents of the exocrine prothoracic gland were unsuccessful.

The gland is located in the ventral anterior portion of the prothorax and consists of a single sac. The gland is small, about 1.7 mm in length in the last instar, and is situated entirely beneath the longitudinal and oblique segmental muscles of the integument. A short duct leads to the orifice located within a transverse invagination in the cervical margin of the prosternite (Figure 1B).

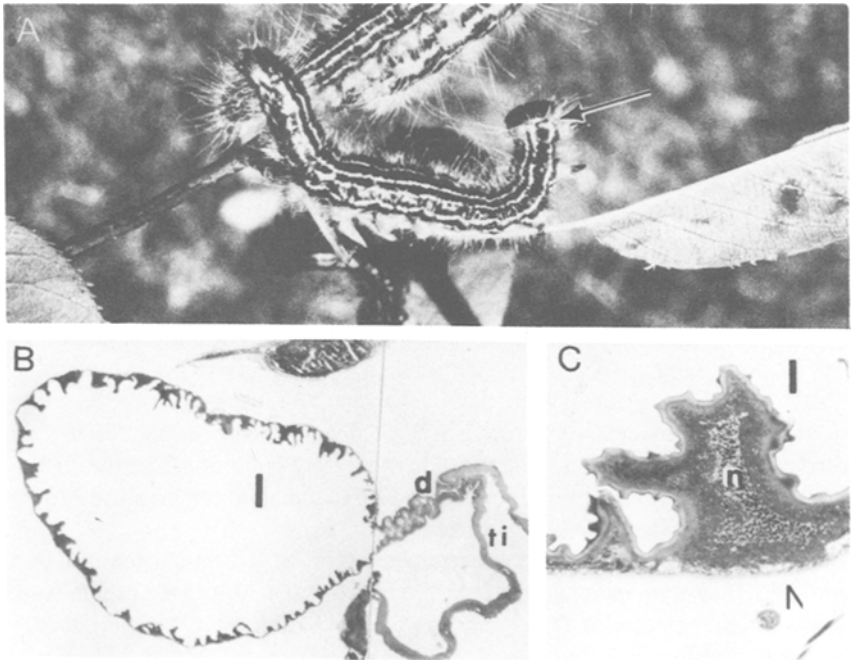

FIG. 1. (A) Late instar *Datana ministra* (Drury) larva in typical warning stance. Arrow indicates the position of the gland. (B) Sagittal section through the prothoracic gland; 38×. Lumen, l; duct, d; transverse invagination, ti. (C) Light micrograph showing irregular shape of gland cell; 720×. Lumen, l, nucleus, n.

The orifice is a simple slit and does not possess the complexity of cuticular structure or musculature seen in *S. concinna* (Weatherston et al., 1979).

The duct between the gland and the orifice has relatively thick cuticle (Figure 1B) and has a corrugated appearance due to annular folds in the cuticular lining. The cuticle is untanned and composed of a thin layer of dense epicuticle and several distinct lamellae of endocuticle. Unlike the analogous structure in *S. concinna*, no mesocuticle is present (Percy and MacDonald, 1979).

The gland lumen is lined with untanned cuticle presenting a surface that is highly convoluted and ridged to accommodate the large irregularly shaped cells (Figure 1C). A thin layer of dense epicuticle overlies an area containing numerous filaments and several lamallae of endocuticle (Figure 2A).

An outstanding feature of the underlying cells is the highly complex configuration of the apical plasma membrane, which is both everted within the cuticle as microvilli and inverted within the cells as folds. At the apices of the folds, there are also finger-like extensions of the plasma membrane which project even further into the cell (Figure 3).

Within the cytoplasm, numerous membranous tubules run parallel to the long axes of the microvilli, folds, and finger-like extensions. Within microvilli, the tubules extend freely in the cytoplasm (Figure 4A). Between the folds, the tubules are sometimes intimately associated with the apical plasma membrane (Figure 4B), and sometimes free within the cytoplasm (Figure 4A). Surrounding the finger-like extensions (fe in Figure 3), are tubules which, when in close association with the plasma membrane and when in cross-section, often present a striking rosette configuration (Figure 4B).

The large, obviously polyploid lobed nuclei, are surrounded by numerous active Golgi complexes and arrays of rough endoplasmic reticulum (Figure 2B). Some smooth tubular endoplasmic reticulum is also present. Numerous mitochondria are found, particularly below the cuticular extensions. Shortly after moulting, glycogen deposits appear peripheral to the nuclei, but quickly disappear. Lipid deposits are rarely present and, although microbodies occur, they are not numerous. Microtubules are observed throughout the cell.

The thick basement membrane is penetrated by numerous tracheoles (Figure 2A) which extend past the area of basal involutions and branch throughout the cells.

Chemical Studies. Absence of significant amounts of formic acid in the insect glands was evidenced by the failure to prepare the *p*-bromophenacyl ester from glands stored in sodium hydroxide by either the methods described by Weatherston et al. (1979) or Durst et al. (1975). The ester could be easily prepared from authentic formic acid. On several occasions the GLC trace of the product derived from insect material contained a trace peak at the retention time of the ester.

Analysis of the gland contents from two glands which had been stored in methylene chloride, by GLC on methyl silicone and Carbowax 20 M revealed

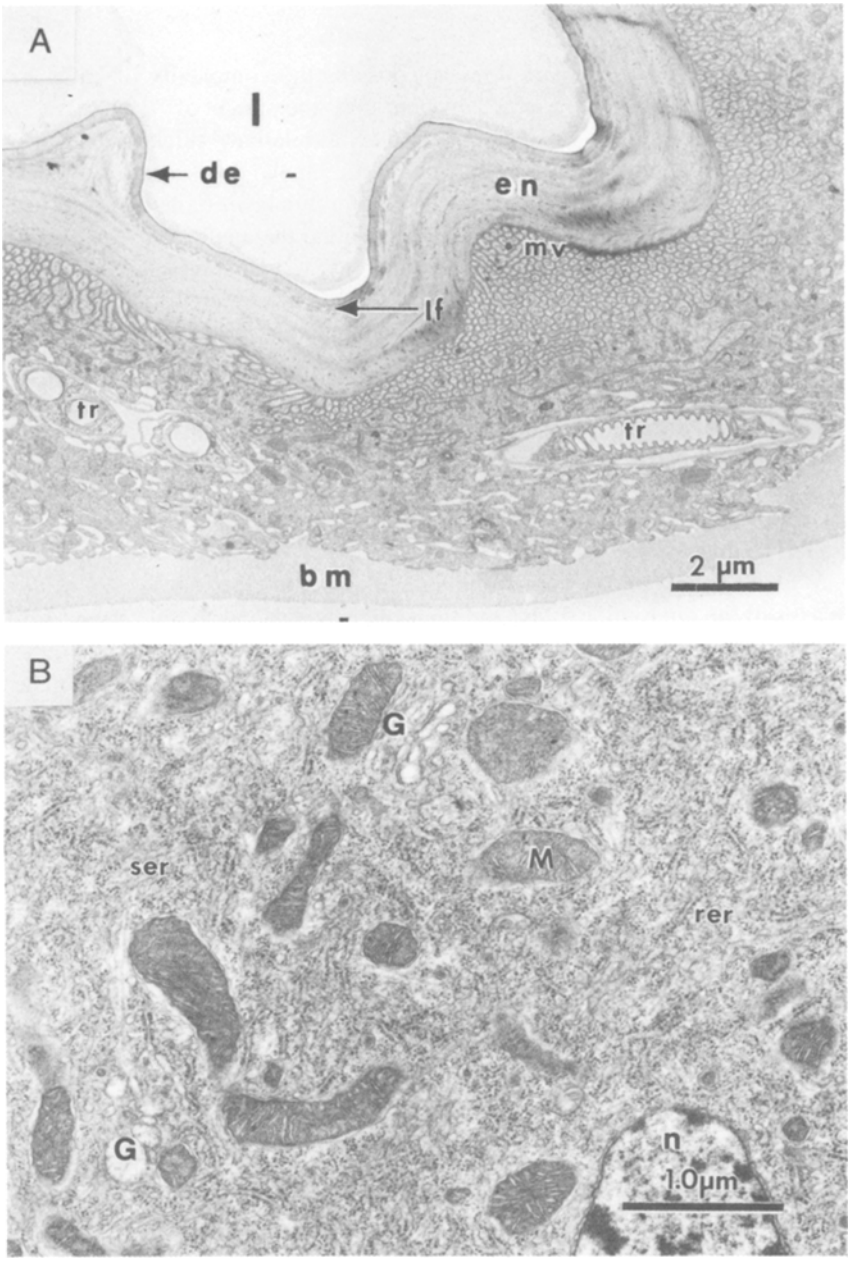

FIG. 2. (A) Low magnification of peripheral area of gland cell. 7000×. Lumen, l, dense epicuticle, de, layer of filaments, lf, endocuticle, en, microvilli, mv, tracheole, tr, basement membrane, bm. (B) Typical example of cytoplasmic organelles in a mature gland cell; 21000×. Golgi body, G; mitochondrion, M; smooth endoplasmic reticulum, ser; rough endoplasmic reticulum, rer; nucleus, n.

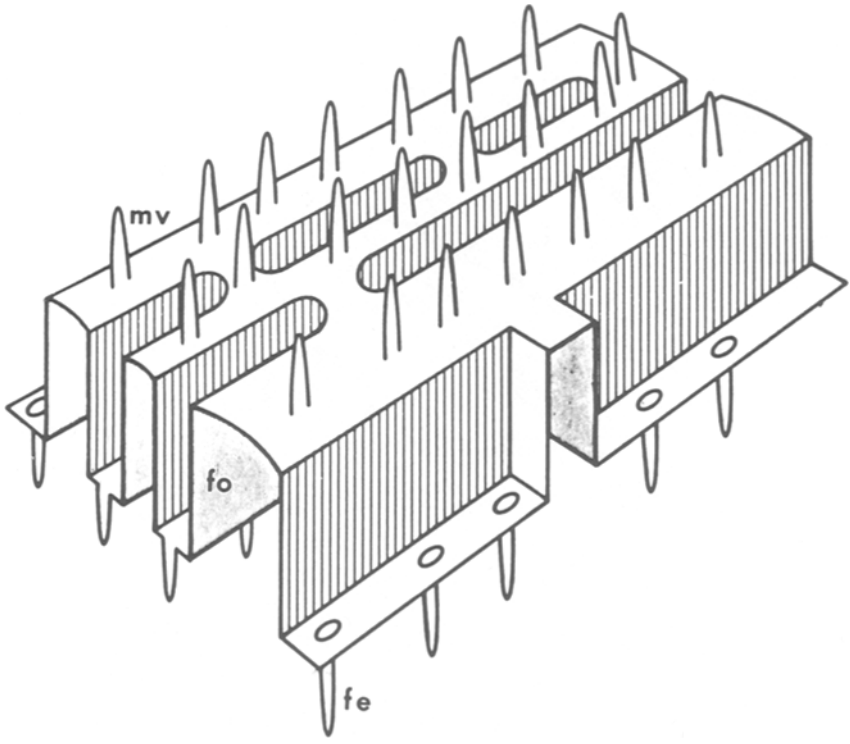

FIG. 3. Diagrammatic representation of apical plasma membrane of gland cells. The membrane is everted within the cuticle as microvilli (mv), and inverted within the cells as folds (fo). At apices of the folds are finger-like extensions (fe) of the plasma membrane which project farther into the cell.

three major components (Figure 5). Their retention times and the ratio in which they occur are given in Table 1.

Base methanolysis of the secretion removed components A and B, indicating that they could be esters. Since component C tailed significantly on methyl silicone, we surmised that it was probably an alcohol. Since acetates have been found in the secretion of *S. concinna* (Weatherston et al., 1979), an homologous series of even-numbered carbon acetates (C_{10}–C_{18}) was chromatographed under the same conditions, and the retention time of authentic dodecyl acetate shown to be 8.34 min and 8.06 min on Carbowax 20 M and methyl silicone, respectively. This suggested that component B was dodecyl acetate.

Dodecanol obtained by base methanolysis of dodecyl acetate was similarly chromatographed, giving retention times of 9.07 min on Carbowax 20 M, and 6.61 min on methyl silicone, indicating that compound C might be dodecanol.

Since base methanolysis of the secretion yielded only one neutral product

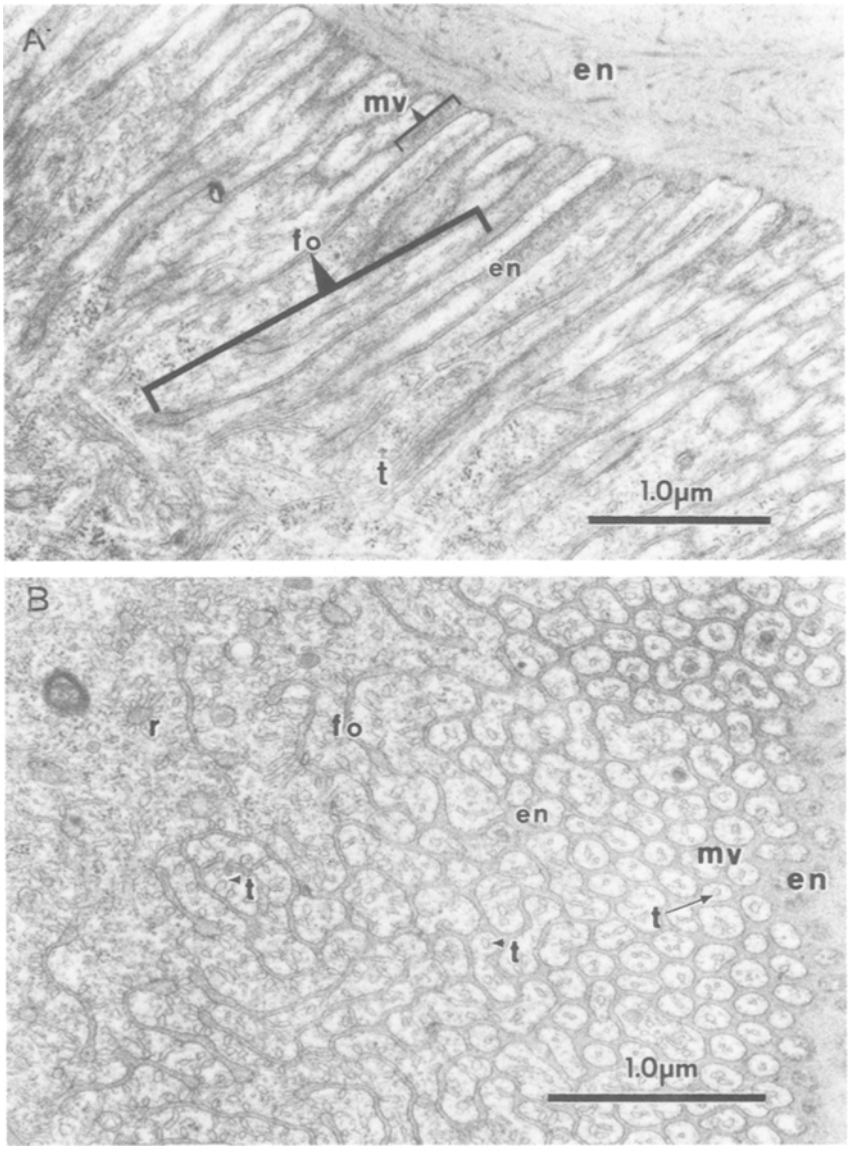

FIG. 4. (A) Longitudinal section through the apex of a gland cell showing microvilli, folds, and tubules free within the cytoplasm; 24000×. Endocuticle, en; microvilli, mv; folds, fo; tubules, t. (B) Cross-section through the convex apex of a gland cell showing microvilli at right, folds and rosettes towards the left; 28,500×. Endocuticle, en; microvilli, mv; rosette, r; tubules, t.

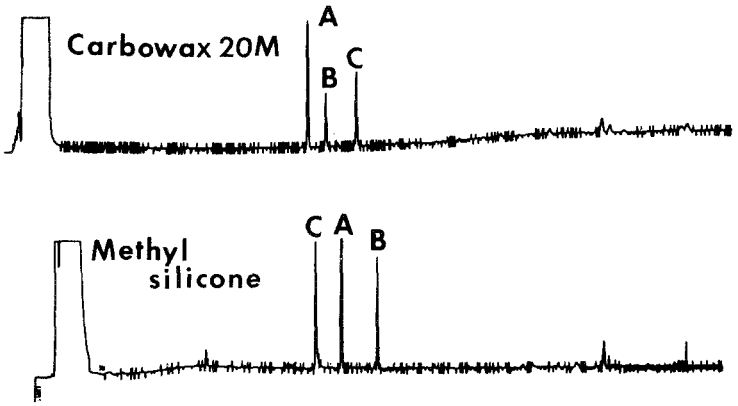

FIG. 5. GLC traces of the gland contents on Carbowax 20 M (upper) and on methyl silicone (lower).

whose retention time on Carbowax 20 M was 9.09 min, it appeared that compound A was also an ester of dodecanol. This, coupled with the fact that we had only been able to detect an extremely small trace of formic acid as described above, led us to deduce that component A might be dodecyl formate. GLC analysis of authentic dodecyl formate gave retention times of 7.97 min and 7.19 min on Carbowax 20 M and methyl silicone, respectively. The tentative assignments of dodecyl formate, dodecyl acetate, and dodecanol to components A, B, and C, respectively, were confirmed by GC-MS.

DISCUSSION

The prothoracic gland systems of the notodontid larvae examined to date possess either one or two sacs (Table 2). From Table 2 it can be seen that this morphological characteristic is not reflected in the nature of the secretion. Al-

TABLE 1. RETENTION TIMES AND RATIO OF GLAND COMPONENTS OF *D. ministra* CHROMATOGRAPHED ON CARBOWAX 20 M AND METHYL SILICONE

	Carbowax 20 M		Methyl silicone	
	R_t (min)	Ratio	R_t (min)	Ratio
Component A	7.92	2.31	7.21	2.33
Component B	8.35	1.00	8.05	1.00
Component C	9.07	1.46	6.59	1.13
Dodecyl formate	7.97		7.19	
Dodecyl acetate	8.34		8.06	
Dodecanol	9.07		6.61	

TABLE 2. MORPHOLOGY AND CONTENTS OF PROTHORACIC GLANDS IN NOTODONTID LARVAE

Species	Morphology	Secretion	References
Datana ministra	one sac	lipophilics	Detwiler, 1922, this paper.
D. angusii	one sac	?	Detwiler, 1922
D. contracta	one sac	?	Detwiler, 1922
D. integerrima	one sac	acidic	Detwiler, 1922
Notodonta anceps	one sac	formic acid	Hintze, 1969
Cerura vinula	one sac	formic acid + amino acids	Hintze, 1969; Schildknecht and Schmidt, 1963
Litodonta hydromeli	two sacs	?	Percy, unpubl.
Macrurocampa marthesia	one sac	formic acid	Packard, 1895; Poulton, 1887
Heterocampa guttivitta	one sac	formic acid + ?	Percy, unpubl.
H. manteo	two sacs	formic acid + lipophilics	Eisner et al., 1972
Schizura concinna	two sacs	formic acid + lipophilics	Weatherston et al., 1979; Percy and MacDonald, 1979
S. unicornis	two sacs	formic acid	Detwiler, 1922; Percy, unpubl.
S. leptinoides	two sacs	20% formic acid, no additives	Eisner et al., 1961; Percy, unpubl.

though we can only report qualitative analytical data for the gland contents of *D. ministra*, the amounts of materials isolated were much less than from *S. concinna*. This is most probably related to the gland size, which in *D. ministra* is very small (1.7 mm in length) so that it is situated entirely beneath the longitudinal and oblique segmental muscles of the prothorax. By contrast, the gland of *S. concinna* (~ 6 mm in length) extends obliquely to the right posterior dorsal margin of the mesothorax (Weatherston et al., 1979); yet final instar larvae of both species are about the same size.

All the notodontids whose gland ultrastructure have been examined in this laboratory, *Litodonta hydromeli*, *S. unicornis*, *S. concinna*, *S. leptinoides*, and *Heterocampa guttivitta* (Percy and MacDonald, unpublished) possess finger-like inversions of the apical membrane surrounded by membraneous tubules parallel to the long axes of the extensions, and which give a rosette configuration in cross-section. Similar-appearing structures have been observed in midgut cells of the aphid *Hyperomyzus lactucae* (O'Loughlin and Chambers, 1972) and in the wax-producing epidermal gland of a scale insect (Waku and Foldi, 1984). The apical membrane configuration of the *D. ministra* gland cells which possess true microvilli is more complex than those of the cells of insect pheromone-

producing glands and arthropod defensive glands which, in general, are merely highly convoluted (Percy and Weatherston, 1974).

All notodontid larvae examined to date in relation to their chemistry have been shown to produce formic acid (Table 2) (Pavan and Valcurone-Dazzini, 1976). Although Detwiler (1922) reported that the gland contents of *D. integerrima* were acidic, *D. ministra* is the first *Datana* species to have the gland contents analyzed. Our analyses indicate that the glands do not contain formic acid but only a mixture of dodecanol and the two corresponding formate and acetate esters. This is the first report of an alkyl formate in an exocrine secretion of a lepidopteran insect, although synthetic formates have been shown to act as sex attractants, synergists, inhibitors, and disruptants for several lepidopteran species (Henneberry et al., 1981).

No tests were carried out which would have given an insight as to the function of the secretion. Analogy to other notodontid species would seem to indicate that the materials were produced for defense; e.g., Eisner et al. (1961) reported that the lipophilic components isolated from *H. manteo*, although acting as spreading agents and aids to cuticle penetration of the formic acid, also have a defensive function themselves. The following indirect and fragmentary evidence leads us to the conclusion that the secretion from the exocrine prothoracic gland of *D. ministra* does not function as a defensive mechanism: (1) Formic acid is absent in the gland. (2) The glands, although present from the second through the fifth instar, are only replete in the last instar; this differs from *S. concinna* in which the glands from the second instar onwards are charged (Percy and MacDonald, unpublished). (3) On provocation, *D. ministra* adopt the warning posture (Figure 1A); however, even on extreme provocation do not spray, but regurgitate their gut contents. This is in contrast to *S. concinna* which, in common with other notodontids of the genera *Cerura* and *Macrurocampa*, rear up and aim their spray towards the source of the annoyance (Weatherston et al., 1979; Herrick and Detwiler, 1919; Poulton, 1887; Packard, 1895). (4) The difference in the complexity between the gland orifice of *D. ministra* (a single slit) and that of *S. concinna* (containing many folds, spikes, and cuticular projections) would indicate that only the latter possesses a mechanism for ejecting the secretion atomized into a fine spray.

The inability of the insects to spray would not contradict the possibility that the secretion functions as an alarm pheromone. However, there was no evidence in the laboratory of dispersal within a colony when an individual was provoked.

D. ministra are gregarious feeders until during the last instar, at which time they begin to disperse, and since it is only during this instar that the glands are charged with secretion, it can be conjectured that the secretion may act as a "dispersal pheromone."

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IDENTIFICATION OF THE SEX ATTRACTANT PHEROMONE OF THE SOUTHWESTERN CORN BORER *Diatraea grandiosella* DYAR[1,2]

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Abstract—We report the identification of the southwestern corn borer, *Diatraea grandiosella* Dyar (Lepidoptera: Pyralidae), female sex attractant pheromone as a mixture of (Z)-9-hexadecenal, (Z)-11-hexadecenal, and (Z)-13-octadecenal in the ratio 21.5:70.6:7.9. Initially, six 16- and 18-carbon aldehydes including *n*-hexadecanal, (Z)-9-octadecenal, (Z)-11-octadecenal, and the three above were isolated from female gland rinses and evaluated as potential pheromone components by GLC-MS and laboratory bioassays. By laboratory flight chamber and field tests, the stated mixture of (Z)-9-hexadecenal, (Z)-11-hexadecenal, and (Z)-13-octadecenal was shown to be as effective as the female for male attraction. Electrophysiological studies confirmed the requirement for these three compounds, but not for *n*-hexadecanal, (Z)-9-octadecenal, and (Z)-11-octadecenal.

Key Words—Southwestern corn borer pheromone, *Diatraea grandiosella* Dyar, Lepidoptera, Pyralidae, (Z)-9-hexadecenal, (Z)-11-hexadecenal, (Z)-13-octadecenal, *Zea mays* L.

INTRODUCTION

The southwestern corn borer, *Diatraea grandiosella* Dyar is an important pest of corn in the southwestern and southeastern United States and in Mexico. It is

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

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one of the major insect pests of irrigated corn in certain areas of Kansas, Oklahoma, Texas, and New Mexico. In these areas, the insect regularly causes heavy yield losses if not controlled by insecticides. For insecticides to be effective, they must be applied when eggs and young larvae are present on the plant. Once larvae enter secluded areas on the plant, such as in ears and stalks, insecticide effectiveness is greatly induced. Thus, timing of insecticide applications is critical and relies on the ability to pinpoint the presence of eggs and young larvae on the plant.

In recent years, pheromones have been used more and more as monitoring tools for determining adult insect flights. The presence of a sex attractant pheromone produced by southwestern corn borer (SWCB) females was indicated by field tests (Davis and Henderson, 1967; Langille and Keaster, 1973). The identification of the sex attractant pheromone of the SWCB and its subsequent commercial availability could have considerable value for monitoring adult populations of this pest and for potential behavioral manipulations within an integrated pest management system.

We report the identification of the female sex attractant pheromone of *D. grandiosella* through chemical, laboratory flight chamber, electrophysiological, and field behavioral experiments.

METHODS AND MATERIALS

Insects. Adult *D. grandiosella* used in chemical analyses, electrophysiological experiments, laboratory behavioral bioassays, and some field studies were obtained from a colony maintained on a wheat germ–casein diet (Davis, 1976) in the Crop Science Research Laboratory, Mississippi State, Mississippi, 39762.

Chemical Analyses. Initially, abdominal tips were excised from ca. 24-hr-old virgin females and ground in batches of ca. 1000 in pentane or hexane. The filtrate was concentrated and chromatographed on a 2 × 25-cm Biosil A® (silicic acid) column from which the active components were eluted with hexane-methylene chloride (50:50). Aliquots were employed for a series of functional group tests and were also subjected to ozonolysis (Beroza and Bierl, 1966).

An active fraction was also collected by preparative GLC of tip extracts from a 0.0032 × 1.5-m stainless-steel column packed with 5% Ap L® on 60/80 Gas Chrom Q, 200°C; flow = 30 ml/min. The active fraction was ozonized, cleaved with triphenylphosphine to aldehydes, and then analyzed by GLC-MS employing the previously described Ap L column.

Later, abdominal tips from calling females (ca. 2 hr into scotophase) were rinsed in heptane (5 μl/tip) for 1 min (Klun et al., 1980a,b) to minimize the occurrence of interfering compounds. After concentration under a N_2 stream, an aliquot equivalent to 5 tips/μl was analyzed on a Hewlett-Packard 5792A capillary GLC equipped with a DB-1 fused silica capillary column (60 m ×

0.253 mm ID, film thickness 1 μM, J&W Scientific Co., Cordova, California). The carrier gas was Helium, 40 cm/sec. The oven temperature was programmed from 70 to 250°C at 10°C/min (injection temperature = 200°C, detector temperature = 250°C). Rinses from ca. 2000 abdominal tips were concentrated and fractionated by isocratic high-performance liquid chromatography (HPLC) on a 10-cm Waters μResolve® silica column using 0.75% toluene in isooctane at a flow rate of 1.5 ml/min. Detection was accomplished with a Waters model 450® variable wavelength detector at 295 nm. Peaks were quantitated with a Hewlett Packard 3390A integrator. HPLC fractions were analyzed by GLC on the DB-1 column. Subsequently, a DB-1 (GLC) column (60 m × 0.322 μm) was interfaced to a Hewlett Packard 5985-B® quadrupole mass spectrometer through an open-split interface for acquisition of mass spectra. Aliquots equal to 50 female tips were generally adequate to obtain spectra of major components.

Identification of pheromone components was accomplished by comparing GLC retention volumes and mass spectra with standard compounds acquired commercially or prepared by synthesis. (*Z*)- and (*E*)-9-hexadecenal and (*Z*)- and (*E*)-11-octadecenal were synthesized by oxidation of the related unsaturated *Z*- and *E*-alkenols (Nu-Chek Prep, Inc., Elysian, Minnesota) using chromic anhydride in pyridine (Holum, 1961). Additionally, a number of aldehydes and acetates were acquired by purchase from Shin-Etsu Chemical Co., Ltd., Tokyo, Japan, and Sigma Chemical Co., St. Louis, Missouri. Purity of purchased compounds typically exceeded 98%.

Electrophysiology. Electroantennogram (EAG) techniques used were similar to those described in detail (Schneider, 1957; Dickens, 1979). Briefly stated, Ag–AgCl electrodes filled with insect physiological saline were used. After prepuncture with a sharpened tungsten needle, the recording electrode was placed in either the terminal or penultimate antennal segment, and the indifferent electrode was implanted in the proximal antennal segment.

Single sensillum recording techniques used are described in detail elsewhere (Boeckh, 1962; Dickens, 1979). In general, recordings were made with 50.8-μm-diameter tungsten wire electrolytically sharpened to a tip diameter of ca. 1–2 μm. The recording electrode was positioned under optical control (100×) with a Leitz high-power micromanipulator near the base of a single sensillum trichodeum. The indifferent electrode was implanted in a nearby antennal segment. Records of EAG and single cell reponses were made on Polaroid film.

Two methods of odor delivery were used in these experiments. In one method, candidate odorants were delivered as 1-μg samples placed on filter paper strips (20 × 7 mm) in 20-ml eccentric tip syringes. From a distance of ca. 1 cm, a 10-ml puff of odor-laden air was delivered to the preparation by an air-driven piston device. Stimulus duration was ca. 1 sec. In a second method of odor delivery, potential pheromone compounds were delivered as 10-μl samples of serial dilutions placed on filter paper (8 × 18 mm) inserted into glass

cartridges (5 × 80 mm ID) and oriented toward the preparation from ca. 1 cm. Hydrocarbon-free air (filtered and dried) carried odor molecules evaporating from the filter paper over the antennal preparation. Stimulus duration was 1 sec as regulated by a solenoid valve. Air flow was 1 m/sec as measured by a thermistor. Serial dilutions of potential pheromone compounds were prepared in nanograde pentane.

Flight Chamber Bioassay. The flight chamber was constructed in the general shape of a half cylinder (length 2.4 m, width 0.91 m, height 0.55 m) and is similar in design to the one used by Miller and Roelofs (1978). A large fume hood was located at the downwind end of the flight chamber to eliminate residual pheromone. Wind speed within the chamber was regulated at 1.4 km/hr. A dim source of light was provided by two banks of red-painted, 7.5-W appliance bulbs located at distal ends of the chamber.

Single males (less than 24 hr postemergence) were placed into 5 × 7.6-cm (H × D) wire mesh cages and preconditioned in the dark within the bioassay room (26.7 ± 1.0°C, relative humidity not regulated) for 2–3 hr prior to assay in large plastic containers. Similarly, two virgin females, used as controls, were placed into each wire mesh cage and preconditioned to induce calling.

Rubber septa (A.H. Thomas, No. 8753-D22) that had been exhaustively extracted in a Soxhlet apparatus with CH_2Cl_2, were used as the pheromone-release substrates. Each septum was treated with 100 μg of pheromone in 100 μl of heptane 24 hr prior to testing and sealed in a glass vial. This concentration was based on a preliminary study conducted to determine the most appropriate concentration to use for flight chamber testing. Pheromone component ratios were based upon triplicate GLC measurements of female abdominal tip rinses as previously described (Table 1). Where one or more of the six components was excluded, the ratios of the others were maintained.

The bioassay procedure consisted of: (1) attaching the screen cage containing the male to the screen covering at the downwind end of the chamber at a predetermined location within the pheromone plume; (2) allowing the male 5 min to adapt to wind tunnel conditions; (3) placing the treated septa or the cage containing the virgin females on a metal wire (27.5 cm high) located in the center of the flight chamber 2.3 m upwind from the male; (4) releasing the male from the cage 30 sec after initiation of wing fanning; (5) observing the male's flight behavior for 5 min (Figure 1); (6) removing pheromone source and male from flight chamber; and (7) exhausting flight chamber for 5 min to remove residual pheromone from the flight chamber before testing the next treatment. Males were used only once.

Bioassays were conducted in a random fashion. One male equaled one observation for each treatment. Each group of treatments was randomized and repeated up to 25 times. The percent landing on the source and the time of flight from initiation to landing on the pheromone source were recorded.

Field Studies. Two studies were conducted at Mississippi State, Missis-

TABLE 1. RELATIVE COMPOSITIONS AND QUANTITY PER INSECT OF SOUTHWESTERN CORN BORER PHEROMONE COMPOUND (BASED ON GLC ANALYSES OF TIP RINSES)

Compound	Composition (%)[a]	Quantity per insect (ng)
(Z)-9-Hexadecenal	17.1	2.3
(Z)-11-Hexadecenal	56.3	10.2
n-Hexadecanal	14.9	2.5
(Z)-9-Octadecenal	4.5	0.9
(Z)-11-Octadecenal	0.9	0.05
(Z)-13-Octadecenal	6.3	1.2

[a] These percentages were subsequently used to establish the ratios of blends used in all flight chamber and field tests.

sippi, in May 1985 and in July–August 1985 to compare the attractiveness of synthetic SWCB pheromone blends and virgin females. A third study was conducted in Castro County, Texas, in July–August 1985. The Mississippi studies were conducted in corn fields infested with laboratory-reared SWCB larvae. Responding males were a mixture of wild insects and those from artificial infestation. The Texas studies were conducted in a corn-growing area with high natural infestations of SWCB. The procedures used in the field studies were similar with only minor modifications. In the May 1985 test at Mississippi State,

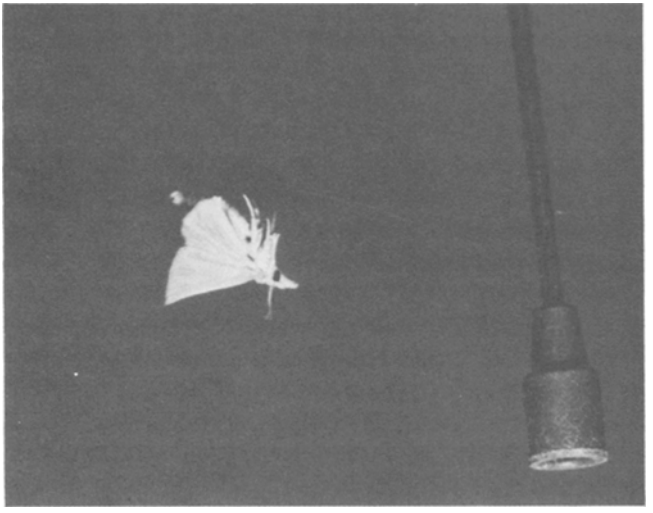

FIG. 1. Male southwestern corn borer moth responding in the flight chamber to a rubber septum impregnated with the synthetic pheromone formulation.

traps were baited with 1500 μg of the three-component formulation found effective in the flight chamber tests: (*Z*)-9-hexadecenal, (*Z*)-11-hexadecenal, and (*Z*)-13-octadecenal (21.5:70.6:7.9); or a four component formulation: (*Z*)-9-octadecenal, (*Z*)-11-hexadecenal, (*Z*)-9-octadecenal, (*Z*)-13-octadecenal (20.4:66.9:5.4:7.5). A solvent control and virgin SWCB females were also included. The treatments were arranged in a 4 $\times$ 4 Latin square within a field of corn that was in the seedling stage of growth. Each treatment was suspended in a small wire cage within Scentry® wing traps held 1 m from the ground by metal stands. The distance between traps was 25 m. A uniform SWCB male population was created around and within the test area by placing 53 SWCB male pupae obtained from our laboratory culture in each of 25 small wire emergence cages suspended 1 m from the ground by metal stands. The experiment was initiated one day after the first adult was observed to have emerged from the field emergence cages.

The number of males captured was recorded, and septa, females, and trap bottoms were replaced daily. The study was conducted for five consecutive nights. Data on the numbers of males captured per night were analyzed using the ANOVA. The Student-Newman-Kuels' test ($P < 0.05$) was employed for separation of means.

In the July–August 1985 tests in Mississippi and Texas, the previously defined three-component formulation only, at 500 and 1500 μg, was employed. In addition to solvent controls, traps holding two Mississippi females (less than 24 hr old) per trap were used in the Mississippi test, and two Mississippi females and two Texas females each were used in the Texas tests. The tests were of RCB design with two replications over a three-week period. The traps were the Pherocon 1C® wing with a metal stand; height, 1 m; distance between traps, 50 m.

The weekly schedule was: Monday, deployment of traps with formulations; Wednesday, remove and record numbers of males and rerandomize treatments; Friday, remove and record numbers of males; remove septa and females; the following Monday, repeat schedule. The statistical analysis was as above.

RESULTS AND DISCUSSION

Chemical Analyses. In the initial work, aliquots of a partially purified, biologically active fraction extracted from the abdominal tip were analyzed to determine the nature of the active substance(s). Silica gel TLC evidence was consistent with a ketone, aldehyde, ester, or similar compound, but not an alcohol or a hydrocarbon. Catalytic hydrogenation (5% palladium on charcoal) destroyed pheromonal activity, suggesting unsaturation. Sodium borohydride and 2,4-dinitrophenylhydrazine destroyed the activity, suggesting a carbonyl function. Because the activity was only slowly decreased by saponification, but

rapidly destroyed by lithium aluminum hydride, an ester was not indicated. Neither prolonged treatment with diazomethane nor reaction with trichloroacetyl isocyanate destroyed the activity; thus an alcohol function was unlikely. Because the activity was slowly destroyed by hydrochloric, sulfuric, and phosphoric acids, the phosphoric acid test for epoxides was inconclusive. GLC retention indices with the Ap L column indicated several components with 16–20 carbon atoms or their equivalents. Thus, the sum of these tests gave presumptive evidence for unsaturated carbonyl compounds.

Ovipositor extracts (100 females, 1 min rinse procedure) were analyzed by GLC and GLC-EI-MS employing the 60-m DB-1 column. The spectra and retention volumes of the major components, (*Z*)-9-hexadecenal, (M^+ 238), (*Z*)-11-hexadecenal (M^+ 238), and *n*-hexadecanal (M^+ 240), were comparable to that of authentic compounds. *n*-Hexadecanal was separated from an obscuring peak of isopropyl myristate (M^+ 270) and identified only after separation by HPLC. Three other peaks in active ovipositor extracts included (*Z*)-9-octadecenal, (M^+ 266), (*Z*)-11-octadecenal (M^+ 266), and (*Z*)-13-octadecenal (M^+ 266), and they accounted for approximately 11% of the total content.

The relative composition of the six candidate pheromone compounds (average of four replicates of 10 insects) was: (*Z*)-9-hexadecenal/(*Z*)-11-hexadecenal–*n*-hexadecanal–(*Z*)-9-octadecenal–(*Z*)-11-octadecenal–(*Z*)-13-octadecenal (17.1 : 56.3 : 14.9 : 4.5 : 0.9 : 6.3 (Table 1). The average recovery of each, respectively, from 1-min heptane tip rinses was 2.3, 10.2, 2.5, 0.9, 0.05, and 1.2 ng/insect. The relative composition of the three (later established) essential pheromone compounds was (*Z*)-9-hexadecenal–(*Z*)-11-hexadecenal–(*Z*)-13-octadecenal: 21.5 : 70.6 : 7.9.

Structural assignments were made for several other compounds using more concentrated ovipositor extracts (approximately 1000 females) by GLC and GLC-EI-MS analysis. These compounds accounted for approximately 9% of the integration count from calling female ovipositor rinses but were present in higher concentrations in less active extracts in which tips were held in solvent for longer periods. Listed in the order of increasing elution times, they are: 2,6-di-tertiary-butyl-4-methyl phenol(probable solvent impurity), isopropyl myristate, (*Z*)-9-hexadecenol, (*Z*)-11-hexadecenol, *n*-hexadecanol, palmitoleic acid, palmitic acid and, perhaps, (*Z*)-9-hexadecen-1-ol acetate.

(*E*)-9-Hexadecenal and (*E*)-11-hexadecenal were prepared by oxidation from their respective *E* alkenols. The *E* aldehydes had slightly longer GLC retention volumes, but there were no apparent differences in the mass spectra of the *E* and *Z* aldehydes. Infrared spectra of the two synthetic *Z* aldehydes did not show the absorption in the 950–1000 cm^{-1} region exhibited by the two synthetic *E* aldehydes, evidence for the isomeric purity and correct assignment of the synthesized aldehydes as *Z* isomers.

Electrophysiology. Electrophysiological studies initially verified the essentiality of (*Z*)-9-hexadecenal and (*Z*)-11-hexadecenal as pheromone compo-

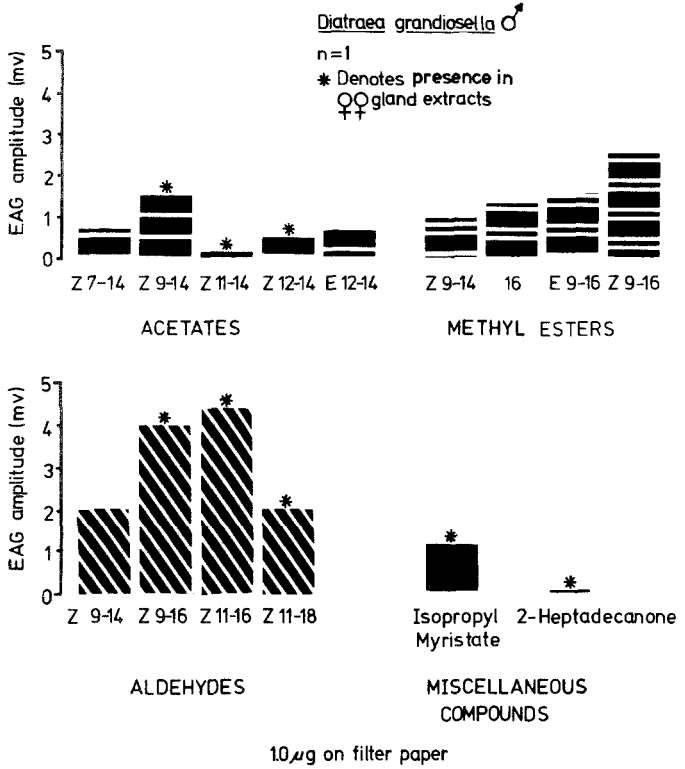

FIG. 2. Electroantennogram (EAG) response of SWCB males to volatiles emanating from 1 μg of several candidate pheromone components (and other compounds) identified in chemical studies from 1976–1983.

nents. Among compounds tested with various functional groups, the antennal receptors were shown to be more responsive to aldehydes than to acetates or methyl esters (Figure 2). This heightened responsiveness was especially clear for the 16-carbon aldehydes with unsaturation at the 9 or 11 position.

Subsequent electrophysiological recordings of cells associated with individual sensilla trichodea revealed the presence of at least two cells with differing spike heights associated with each sensillum (Dickens, unpublished). In each of four recordings made from the sensilla, the cell with the larger amplitude spike responded to the (Z)-11-hexadecenal, while the cell with the smaller amplitude spike was reliably activated by the (Z)-9-hexadecenal.

Further EAG investigations revealed antennal receptors of SWCB males to be highly responsive not only to (Z)-9-hexadecenal and (Z)-11-hexadecenal, but also verified the essentiality of a third compound, (Z)-13-octadecenal (Figure 3). However, *n*-hexadecanal, (Z)-9-octadecenal, and (Z)-11-octadecenal

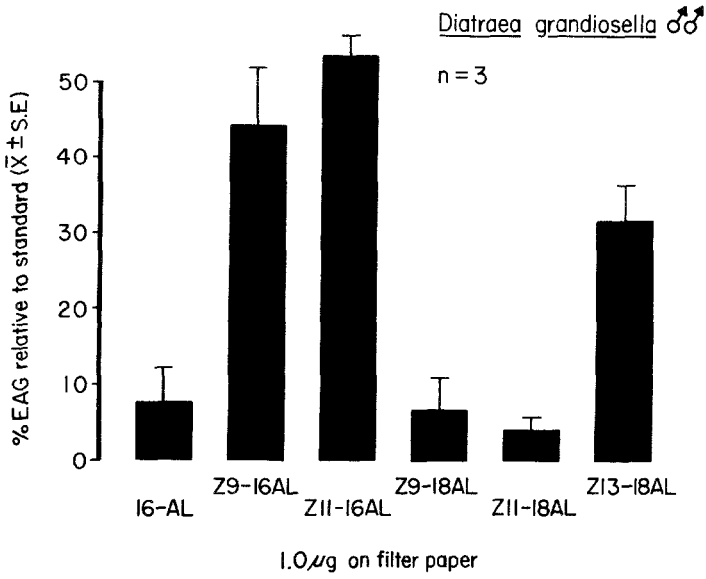

FIG. 3. Mean EAGs of SWCB males to volatiles emanating from 1 μg of six potential pheromone components identified from female abdominal tip extracts in 1983–1984.

also elicited significant EAGs, but only at higher dosages. Thus (Z)-9-hexadecenal, (Z)-11-hexadecenal, and (Z)-13-octadecenal were indicated as the major pheromone components.

Laboratory Flight Chamber Tests. Studies were conducted to determine which pheromone blends were most effective in attracting males. The blends were formulated with each appropriate compound included at the same relative ratios as established by GLC analyses (Table 1). There were at least 16 males/treatment used in testing each formulation. The results are given in Table 2.

Females were effective in attracting males, with 96% landing on the source with an average time of flight of 60 sec. Formulation A containing all six of the candidate components attracted 76% of the males tested in an average time of 60 sec. Failure to include (Z)-9-hexadecenal, (Z)-11-hexadecenal, or (Z)-13-octadecenal (in turn) with the other five components resulted in no attraction (B, C, D). Elimination of *n*-hexadecenal, (Z)-9-octadecenal, and (Z)-11-octadecenal, in turn, from five-component mixtures (E, F, G) did not prevent male performance although the attractiveness was somewhat poorer than that of females. Elimination of *n*-hexadecenal, (Z)-9-octadecenal, and (Z)-11-octadecenal (in pairs) from four-component mixtures also did not prevent male attractiveness (H, I, J). Three two-component mixtures which did not include, in turn, (Z)-9-hexadecenal, (Z)-11-hexadecenal, or (Z)-13-octadecenal were not attractive (K, L, M). Finally, the three-component mixture consisting of (Z)-

TABLE 2. FLIGHT CHAMBER RESPONSES OF SOUTHWESTERN CORN BORER MALES TO 14 SYNTHETIC FORMULATIONS; PERCENT SUCCESSFUL FLIGHTS AND TIME REQUIRED.

Treatment	Formulation[a]														
	♀	A	B	C	D	E	F	G	H	I	J	K	L	M	N
(Z)-9-Hexadecenal		+		+	+	+	+	+	+	+	+	+	+		+
(Z)-11-Hexadecenal		+	+		+	+	+	+	+	+	+	+		+	+
n-Hexadecanal		+	+	+	+		+	+			+				
(Z)-9-Octadecenal		+	+	+	+	+		+	+						
(Z)-11-Octadecenal		+	+	+	+	+	+			+					
(Z)-13-Octadecenal		+	+	+		+	+	+	+	+	+		+	+	+
Landing on source (%)	96	76	0	0	0	68	64	80	72	80	64	8	0	0	96
Flight time from initiation to landing ($\bar{X}$ sec)	60	60	—	—	—	58	78	81	77	74	76	56	—	—	46

[a] 100 μg of formulations employing the percentage ratios given in Table 1. Where one or more components are excluded, the total of the percentages is less than 100.

9-hexadecenal, (Z)-11-hexadecenal, and (Z)-13-octadecenal (N) was as efficient as the virgin female in attracting males to the pheromone source (96%), and the flight time was somewhat shorter (46 sec).

Field Tests. The May 1985 Mississippi test employed laboratory-reared, released males because the native population was very low at that time. The results of the test showed that the three-component blend was as effective as females in attracting males. A four-component blend that additionally included (Z)-9-octadecenal was equally effective (Table 3, and Figure 4). The four-component blend was evaluated to determine whether an additional component might

Table 3. SOUTHWESTERN CORN BORER MALES CAPTURED IN LATIN-SQUARE FIELD TEST CONDUCTED INDUCTED IN MISSISSIPPI, MAY 1985 (MEAN OF CAPTURES PER TRAP PER NIGHT)

Treatment	Mean[a]
3-Component blend[b]	7.30a
4-Component blend[c]	7.25a
Virgin females	6.30a
Solvent control	0.006

[a] Means followed by the same small letter are not significantly different at the $P < 0.05$ level according to the Student-Newman-Kuels' test.
[b] 1500 μg (Z)-9-hexadecenal–(Z)-11-hexadecenal–(Z)-13-ocatadecenal (21.5:70.6:7.9).
[c] 1500 μg of (Z)-9-hexadecenal–(Z)-11-hexadecenal–(Z)-9-octadecenal–(Z)-13-octadecenal (20.4:66.9:5.4:7.5).

either decrease or improve the attractiveness. The possibility of decreased attractiveness had been suggested from the results of the flight chamber bioassays (Table 2).

The results of the July–August 1985 Mississippi and Texas tests which were conducted in corn-growing areas with high natural infestations are summarized in Table 4. The tests show that the defined three-component formulation was as effective as either native Mississippi or Texas females in attracting males from either geographical location. Although not statistically significant, there appeared to be an indication that the 1500-μg formulation is more attractive than the 500-μg formulation and may out perform it over an extended pe-

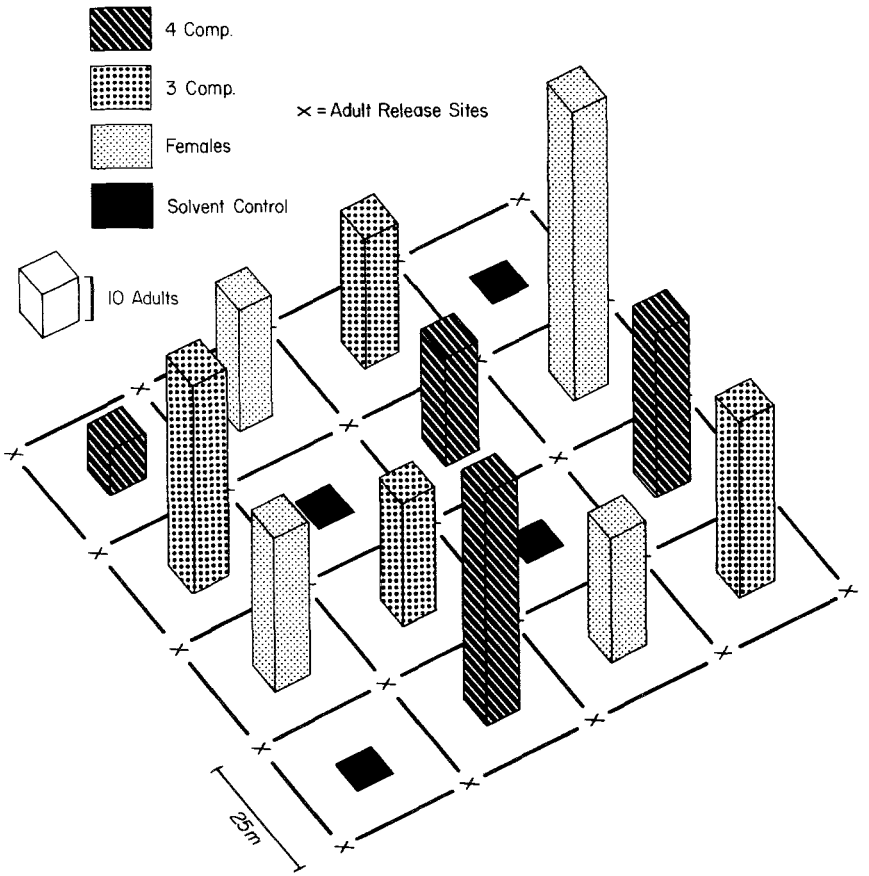

FIG. 4. Southwestern corn borer males captured during a five-night period in traps baited with 1500 μg of defined three- and four-component pheromone blends (see Table 1 for ratios), or virgin females; field-released insects responding in a Latin-square designed test.

TABLE 4. SOUTHWESTERN CORN BORER MALES CAPTURED IN MISSISSIPPI AND TEXAS FIELD TESTS, JULY–AUGUST 1985 (MEAN OF CAPTURES PER TRAP PER 2 NIGHTS)

Treatment	Mean[a]	
	Mississippi	Texas
3-Component, 500 μg[b]	8.67a	8.17a
3-Component, 1500 μg[b]	16.92a	10.83a
Virgin Mississippi females	15.92a	10.17a
Virgin Texas females		5.75a
Solvent control	0.08b	

[a] Means followed by the same small letter are not significantly different at the $P < 0.05$ level according to the Student-Newman-Kuels' test.
[b] (Z)-9-Hexadecenal-(Z)-11-hexadecenal-(Z)-13-octadecenal (21.5:70.6:7.9).

riod. There also was no statistically significant difference in the ability of Texas or Mississippi females to attract Texas males.

In subsequent field tests (to be reported in detail later) using a pheromone-baited, nonsaturating *Heliothis* Scentry trap, rather than the sticky trap, much higher captures have been recorded, in fact, as many as 724 males per night. Also evidence has been obtained that the 1500-μg formulation retains a degree of effectiveness for two to four weeks.

In summary, the sex attractant pheromone of the southwestern corn borer has been identified and shown to be very potent and effective for attracting males over its geographical range.

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SEX-IDENTIFYING URINE AND MOLT SIGNALS IN LOBSTER (*Homarus americanus*)

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Abstract—During courtship, premolt female lobsters, *Homarus americanus*, choose a male and initiate a pair bond by repeated approaches to his shelter. The male allows such a female to share his shelter for about one week. This knowledge formed the basis to search for quantitative evidence for lobster sex pheromone(s) used in courtship: male cues to allow premolt females to identify a preferred male, and female cues to allow males to identify a premolt mature female. In each of four 1500-liter naturalistic aquaria, the behavioral responses of one female and two male lobsters to male and female lobster urine (0.5 ml) and body odor (20 ml) stimuli were observed. These stimuli were injected once or twice per day into a continuously flowing delivery tube attached to lobster shelters. Habituation to stimulus introduction—a serious problem in earlier experiments—was apparently avoided in the more natural social and physical environment we employed in these experiments. We demonstrated that male and female molt body odors contain different chemical substances: females responded to male molt body odor and males responded to female molt body odor but not vice versa. In general, male and female intermolt urine caused strong responses; however, females responded only weakly to male urine. This suggests that male and female urine are chemically different. Female urine and molt body odor caused a typically male "high-on-legs" response. These results show that molt body odors and intermolt urine contain sex-specific substances, which may be used in lobster courtship as well as other social interactions.

Key Words—Lobster, *Homarus americanus*, sex pheromone, urine odor, body odor, courtship, chemical communication.

INTRODUCTION

Behavioral evidence for female sex pheromones has been obtained in various Crustacea (Ryan, 1966; Dahl et al., 1970; McLeese, 1970; Atema and Engs-

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trom, 1971; Teytaud, 1971; Kamiguchi, 1972; Katona, 1973; Kittredge et al., 1971; Eales, 1974; Ameyaw-Akumfi and Hazlett, 1975). In a review of crustacean sex pheromone research, Dunham (1978) argued convincingly that important aspects of experimental design had often been lacking in these studies. However, in many of these studies the possibility of a sex pheromone acting other than as an attractant must be considered, as well as the role of behavioral reinforcement by social interactions, habituation to repeated presentation of unreinforced stimuli, natural biological rhythms, etc. (Atema et al., 1979; Atema and Cobb, 1980; Dunham, 1985). Despite a rather large number of studies by several authors on sex recognition in crayfish, Dunham's recent review (1985) concludes that it remains debatable whether these animals are capable of sex recognition or even species recognition by chemical stimuli, since results are inconsistent and inconclusive.

Early attempts to identify and isolate a female sex pheromone in *Homarus americanus* failed (Atema and Gagosian, 1973; Gagosian and Atema, 1973; McLeese et al., 1977; Atema, unpublished) probably due to the lack of understanding of its biological function and context. It was formerly believed that female lobsters—like moths—were sedentary dispersers of pheromones. Through further behavioral studies in the laboratory (Atema et al., 1979; Cowan and Atema, 1985) and in the field (Karnofsky and Atema, 1979) we have learned that females, not males, are the active partners in sexual selection, seeking mature male lobsters in their shelters. This search and selection takes place in the premolt period and involves courtship and pheromones. Therefore, a bioassay requiring a male to locate the source of release is unnatural and, as experience showed, is doomed to failure. Under conditions where single males were presented with a social odor in the absence of other stimuli, responses often habituated or did not allow discrimination between odors (Atema, unpublished).

Based on new understanding of the role of the female lobster sex pheromone, we designed a naturalistic odor testing situation in which natural context was preserved and habituation was avoided. Similarly, Gleeson (1980) and Gleeson et al. (1984) designed a successful bioassay for blue crab sex pheromone(s) based on the male's natural response to premolt females. We use the term "pheromone" to describe a body odor produced in the context of communication; it may be a mixture of compounds. "Body odor" refers to the entire chemical output which any organism produces as a result of metabolism; body odor may include urine, urine pheromones, and/or other pheromones. Many body odors may be detected and used in predation or social behavior without there being evidence for communication (Atema et al., 1980; Atema and Stenzler, 1977; Bryant and Atema, 1986).

We chose three potential sources of chemical signals that may have social significance: intermolt urine, molt body odor, and intermolt body odor. Urine was shown to be the source of female sex pheromones in crabs (Ryan, 1966; Gleeson, 1980). Crustacean urine, including in lobsters, is stored in bladders,

an indication of the need for controlled release. Control of urine release was implied in a study of female crab pheromones (Christofferson, 1978). Urine is released from a bilateral pair of papillae (nephropores) at the base of the large (second) antennae into the powerful gill current (McPhie and Atema, 1984; Atema 1985), suggesting that the gill current is used—among other functions—as a broadcast system for chemical signals.

Molt body odor is the mixture of metabolites in the water collected from the tank of a molted animal. Mature females molt about one half hour before mating. Molting and mating take place about midway during a cohabitation period of about one week in the male shelter (Atema et al., 1979). Mature female "molt water" contains the female lobster sex pheromone (McLeese, 1970; Atema and Engstrom, 1971) to which males show a typical posture variously described as "mating dance" or "high-on-legs" (Atema and Engstrom, 1971; Atema and Cobb, 1980). Similarly, intermolt body odor is the mixture of metabolites in the water collected from intermolt lobsters. There is no evidence for specific pheromones in intermolt water, but field and laboratory observations (Karnofsky and Atema, 1979; Cowan and Atema, 1985) suggest that lobsters can distinguish between intermolt individuals.

One other aspect of odor signals is important to consider: dispersal and dilution. In mature lobsters, the gill current projects the animal's body odor some seven body lengths forward (McPhie and Atema, 1984; Atema, 1985) with or without the addition of urine. After about seven body lengths, the gill current has lost most of its momentum due to viscous drag (vortices and turbulence) and "dissolves" in a large dilute odor cloud. Ambient currents pick up this cloud and disperse and dilute it further. Reasonable estimates of odor dilution are not known in this or most other cases. Rough estimates can be obtained from measurements of dye diluting away from a stationary source, where dilution of 3–4 orders of magnitude were regularly observed at distances of 3–10 cm away from the source. These figures refer to the relevant parameter, odor peaks, not the commonly used but less significant parameter, average odor dilution. For a discussion of aquatic odor dispersal see Atema (1985).

With our naturalistic bioassay (see Methods and Materials), we provide evidence for special molt substances in male and female molt body odor and for chemical differences between male and female urine. Our focus was on males responding to female cues, since the best evidence thus far was for female pheromones. This focus is reflected in the experimental design using intermolt males in shelter as the primary group of receivers.

METHODS AND MATERIALS

Four groups of three mature lobsters, two males and one female each, were kept in four 1500-liter aquaria under seasonally adjusted artificial light–dark cycles and ambient flow-through seawater conditions (Figure 1). The three lob-

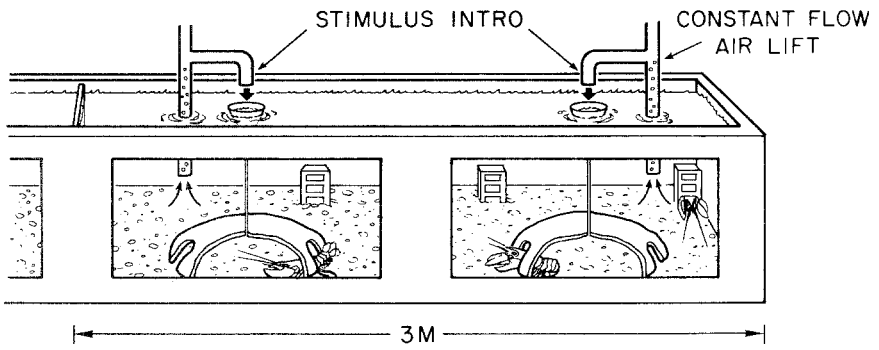

FIG. 1. Diagram of one of four similar naturalistic observation tanks used in these experiments. Observation shelters were placed in front of each window. A constantly flowing air-lift water circulation system with funnel interruption delivered a slow irregular flow of home tank water into these shelters at a mean flow rate of about 320 ml/min. Test and control stimuli were injected into the funnel. Ambient, unfiltered seawater flowed through each of the four tanks at a rate of about 25 liters/min (not drawn in figure). Substrate consisted of sand, gravel, shells, and rocks. The tank sketched here was one half of a 6-m-long tank. The two halves were separated by a solid barrier; each half had its own inflows and drains. Lobsters are drawn in to scale. The lobster in the left shelter is shown fanning his pleopods (FAN) at one of the shelter entrances; the lobster in the right shelter is shown checking one of the shelter entrances (CE). See Table 1 for description of behaviors.

sters per tank interacted normally, allowing each male lobster to recognize the presence of a male and female lobster. Thus, male and female odor stimuli would not be presented in a social vacuum. This feature was designed to prevent habituation to repeated odor introductions. Normal social interactions, including courtship and mating behavior, occurred in the tanks. Odor tests were done only when a lobster was alone in one of the observation shelters. These hand-molded concrete shelters had two entrances and dimensions comparable to those of lobster-made shelters in the field (personal observation). Such shelters were placed against the two observation windows in each tank to record behavior inside the shelter. Cinderblocks provided less preferred shelter space to the rear of each aquarium (Figure 1). The aquaria were supplied with fresh dead fish or squid daily in addition to the constant availability of a variety of live mussels, worms, crabs, fish, and squid. Uneaten dead food was removed the next day. Ample availability of food should keep the lobsters food-satiated to avoid the possibility that hunger motivated responses to social odors. Food-satiated lobsters responded to social odors but not to food odors (McLeese, 1973).

Experiments were begun on May 24, 1984, when the water temperature reached 13°C and terminated on November 13, 1984, at the same temperature. Temperature was recorded daily and reached its summer peak of 23°C in mid-

August. However, no experiments were done at temperatures above 21.5°C (August 2–27).

Each observation shelter was provided with a constant-flow stimulus introduction system (Figure 1). Urine (0.5 ml) or body odor (20 ml) were introduced up to twice a day. We allowed at least 4 hr between tests for an individual lobster to minimize possible interaction between responses. The introduction system caused dilution of the stimuli; dye experiments showed urine to be diluted at least 10,000-fold and body odor at least 1000-fold before reaching the receiving animal. Most likely further dilution due to tank circulation and lobster-generated currents would result in negligible signal-to-noise ratios within minutes. Body odor stimuli were prepared by keeping a donor lobster in a 10-liter aquarium with aeration and flow-through ambient seawater.

After an hour of acclimation, the flow was shut off for 4 hr with the lobster in place. Aliquots were taken to serve as the body odor stimulus within 1 hr after collection. Control stimuli for body odor tests were either 20 ml home tank water (i.e., taken fresh from the tank of the recipient), or 20 ml fresh ambient seawater stored for 4 hr in a 10-liter aquarium with aeration. Home tank water was used as a control for experimental manipulations since, due to habituation, animals should show no novelty response to the water they were inhabiting. Fresh ambient seawater prepared in a manner identical to the test stimulus was the other necessary solvent control. Urine was collected by taking a lobster from a communal holding tank and holding two small vials up to the nephropores. An individual lobster could produce urine from a few drops to a powerful fine jet from both nephropores, occasionally providing as much as 5.0 ml from one nephropore. Often no urine was collected with this method. Urine samples (0.5 ml) were used within 1 hr after collection, usually within 20 min. For urine tests, 0.5-ml samples of home tank water served as controls.

Durations of seven behaviors were recorded to the nearest second (Table 1). These behaviors were chosen from a longer list (Atema and Cobb, 1980; Cowan and Atema, unpublished). Nine different stimuli were tested for both males and females; 0.5 ml male or female intermolt urine; 20 ml male or female intermolt body odor; 20 ml male or female molt body odor (collected in a five-day postmolt period); 0.5 ml and 20 ml home tank water controls; 20 ml fresh seawater control. All experimental lobsters, donors and recipients, were individually marked to follow their molt cycles. All animals were sexually mature (75–90 mm carapace length). Recipient animals never received any one stimulus more than 10 times; molted animals were replaced. The total number of all tests (including controls) done with any individual lobster ranged from 1 to 33, with a median of seven and a mean of eight. A test consisted of two continuous 5-min observation periods: (1) no stimulus and (2) control or test stimulus. A condition for doing a test was that in the no-stimulus observations the lobster was quiet in a shelter and not performing any of the behaviors listed in Table 1. The observer was blind to the type of stimulus being introduced.

TABLE 1. DESCRIPTION OF LOBSTER BEHAVIORS IN STUDY[a]

Behavior	Description	Comments
Locate source (LS)	Reach up the glass at the front of the shelter and probe the stimulus inflow tube with chelae or clasp the tube with the dactyls of the pereiopods	Searching for the source of release; often the basis of experimental designs in other studies of crustacean chemical communication; follows from "check glass"; always accompanied by antennule flicking (an odor sniffing behavior)
Check glass (CG)	Turn to face the front of the shelter and place tips of the chelae at the window below the stimulus inflow tube.	Always precedes "locate source"; sometimes accompanied by antennule flicking and by scanning the shelter from entrance to entrance.
Check entrance (CE)	Walk over to and stand still at one of the two entrances (see Figure 1)	Generally accompanied by antennule flicking; common response to an approaching animal both in nature and in large aquaria (personal observation)
Pleopod fan (FAN)	Slow to rapid beating of the pleopods, almost always with tail extended (see Figure 1)	Creates (strong) current from front to rear of lobster; when standing with tail in shelter entrance, water is drawn into one entrance and blown out the other
Seizer open (SO)	Seizer claw fully opened (half open is the relaxed position)	Aggression (both offense and defense)
Seizer closed (SC)	Seizer claw fully closed	Seen in a variety of social contexts
High-on-legs (HOL)	Stand with all pereiopods (fully) extended	Described as a male response to female molt water (Atema and Engstrom, 1971); resembles male blue crab behavior in response to female sex pheromone (Gleeson, 1980); often accompanied by FAN

[a] For more complete behavioral context see Atema et al. (1979), Atema and Cobb (1980).

After normalizing the raw scores ($\sqrt{x + 0.5}$), data were analyzed with a three-way ANOVA for unequal replicates, comparing two sexes, seven behaviors, and nine stimuli simultaneously. This analysis was followed by Duncan's multiple-range test ($\alpha = 0.05$) to determine differences in duration of individual poststimulus behaviors by comparison with the appropriate control tests and to establish differences between the six test stimuli, and between male and female responses.

RESULTS

The mean durations of all behavioral responses recorded are presented as raw duration scores in Figure 2. Statistical analysis of the results showed a strong three-way interaction between sex, behavior, and stimuli (ANOVA, $P < 0.0005$). Responses to tank water control and fresh seawater control were not different (test for least significant differences, $P > 0.05$). Subsequently all social odor tests were analyzed by comparing the behavioral responses to test stimuli with responses to ambient seawater controls (Duncan's multiple-range test, $\alpha = 0.05$). Based on these comparisons (Table 2), it is evident that some stimuli, such as female urine, elicited the appearance of a number of different behaviors, while other stimuli, such as female molt body odor presented to females, did not. "Locate source" (LS) by itself was rarely a significant response. Since LS is an escalation of "check glass" (CG) (Table 1), we combined the two units into LS/CG for further analysis. The unit "seizer open" (SO) by itself showed no significant differences from control and is not listed in Tables 2–4.

Overall, males responded more than females to each of the stimuli except to male molt body odor (Tables 2 and 3). Males responded to five of the six odors tested, whereas females showed no response to half the stimuli: i.e., both male and female intermolt body odor, and female molt body odor (Table 2). Males responded more strongly than females to female molt body odor and male urine (Table 3).

The strongest response—as indicated by the largest number of different behaviors being significant—was elicited from males responding to female urine (Table 2). This response included the "high-on-legs" (HOL) behavior that turned out to be a unique male response displayed only to this stimulus and perhaps to female molt body odor (Figure 2); females never showed this behavior to a significant degree (Table 2). When compared with controls, female molt body odor elicited the greatest contrast between male and female responses: males showed a number of different behavioral responses to it, whereas females did not respond to it at all (Table 2). Conversely, male molt body odor caused stronger responses in females than in males (Tables 2 and 3). Male urine

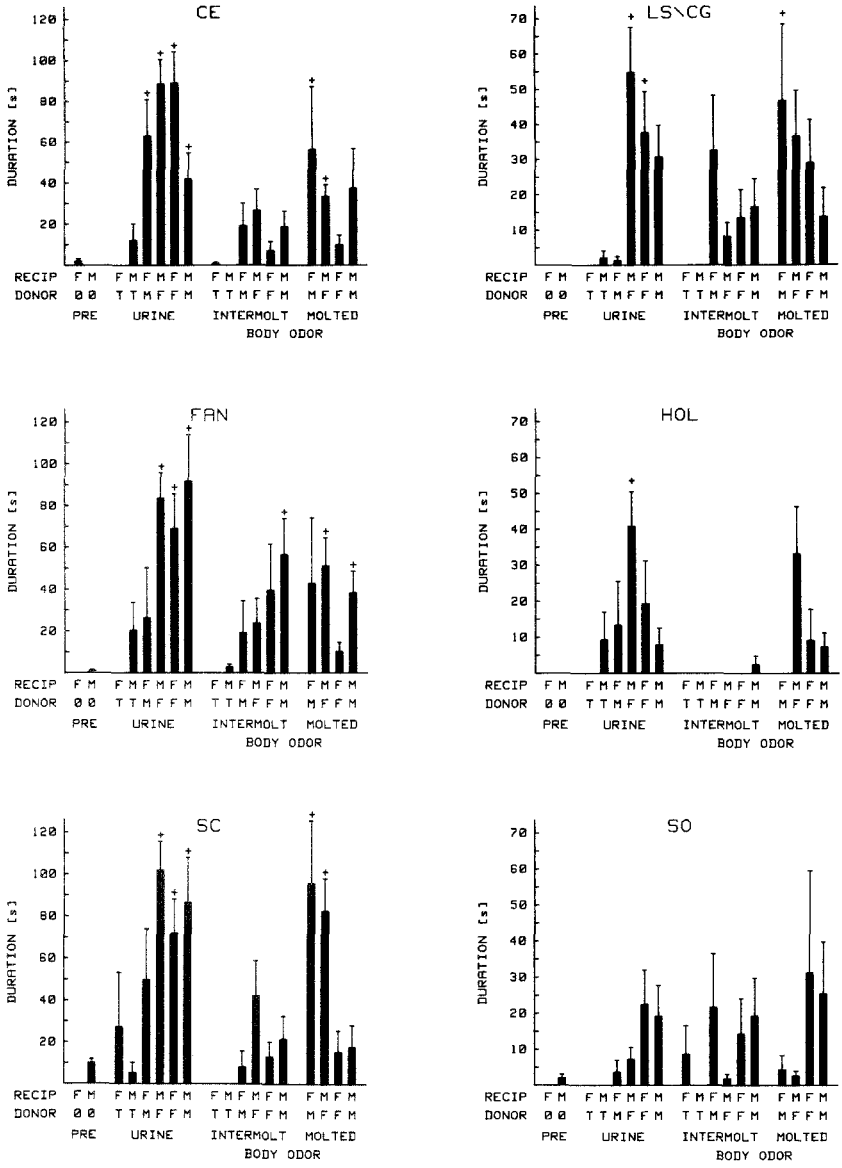

FIG. 2. Mean durations ± SEM of six behaviors (abbreviations and descriptions in Table 1) observed in 5 min following stimulus introduction. Histograms represent raw behavior duration scores approximated to the nearest second. There are two groups of recipients (RECIP): males (M) and females (F); three categories of donors (DONOR): males and females and control tank water (T); and eight stimuli: male and female urine and its 0.5-ml home tank water control, male and female intermolt body odor, male and female molt body odor, and 20-ml ambient seawater control for body odors. Also presented is behavior observed immediately preceding stimulus introduction (PRE). Subsequent data analysis showed behaviors marked (+) to be significantly different from their controls (see Table 2).

TABLE 2. MALE AND FEMALE RESPONSES TO SIX STIMULI[a]

Response	Stimulus		N	Behavior units				
				LS/CG	CE	FAN	SC	HOL
M	Urine	F	(60)	*	*	*	*	*
	Mobo	F	(28)	—	*	*	*	—
	Ibo	F	(16)	—	—	—	—	—
	Urine	M	(21)	—	*	*	*	—
	Mobo	M	(13)	—	—	*	—	—
	Ibo	M	(15)	—	—	*	—	—
F	Urine	F	(25)	*	*	*	*	—
	Mobo	F	(10)	—	—	—	—	—
	Ibo	F	(8)	—	—	—	—	—
	Urine	M	(6)	—	*	—	—	—
	Mobo	M	(6)	*	*	—	*	—
	Ibo	M	(8)	—	—	—	—	—

[a] The asterisks indicate, for five different behavior units, that the test stimulus caused a stronger response (Duncan's multiple-range test $\alpha = 0.05$) than seawater control stimuli. For behavior units see Table 1. N, sample size; M, male; F, female; Mobo, molt body odor; Ibo, intermolt body odor.

was a strong stimulus for males, but not for females (Tables 2 and 3). Males responded weakly to male molt and intermolt body odor (Table 2). Intermolt body odors did not elicit other significant responses (Table 2).

Because males responded more and generally more strongly than females to the same stimuli, comparisons between the six stimuli can be made only by

TABLE 3. RESPONSE DIFFERENCE BETWEEN MALES AND FEMALES[a]

Stimulus		Behavior units				
		LS/CG	CE	FAN	SC	HOL
Urine	F	—	—	—	—	—
Mobo	F	—	—	—	*	—
Ibo	F	—	—	—	—	—
Urine	M	—	—	*	—	—
Mobo	M	—	—	—	(*)	—
Ibo	M	—	—	—	—	—

[a] The asterisks in this table indicate that males responded significantly more than females (Duncan's test $\alpha = 0.05$), except asterisk in parentheses where females responded significantly more than males. See Table 2 legend for abbreviations.

TABLE 4. STIMULUS COMPARISON[a]

Stimulus	Receiver	Behavior units				
		LS/CG	CE	FAN	SC	HOL
Urine F Urine M	M	—	*	—	—	—
	F	—	—	—	—	—
Urine F Mobo F	M	—	*	—	—	—
	F	—	*	*	*	—
Urine F Ibo F	M	*	*	*	—	*
	F	—	*	—	*	—
Urine M Mobo M	M	—	—	—	*	—
	F	—	—	—	—	—
Urine M Ibo M	M	—	—	—	*	—
	F	—	—	—	—	—
Mobo F Mobo M	M	—	—	—	*	—
	F	—	—	—	(*)	—
Mobo F Ibo F	M	—	—	—	—	—
	F	—	—	—	—	—
Mobo M Ibo M	M	—	—	—	—	—
	F	—	—	—	*	—
Ibo F Ibo M	M	—	—	—	—	—
	F	—	—	—	—	—

[a] The asterisks in this table indicate that the stimulus listed on the left caused longer durations of the particular behavior than the stimulus on the right (Duncan's test $\alpha = 0.05$), except asterisk in parentheses where this is reversed. For example, Mobo F caused significantly more SC in male receivers than Mobo M, but female receivers showed significantly more SC to Mobo M than to Mobo F. See Table 2 legend for abbreviations.

considering male and female responses separately. Stimulus comparison (Table 4) shows that female urine was a stronger stimulus than male urine for males. In addition, for both sexes, female urine was a stronger stimulus than both female molt and intermolt body odor (Table 4). Male urine caused stronger responses than both male molt and intermolt body odor in males. Males and females responded more strongly to the molt body odor of the opposite sex than to molt body odor of their own sex. Male molt body odor was a stronger stimulus than male intermolt body odor for females; males made no distinction between these male stimuli (Table 4) and showed only weak responses to both (Table 2).

While SO did not occur with sufficient consistency to be significantly different from controls, the raw mean scores (Figure 2) show that SO and "seizer closed" (SC), the two independent changes from the relaxed half-open claw

TABLE 5. CONTINGENCY TABLE FOR TESTING DISTRIBUTION OF SO AND SC IN RESPONSE TO MOLT BODY ODORS (MOBO) IN TWO SITUATIONS: INTERSEXUAL AND INTRASEXUAL RESPONSES

Mobo	Intersexual	Intrasexual	
SO	7	57	$\chi^2 = 118$
SC	179	33	$P < 0.001$

TABLE 6. CONTINGENCY TABLE FOR TESTING DISTRIBUTION OF SO AND SC IN RESPONSE TO INTERMOLT URINE IN TWO SITUATIONS: INTERSEXUAL AND INTRASEXUAL RESPONSE

Urine	Intersexual	Intrasexual	
SO	11	42	$\chi^2 = 13$
SC	151	158	$P < 0.001$

TABLE 7. CONTINGENCY TABLE FOR TESTING DISTRIBUTION OF SO AND SC IN RESPONSE TO INTERMOLT BODY ODOR (IBO) IN TWO SITUATIONS: MALES RESPONDING TO FEMALE IBO (M TO F) AND FEMALES RESPONDING TO MALE IBO (F TO M)

Ibo	M to F	F to M	
SO	2	22	$\chi^2 = 35$
SC	42	8	$P < 0.001$

position (Table 1), are not randomly distributed. In particular, intrasexual responses to molt body odor (i.e., males responding to male molt body odor, and females to female molt body odor) show longer SO than SC, while intersexual responses show the reverse effect. From χ^2 analysis of contingency tables (Tables 5 and 6), we conclude that for both sexes SO is a behavior associated more than expected with introductions of molt body odor and intermolt urine of animals of the same sex, while SC is associated with molt body odor and urine of the opposite sex ($P < 0.001$). This result is partially reflected also in Table 3, showing that males give stronger SC responses to female molt body odor, and vice versa. In contrast, intermolt body odors cause sex differences in claw responses: analysis of Table 7 shows that males are more likely to respond with SC to female intermolt body odor, while females are likely to respond with SO to male intermolt body odor ($P < 0.001$).

DISCUSSION

In interpreting these results, we must consider both the natural biological context of social interactions in lobsters and the experimental design of this study. The study was focused on male responses to female odors and employed two males and one female per tank as odor recipients. In addition, females consistently yielded more urine than males despite our attempts to collect urine from comparable numbers of males and females each day. This resulted in unequal sample sizes. Finally, our experimental design was based on observing responses of males in shelters receiving male and female urine and body odors. This is the natural context in which a male discriminates between lobsters approaching his shelter entrance, and by which he allows premolt females to enter and pair bond (Atema et al., 1979; Karnofsky and Atema, 1979; Atema and Cobb, 1980; Atema, 1986). Courting females, however, do not sit in shelters; they walk around and approach male shelters selecting a mate. For females, the situation of receiving social odors while inside a shelter may not be conducive to producing behavioral responses meaningful to courtship. A bioassay focused on female behavior should be based on her natural courtship behavior; this was not the aim of this study. Nevertheless females showed interesting responses and differentiated well between various stimuli presented.

Our interpretation of the results is based on a statistical comparison between responses to test and control stimuli to avoid being misled by the greatly unequal sample size of the different stimuli presented to males and females. In discriminating between responses, we are not placing great weight on the meaning of the different behaviors measured (see also Rose, 1984). Initially, we focus on the number of behavioral changes. In the interpretation of results, we will not specifically consider the possibility of masking "pheromones" that might suppress responses, although their existence is of theoretical interest.

Overall, males responded more strongly than females when presented with the same stimuli. The six test stimuli caused greatly different responses in both sexes, both qualitatively and quantitatively. But can we conclude that there are qualitative chemical differences in the six stimuli? Body odors may contain various amounts of urine; males and females may produce different amounts of urine; recently molted animals may produce different amounts of urine than intermolt animals. Moreover, the response difference between males and females may be merely a result of a sex difference in response threshold, not the recognition of qualitatively different compounds in the six stimuli. Thus, one extreme hypothesis is that all stimuli are various dilutions of lobster urine, regardless of sex or molt state. On the other extreme one might postulate different chemical compositions of male and female urine and special molt signals for both sexes. We argue that our results contradict the urine dilution hypothesis, and we provide evidence for special male and female molt substances.

Females showed consistently strong responses to male molt body odor (Tables 2, 3, and 4). Whereas in general females were less responsive than males to the same stimuli, in the case of male molt body odor female response was greater than male response (Table 3). Additionally, in contrast to males, females responded more strongly to male molt body odor than to either female molt body odor, or male intermolt body odor or urine (Tables 2 and 4). These results support the hypothesis of a qualitatively different (set of) substance(s) in male molt body odor to which females but not males respond. There may or may not be other compounds in male molt body odor that caused the weak male response.

Female intermolt urine caused similarly strong responses in males and females (Tables 2 and 3), although males showed HOL (Table 2). However, males responded more strongly than females to female molt body odor (Tables 2 and 3). If female molt body odor were merely a dilute female urine, one would expect it to cause weaker but still similar responses in both males and females. However, in reality males responded strongly to female molt body odor and females not at all (Table 2). This result is also evident in the direct stimulus comparisons of Table 4, where female urine and female molt body odor cause greater response differences in females than in males: females responded strongly to female urine but not to female molt body odor. These results support the hypothesis that females produce a (set of) special molt substance(s) to which males but not females respond.

In addition to evidence for special molt substances in male and female molt body odor, the results point to differences between male and female intermolt urine. Female urine caused stronger responses than male urine in both sexes. Thus, according to the dilution hypothesis, female urine may contain a higher concentration of the same active fraction than male urine. However, based on male and female response similarity to female urine (Tables 2 and 3 and Figure 2), the dramatic difference between males and females in their responses to male urine (Tables 2 and 3 and Figure 2) would not be expected, if male urine were merely a dilute form of female urine. While these latter results cannot exclude the dilution hypothesis, it seems more likely to suspect some qualitative rather than merely quantitative sex differences in intermolt urine.

The demonstration of a female-specific molt odor was expected based on previous work (Atema and Engstrom, 1971; Atema et al., 1979). The present experiments provide an indication of how a quantified bioassay might be designed to pursue chemical isolation and identification of the active compound(s). These and the female blue crab sex pheromone(s) (Gleeson et al., 1984) would become the first marine animal sex pheromones to be identified chemically.

In contrast, we did not expect to demonstrate the presence of a male-specific molt odor causing particular excitement for females (Table 2: locate the source of release, check the shelter entrances, and close seizer claw), nor can

we at present provide a natural context for such a response. Females residing in shelters are not likely to ever receive male molt odor and, if they did, we would not know the biological significance of responding to it. The possibility exists that molting in all lobsters is accompanied by a molt-specific odor which might contain, for instance, the arthropod molting hormone, crustecdysone (Atema and Gagosian, 1973) or a metabolite of it. However, the present experiments showed clearly that males and females responded differently to male and female molt odors. One might argue that if intrasexual competition were severe, then both sexes should respond to intrasexual molt odors. This would allow each sex to eliminate a freshly molted and thus vulnerable competitor. Similarly, one might argue that intermolt urine and body odor could act as warning signals announcing the presence of a potentially dangerous competitor, particularly for shelter possession. (It should be remembered that all recipients were tested in their shelters.)

These expectations based on sexual selection theory may find some confirmation in the results of the analysis of SC and SO. Both sexes showed higher than expected durations of wide-open seizer claw (SO) in response to intrasexual molt body odor and intermolt urine. A wide-open seizer claw is seen in defensive and offensive agonistic interactions. Similarly, a higher than expected incidence of closed seizer claw (SC) was observed in response to intersexual molt body odor and intermolt urine. (It should be emphasized that SC and SO are, of course, mutually exclusive, but not dependent, because the relaxed seizer claw position is half open.) Finally, intermolt body odor also caused a sex difference in responses: males tended to close their seizer claw when presented with female body odor, whereas females more often opened their seizer claw toward male body odor. These results seem to indicate that intermolt females are wary of intermolt males, but that males are appeasing to females. Such an interpretation would fit data collected in the field showing that smaller males could successfully defend their shelters against larger females, but not the reverse (E.B. Karnofsky, J. Atema and R.H. Elgin, unpublished observation). To interpret these results properly will require further behavioral-ecological investigation in the field and in naturalistic environments.

The pronounced responses to intermolt urine (0.5 ml diluted several orders of magnitude before reaching the lobster's chemoreceptors) indicate that urine may be used for social communication in general. The fact that male and female intermolt body odors were weak stimuli for males, and caused no response in females, may indicate that body odor contained little urine. Perhaps lobsters do not release urine constantly or under the conditions of confinement in our body odor collecting tank, as argued for crabs (Christofferson, 1978). Together with the presence of a urinary bladder and the positioning of the nephropores just above the gill current, these results argue for controlled urine release and the use of urine as a distinct chemical signal. The urine signal could be injected under the animal's control into the gill current, which would carry it up to seven

animal lengths forward (McPhie and Atema, 1984; Atema, 1985) providing identification of species, sex, and perhaps dominance status, and individual identity. The gill current can also be abruptly reversed by fanning the exopodites of the maxillipeds (McPhie and Atema, 1984; Atema, 1985), thus allowing the animal to not project its body odor with or without urine and instead to sample water chemistry ahead of itself. Such control is required for a communication function. Identifying potential mating partners and competitors appears to be an important aspect of the seemingly loose social structure of a resident lobster population (E.B. Karnofsky, J. Atema and R.H. Elgin, unpublished observation). Under the normally dark, nocturnal conditions of their circadian activity period, chemical identification may well be the best social cue available, and considering the different control mechanisms available to the lobster, urine would be a convenient vehicle for chemical signals.

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CORRELATION OF RETENTION TIMES ON LIQUID CRYSTAL CAPILLARY COLUMN WITH REPORTED VAPOR PRESSURES AND HALF-LIVES OF COMPOUNDS USED IN PHEROMONE FORMULATIONS

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Abstract—A method has been developed to determine by capillary gas chromatography on liquid crystal stationary phases the relative vapor pressures and half-lives of many compounds used as insect pheromones. This study demonstrated that the retention time of seven acetates on a liquid crystal column (cholesteryl-*p*-chlorocinnamate) could be correlated closely to the reported vapor pressures of the compounds. For 13 additional pheromonal acetates and alcohols, reported half-lives showed a high degree of correlation with their retention times on the liquid crystal column. Thus chromatography on capillary liquid crystal gas chromatographic columns appears to be a useful method for determining the relative volatilities of many pheromones to facilitate the development of more precise formulations.

Key Words—Insect pheromone formulations, liquid crystal capillary columns, half-lives of compounds, vapor pressure.

INTRODUCTION

Considerable effort during the last several years has resulted in the identification of a large number of attractants for economically important insect pests (see for example, Klassen et al., 1982). The utility of these potent chemicals in survey and monitoring traps has been well documented, and rubber septa, which are convenient and proven reliable, have been used extensively to formulate single compounds and pheromone blends. However, most pheromone formulations on rubber septa are developed empirically, and little is known about the release characteristics of these formulations. The only research addressing this problem

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has been the determination of the half-lives of numerous aliphatic acetates and alcohols impregnated into rubber septa (Butler and McDonough, 1979, 1981; McDonough and Butler, 1983). Both the release rates and, in the case of blends, the release ratios of pheromone components from septa are probably governed, at least partially, by the vapor pressures of the compounds.

In this regard, Olsson et al. (1983) recently reported a method for determining the vapor pressure of selected pheromonal compounds because they were interested in determining the release ratio of multicomponent pheromones volatilized from female glands. Additionally, Olsson et al. suggested that their vapor pressure data were in good agreement with Butler's and McDonough's half-life determinations. However, the degree of correlation was not provided.

In both Butler's and Olsson's methods, considerable effort is required to obtain the necessary measurements for the half-life and vapor pressure data, respectively. Thus, we sought a method that would expeditiously provide both the relative vapor pressures and half-lives of compounds typically encountered in lepidopterous pheromones. Our previous experience with liquid crystal stationary phases in capillary gas chromatography (Heath et al., 1979; Heath and Doolittle, 1983) suggested that the elution order of olefin isomers of aliphatic acetates could be correlated with the relative vapor pressure of the compounds. Based on this premise, we evaluated the degree of correlation between the retention time on a liquid crystal phase such as cholesteryl-*p*-chlorocinnamate with the reported vapor pressure data of seven compounds. Then, by correlation with retention times on the same column, we determined the relative vapor pressure of 13 additional compounds whose half-lives have been reported. Finally, we evaluated the degree to which relative vapor pressure, measured half-lives, and retention time on a liquid crystal column might be correlated.

METHODS AND MATERIALS

The unsaturated decyl acetates (*Z*)-3-decenyl (*Z*3–10 : Ac), (*Z*)-4-decenyl (*Z*4–10 : Ac), (*Z*)-5-decenyl (*Z*5–10 : Ac), (*E*)-5-decenyl (*E*5–10 : Ac), and (*Z*)-6-decenyl (*Z*6–10 : Ac) were obtained from Christer Löfstedt (University of Lund, Lund, Sweden). Other compounds used in this investigation were obtained from Chemical Samples Co. (Columbus, Ohio). In several instances, synthetic modification was required to obtain the desired compound. Authenticity of the compounds was established using mass spectroscopy and infrared and nuclear magnetic resonance analyses when required.

Three different stationary phases coated on capillary columns were used in the analysis of the compounds. A 50-m polar cyano silicone phase CPS-1 (0.25 mm ID, 0.25 μm film) and a 35-m apolar methyl silicone (0.32 mm ID, 0.5 μm film) were purchased from Quadrex Corporation (New Haven, Connecticut). The efficient use of liquid crystal columns is limited to a narrow temper-

ature range (ca. $\pm 10°C$ about the mesophase transition temperature). To provide reasonable retention times for the compounds investigated, we coated four different capillary GC columns with cholesteryl-*p*-chlorocinnamate (a cholesteric liquid crystal phase) by techniques described previously (Heath and Doolittle, 1983). The columns were prepared with varying film thicknesses (0.25, 0.5, 1.5, 2.5 μm), and column lengths, 35, 37, 42, and 27 m, respectively. Less than 1% variation in a compound's retention was noted with the various liquid crystal columns when the retention time was converted to equivalent chain length units (ECLU) (Swoboda, 1962) relative to saturated acetates. Thus, all retention data in this investigation are expressed in ECLUs.

A Hewlett Packard 5792® gas chromatograph equipped with flame ionization detectors and interfaced to a Nelson Analytical Data Station (Nelson Analytical, Cupertine, California) was used for all analyses. A sampling rate of eight data points per second was used. Helium was used as the carrier gas at a linear flow of 18 cm/sec. Samples were chromatographed isothermally at a temperature that resulted in a partition coefficient (k') of 2.9 or greater.

Data were analyzed using a HP-87 (Hewlett Packard, Corvallis, Oregon) desktop computer. Regression analyses using linear and nonlinear curve fitting (logarithmic, exponential and power) were performed routinely on all data.

RESULTS AND DISCUSSION

Correlation of Vapor Pressure with Retention Time of Acetates on Liquid Crystal Column. Previous analysis of an analogous series of tetradecenyl acetates suggested that the elution order on liquid crystal phases is determined by the length-to-breadth ratio of the compounds (Dewar and Schroeder, 1965; Lester, 1978; Heath et al., 1979). A molecule with a decreased length-to-breadth ratio (distortion of linearity) should have a shorter retention time because of its inability to penetrate the crystal lattice of the phase. Thus, for the tetradecenyl acetates, it was found that elution time was shortest for the (*Z*)-4-tetradecenyl acetate (*Z*4–14 : Ac), followed by *Z*5, *Z*6, and *Z*7; with *Z*3–14 : Ac eluting last (Heath et al., 1979). Olsson et al. (1983) showed, with computer-generated models of the compounds they investigated, that the midpoint of the decenyl acetates lies near the 4 position. Thus the smallest length-to-breadth ratio and the smallest molecular size would occur when the double bond is in the 4 position and *Z*4–10 : Ac would have the highest vapor pressure. Although only one isomeric pair was studied, the vapor pressure of the *cis*-5-decenyl acetate was found to be higher than the *trans*-5-decenyl acetate. Again this is in agreement with our findings and those of Lester (1978) in that the distortion of length-to-breadth ratio is greater for the *cis* isomer than for the *trans* isomer.

Using decyl acetate and four of the decenyl acetates used by Olsson et al. (1983) (Table 1), we compared the elution order of these compounds on the

TABLE 1. RETENTION TIME AND VAPOR PRESSURE DATA USED IN REGRESSION ANALYSIS AND RESULTANT RESIDUALS

Compound	Vapor pressure in P_a (ln vp)[a]	Retention time (ECLU)[b]	Residual[c]
Z3-10:AC	4.42 (1.49)	973	0.96 (−1.47)
Z5-10:AC	4.29 (1.46)	976	−0.52 (−1.57)
Z6-10:AC	4.29 (1.46)	977	0.63 (−0.47)
E5-10:AC	4.07 (1.40)	982	−1.88 (−0.66)
Sat 10:AC	3.66 (1.30)	1000	0.81 (6.75)
Z7-12:AC	0.532 (−0.632)	1181	(−5.00)
Z9-14:AC	0.090 (−2.41)	1366	(2.42)

[a] Reported by Olsson et al. (1983), 1 $P_a = 0.987 \times 10^{-5}$ atm, at 30°C.
[b] Retention time relative to saturated acetates.
[c] Residual ECLU using ECLU = 1187 − 144.4·ln vapor pressure. Numbers in parenthesis refer to residuals when the seven compounds were used and the equation was ECLU = 1123 − 99.9·ln vapor pressure.

cholesteryl-*p*-chlorocinnamate liquid crystal phase with the reported vapor pressures values. The retention times of the compounds were converted to their equivalent chain length units (ECLU) relative to saturated acetates (Swoboda, 1962). The ECLUs of the 10-carbon acetates were plotted against the natural logarithms of their reported vapor pressures (Figure 1). The equation of the curve was

$$\text{ECLU} = 1187 - 144.4 \cdot \ln \text{ vapor pressure} \quad (1)$$

and had a coefficient of determination (r^2) of 0.988. The difference between actual and curve fit values for each data point (residuals) are reported in Table 1. Because only two points were reported for the vapor pressure determination of the Z4-10:Ac, we did not use this compound in our comparison. It should be noted that in accordance with the findings of Olsson et al. (1983), the Z4-10:Ac, which has the highest vapor pressure, elutes well before (ECLU = 969) the other decenyl acetates on the liquid crystal column.

Attempts to curve fit vapor pressure with retention time on nonpolar and polar isotropic phases were unsuccessful. Previous comparison of retention times on isotropic phases with the retention times on a liquid crystal phase using an analogous series of acetates documents the diverse nature of the mesomorphic phases (Heath et al., 1979). Using the decyl and decenyl acetates, the coefficients of determination (r^2) obtained with the nonpolar (methyl silicon) and polar (90% cyano) phases were found to be 0.724 and 0.446, respectively.

Having obtained a significant correlation of the ECLUs of the positional and configurational isomers of the 10 carbon acetates with their vapor pressures, we extended this study to evaluate the correlation of vapor pressure with the

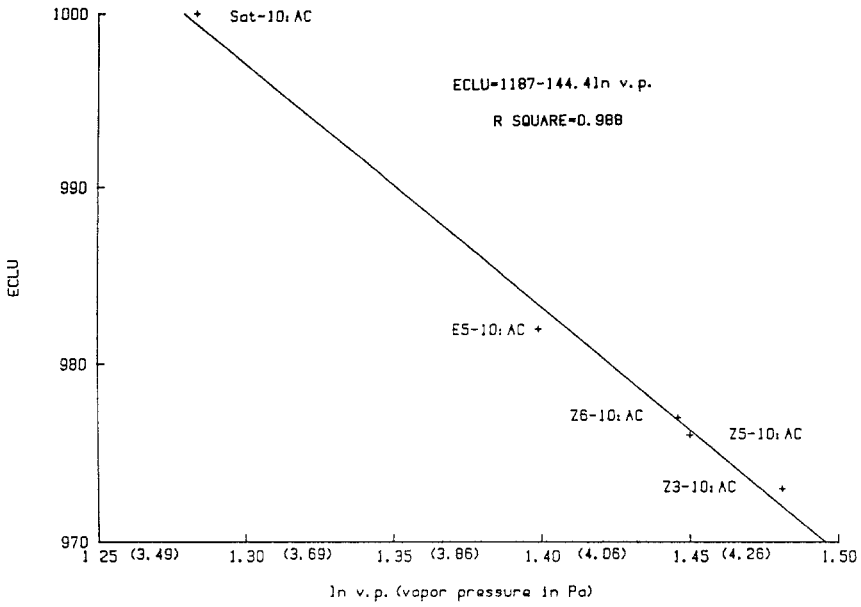

FIG. 1. Plot of retention time (in ECLU) on a 21-m (2.5 μm film) cholesteryl-*p*-chlorocinnamate capillary column of five acetates vs. the natural logarithm of their reported vapor pressures.

retention times of these compounds and (Z)-7-dodecenyl acetate and (Z)-9-tetradecenyl acetate. In this case, while the number of data points between the decenyl acetates and the latter two compounds is limited, we found that using the vapor pressure of seven compounds covering the range of acetates from 10 to 14 carbons still resulted in a high degree of correlation. A curve fit with a coefficient of determination of 0.999 was obtained, and the equation of the curve was determined as

$$\text{ECLU} = 1123 - 99.9 \cdot \ln \text{ vapor pressure} \qquad (2)$$

Residuals from the retention times obtained (in ECLU) are given in Table 1.

Correlation of Vapor Pressure Determined by Retention Time with Half-Life of Compound on Rubber Septa. Using equation 2, which correlates retention time with relative vapor pressure, we determined the vapor pressure of 13 compounds (Table 2) whose half-lives had been determined previously by Butler and McDonough (1979, 1981) and McDonough and Butler (1983). The compounds were chosen because of their availability. They included five saturated acetates (10–14 carbons), four monounsaturated acetates, and four unsaturated alcohols (12–16 carbons). A plot of the curve fit of the reported half-lives vs. the calculated vapor pressure is shown in Figure 2. The coefficient of

TABLE 2. RETENTION TIME (ECLU), CALCULATED VAPOR PRESSURES (VP), AND REPORTED HALF-LIVES (*t*1/2) USED IN REGRESSION ANALYSIS AND RESULTANT RESIDUALS

Compound	ECLU	Calculated (VP)[a]	Half-lives (days)[b]	Residuals	
				*t*1/2 vs. VP[c]	*t*1/2 vs. ECLU[d]
Sat 10:AC	1000	3.42	5.0	−0.2	−0.2
Sat 11:AC	1100	1.26	14.6	0.1	0.3
*Z*7-12:OH	1101	1.25	13.5	−1.1	1.0
*Z*7-12:AC	1181	0.559	34.8	2.0	2.3
*Z*9-12:AC	1198	0.472	44.8	5.8	6.2
Sat 12:AC	1200	0.463	37.3	−0.4	−1.9
*E*9-12:AC	1202	0.453	38.4	−2.0	−1.6
Z9-14:OH	1296	0.177	117.0	12.2	11.1
Sat 13:AC	1300	0.170	97.9	−11.3	−10.3
Z11-14:OH	1306	0.160	117.0	1.2	1.2
*Z*9-14:AC	1366	0.0877	189.0	−24.0	−21.9
Sat-14:AC	1400	0.0624	350.7	51.9	51.8
Z11-16-OH	1447	0.0390	432.0	−50.3	−45.5

[a] Calculated using ln vapor pressure = (ECLU − 1123)/−99.9, equation 2, in text. Vapor pressure units are in P_a.
[b] Half-lives reported by Butler and McDonough (1979, 1981) and McDonough and Butler (1983).
[c] Residual in days. Curve fit equation 3: ln vapor pressure = 2.90 − 1.011 · ln half life.
[d] Residual in days. Curve fit equation 4: ln half life = −8.48 + 0.0101 · ECLU.

determination was 0.995, and the curve was plotted using the natural logarithm of the half-lives and vapor pressures for clarity. The equation of the curve was determined as

$$\ln \text{ vapor pressure} = 2.90 - 1.011 \cdot \ln \text{ half life} \quad (3)$$

The residuals from this curve fit are reported in Table 2.

Since a high degree of correlation of measured half-lives with calculated vapor pressure exists, it is obvious that the same degree of correlation exists between half lives and liquid crystal ECLUs. The residuals (days from the reported half-lives) are given in Table 2, and the equation was determined as

$$\ln \text{ half life} = -8.48 + 0.0101 \cdot \text{ECLU} \quad (4)$$

The coefficient of determination (r^2) for this curve fit was 0.995.

CONCLUSIONS

We found that a high degree of correlation exists between the retention times of seven acetates on a cholesteryl-*p*-chlorocinnamate liquid crystal col-

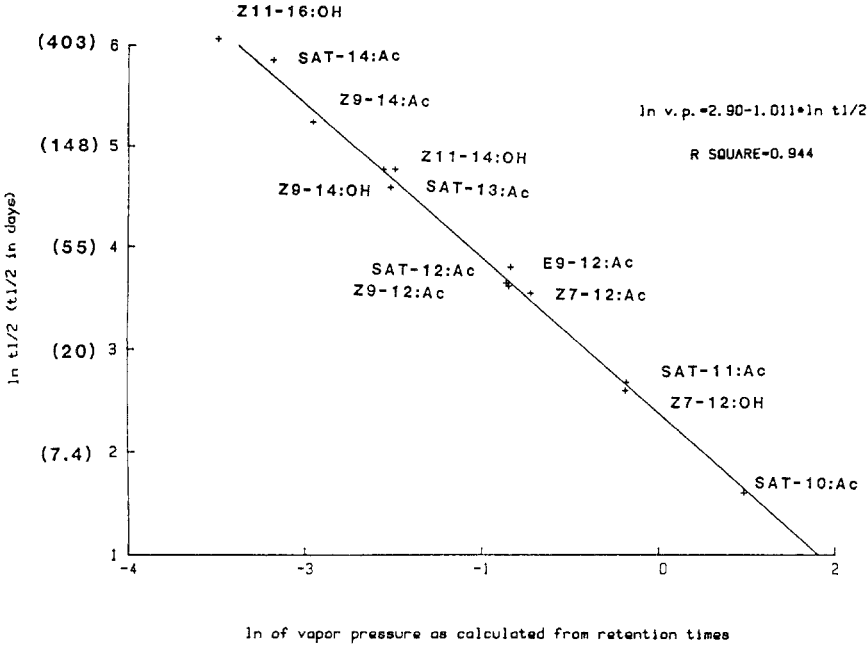

FIG. 2. An ln/ln plot of the reported half-lives of 13 compounds vs. their calculated vapor pressures using ln vapor pressure = (ECLU − 1123)/(−99.9). ECLU were obtained on a 42-m (1.5 μm film) cholesteryl-*p*-chlorocinnamate capillary column.

umn and their reported vapor pressures. The coefficient of determination was 0.999, while the degree of correlation obtained using apolar and polar capillary columns was severely reduced (0.724 and 0.446, respectively). Use of the retention times obtained on the liquid crystal column of 13 compounds (acetates and alcohols) showed a high degree of correlation to their previously reported half-lives. In a subsequent paper, (Heath et al. 1986). The use of the approximate relative vapor pressure of compounds to predict the ratio of components released from rubber septa loaded with multicomponent pheromone blends will be demonstrated. Additionally, investigations are currently in progress using the retention time data obtained on the liquid crystal column to predict release rates of multicomponent blends.

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PERSISTENCE OF NONPROTEIN SEED AMINO ACID S-(β-CARBOXYETHYL)-CYSTEINE IN YOUNG LEAVES OF *Calliandra rubescens:* Ecological Implications

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Abstract—The insecticidal nonprotein amino acid S-(β-carboxyethyl)-cysteine (S-CEC) is the major free amino acid in the seeds of several *Calliandra* spp. where it accounts for up to 2.9% of dry weight. Lesser amounts of other related S-containing amino acids and an array of nonprotein imino acids derived from pipecolic acid are other constituents. High concentrations of imino acids, which also show insecticidal activity, are maintained in the mature leaves, but sulfur compounds are lacking. In this study the disappearance of S-CEC from the germinating seeds and young seedlings of *C. rubescens* was monitored over time. After 10 weeks, S-CEC continues to be found in high concentrations in the stems and new leaves. As young leaves mature, sulfur compounds quickly decrease in concentration. Traces of S-CEC are found in new leaves of plants up to nine months after germination. Whether high concentration of S-CEC in young leaves is due to transportation from the seed or de novo synthesis is unclear. The ecological implications are discussed.

Key Words—Nonprotein amino acid, sulfur amino acid, S-(β-carboxyethyl)-cysteine, imino acid, seedling metabolism, insecticidal compounds, *Calliandra rubescens*, legumes.

INTRODUCTION

The nonprotein sulfur-containing amino acid S-(β-carboxyethyl)-cysteine (S-CEC) was first isolated from seeds of *Albizzia julibrissin* (Gmelin et al., 1958). Its distribution in the plant kingdom appears to be largely restricted to members of the Mimosoideae (Bell, 1971; Evans and Bell, 1979). In *Calliandra*, it is the major free amino acid accumulated in the seeds of all species except those belonging to Series Laetevirentes (Romeo, unpublished). Insecticidal activity of S-CEC against larvae of the polyphagous herbivore *Prodenia eridania* and

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the seed beetle *Callosobruchus maculatus* has been demonstrated (Rehr et al., 1973; Janzen et al., 1977). Similar activity was observed in our laboratory against the leaf herbivore *Spodoptera frugiperda* (Shea and Romeo, unpublished). Although it is presumably derived from cysteine, essentially nothing is known of the biogenesis or metabolism of S-CEC in plants (Krauss and Reinbothe, 1970; Mazelis, 1980). The insecticidal nature of the compound against common seed and leaf herbivores and the large amounts accumulated in seeds of *Calliandra* coupled to the fact that S-CEC is virtually absent from mature leaves of *Calliandra* led us to wonder when the compound disappears from the germinating seed or young seedling.

METHODS AND MATERIALS

Plant Material. Mature *Calliandra rubescens* seeds were collected in December 1983 from dehiscing fruits on plants growing along the northeast boundary of Santa Rosa National Park, northwestern Guanacaste Province, Costa Rica (D.H. Janzen Col. No. 12273). Seeds were germinated and grown in pots in a 50:50 mixture of perlite and vermiculite and kept in a growth chamber maintained at constant temperature (27°C), humidity (85%), and light exposure (12 hr/day). Plants were irrigated daily with $\frac{1}{2}$ strength Hoagland's solution and harvested at regular intervals for analysis of amino acids. The experiment ran for 10 weeks. Individual leaves were harvested and numbered. The first nonembryonic leaf was assigned the number 1, and subsequent leaves were numbered according to the order of appearance on the plant (i.e., a leaf assigned the number 10 would be the tenth leaf to emerge on that plant).

Extraction of Amino Acids. Samples were extracted by the method of Singh et al. (1973). Freshly harvested leaves or seeds from which seed coats had been removed were extracted three times with MeOH–$CHCl_3$–H_2O (12:5:1), and the combined supernatant separated into an upper aqueous phase and a lower nonpolar phase by the addition of H_2O and $CHCl_3$. The aqueous phase was removed, evaporated to dryness, and the residue redissolved in 25% EtOH and used for chromatographic and electrophoretic analysis. Samples for quantitative analysis on the amino acid analyzer were prepared by evaporating aliquots of the above solution and redissolving them in 0.2 M Na^+ buffer (Dionex Femto buffer 1A, pH 2.0).

Quantitative Determination of Amino Acids. Amino acids were quantified using a modified Dionex D-300 Amino Acid Analyzer (Bleecker and Romeo, 1982) interfaced via an A/D converter with a Hewlett-Packard desk top computer (model 9825A). Additional amino acid data was obtained by subjecting the 25% EtOH extracts to high-voltage paper electrophoresis (HVE) for 30 min at 5000 V in a pH 1.9 buffer (Romeo et al., 1983). Papers were oven dried and developed with 0.2% ninhydrin in acetone.

Resolution of S-(β-Carboxyethyl)-cysteine. Since S-CEC coeluted with glutamic acid in our HPLC system, additional steps were taken to determine concentrations of S-CEC. Known amounts of the extracts were run on HVE and developed. The intensities of the S-CEC bands were then compared to bands of known amounts of the compound and a range of concentration of S-CEC for each sample was determined.

RESULTS

S-CEC is the major free amino acid of seeds of *C. rubescens*, accounting for as much as 2.9% total seed weight. As the young seedling grows, the total amount of S-CEC remains essentially constant for two weeks and then gradually decreases to day 70 (Table 1). The rapid decrease in percentage of total plant weight is seen very early (Table 1). After the emergence of the cotyledons from the seed coat, S-CEC is found in all parts of the embryo plant and, with the appearance of young leaves, stem, and roots, it continues to be present in all parts. The distribution within the plant, however, changes with development (Tables 2 and 5). Table 2 shows the distribution of S-CEC in leaves up to day 70. Reading down the diagonal, one sees that high levels are consistently found in young emerging leaves. For example, at day 42 new leaf number 5 has a S-CEC content of 2.6–3.3 mg/g fresh weight. The concentration in new leaf 6 at day 54 is similar. Even at day 70 relatively high amounts of S-CEC are still seen in the new leaves 9 and 10. Reading down the vertical columns one observes that these initial high concentrations in new leaves rapidly decrease as the leaves age. Leaf 3 for example has dropped from 0.9–1.9 mg/g fresh weight on day 28 to 0.2 mg/g fresh weight on day 42. All of the compound is gone by day 54. The large amount in new leaf 6 seen on day 54 is completely gone by

TABLE 1. CONCENTRATION OF S-(β-CARBOXYETHYL)-CYSTEINE AND TOTAL IMINO ACIDS IN SEEDS AND YOUNG SEEDLINGS OF *Calliandra rubescens*

		S-CEC		Imino acids	
Seedling age (days)	Plant wt (mg)	Total (mg)	% Total plant wt	Total (mg)	% Total plant
0 (seed)	85	2.5	2.9	1.2	1.4
14	716	1.9-2.5	0.35	3.8	0.50
24	602	1.2–1.8	0.30	Not sampled	
42	634	1.2–1.8	0.28	2.2	0.33
54	1253	0.6–1.0	0.08	4.3	0.34
70	2456	0.8–1.6	0.07	8.1	0.33

TABLE 2. DISTRIBUTION OF S-(β-CARBOXYETHYL)-CYSTEINE IN LEAVES OF 14- TO 70-DAY-OLD *C. rubescens* SEEDLINGS (mg/g FRESH WEIGHT)

		Leaves									
Seedling age (days)	Seed	1	2	3	4	5	6	7	8	9	10
0	29.0										
14		3.3–4.4									
28		0.4–1.1	0.5–1.2	0.9–1.9							
42		0.4–0.9	–[a]	<0.2	0.9–1.7	2.6–3.3					
54		0.3–0.7	–	–	<0.2	1.6–2.2	2.7–3.3				
70		0.3–0.8	–	–	–	–	–	–	0.2–0.5	1.6–2.2	1.9–2.4

[a] indicates compound was not detected.

TABLE 3. DISTRIBUTION OF S-(β-CARBOXYETHYL)-CYSTEINE AND IMINO ACIDS IN LEAVES OF 70-DAY-OLD *C. rubescens* SEEDLINGS, LEAVES OF MATURE PLANTS, AND SEEDS[a]

	Leaves (order of emergence)										Mature	
	1	2	3	4	5	6	7	8	9	10	leaves	Seeds
S-CEC	+[b]	–	–	–	–	–	–	+	++	+++	–	+++++
CT	+	+	+	+	++	+	+	++	++	++	++	+++
C5	++	+	++	++	++	+	++	++	++	++	++	++
T5	+	+	+	+	++	+	+	+	++	++	++	+++++
PIP	+	+	+	+	+	+	+	+	+	+	VAR.	+

[a] S-CEC = S-(β-carboxyethyl)-cysteine; CT = *cis-trans*-4,5-dihydroxypipecolic acid; C5 = *cis*-5-hydroxypipecolic acid; T5 = *trans*-5-hydroxypipecolic acid; PIP = pipecolic acid.
[b] –, not detected; +, 0–1.0 mg/g; ++, 1.1–2.0; +++, 2.1–4.0; ++++, 4.1–6.0; +++++, >6.1.

day 70. Even more striking is the situation at day 70 where the oldest "new" leaves (those appearing since day 42) 7 and 8 have already lost all or most of their S-CEC, whereas leaves 9 and 10 which appeared later still have relatively large amounts. The only exception to this pattern is in the first leaf where the decrease over time is constant but considerably slower.

Imino acids derived from pipecolic acid also are found at high concentrations in both seeds and leaves. In contrast to S-CEC, total imino acids increase slowly with development. The percentage of total plant weight remains virtually constant, although somewhat less than in seeds (Table 1). The concentration of S-CEC in different leaves relative to various imino acids at day 70 is seen in Table 3. While there is some modest variation between leaves, imino compounds are evenly distributed in the foliage in a 10-week-old seedling. There is no distinct difference between young and old leaves as for S-CEC. The imino

TABLE 4. DISTRIBUTION OF IMINO ACIDS AND S-(β-CARBOXYETHYL)-CYSTEINE IN FIFTH LEAF OF *C. rubescens* SEEDLINGS (mg/g FRESH WEIGHT)[a]

Seedling age (days)	COMPOUND				Total imino acids	S-CEC
	CT	T5	C5	PIP		
42	0.7	1.4	1.8	0.1	4.1	2.6–3.3
54	0.7	1.1	1.6	<0.1	3.5	1.6–2.2
70	1.3	1.1	1.7	<0.1	4.3	—

[a] CT = *cis-trans*-4,5-dihydroxypipecolic acid; T5 = *trans*-5-hydroxypipecolic acid; C5 = *cis*-5-hydroxypipecolic acid; PIP = pipecolic acid; S-CEC = S-(β-carboxyethyl)-cysteine.

TABLE 5. DISTRIBUTION OF S-(β-CARBOXYETHYL)-CYSTEINE IN ROOTS, STEMS, AND YOUNG LEAVES OF *C. rubescens* SEEDLINGS

Seedling age (days)	Root	Stem	Young leaves
5	++++[a]	++++	+++++
10	++	++++	++++
20	−	++++	+++
30	−	++++	+++
40	−	++++	+++
50	+	++++	+++
60	+	++++	+++
70	+	+++	+++

[a] − = not detected; + = 0–1.0 mg/g fresh wt; ++ = 1.1–2.0 mg/g fresh wt; +++ = 2.1–4.0 mg/g fresh wt; ++++ = 4.1–6.0 mg/g fresh wt; +++++ = >6.1 mg/g fresh wt.

compounds remain essentially unchanged within a particular leaf over time. This is seen in Table 4 where imino acid concentrations are compared within a leaf from its appearance to termination of the experiment at day 70. By the time the fifth leaf has appeared at day 42, imino acid concentrations in the plant are approaching those which are found in mature leaves and seeds. Successive young leaves all produce similar high concentrations and maintain them to maturity.

Concentrations of S-CEC in stems over time generally parallel those found in new leaves, while in the root S-CEC decreases steadily to day 20 at which time it disappears only to reappear in moderate concentrations by day 50 (Table 5). Although the experiment was terminated after 10 weeks, traces of S-CEC continued to persist in very young leaves up to nine months after germination.

DISCUSSION

As Clegg et al. (1979) have pointed out, the developmental fate of defensive secondary compounds present in seeds is an important consideration in the pattern and budget of chemical defense of plants against herbivores. In their study, the cyanogenic glucoside linamarin, which is present in large amounts in seeds of *Phaseolus lunatus*, was shown to be a defensive resource as well for the developing seedling. In our study, the restriction to the stem and young leaves only of large amounts of an insecticidal nonprotein amino acid which is the major soluble nitrogen component of seeds is the interesting finding. Whether S-CEC is simply retained in the young leaves, having been transported from the seeds, or is synthesized de novo in the leaves or roots is uncertain.

One of the most often proposed secondary functions for nonprotein amino acids is that of serving as an additional nitrogen reserve for the germinating seedling. Labeling studies have shown that considerable interconversion and metabolism of amino acid carbon occurs in storage tissue during germination (Lea and Joy, 1983). Particular protein amino acids, usually glutamine or asparagine, accumulate and are involved in transport of nitrogen to the growing seedling for further conversion to supply the full range of amino acids and nitrogenous compounds needed for protein synthesis and growth. Often, however, the amides (ASN and GLN) are supplemented or replaced by other amino acids. For example, ornithine has a transport role in pumpkins, and homoserine in peas (Larson and Beevers, 1965). Since total S-CEC does not drop significantly until two weeks after germination, such a role does not seem likely for S-CEC in *C. rubescens*. While transport remains a viable explanation for the concentration of S-CEC in the stems and emerging leaves, the lengthy persistence (70 days) of significant amounts raises the possibility that S-CEC is being synthesized somewhere in the young seedling. The relatively high and constant concentrations in stems and the varying concentrations in roots permit the hypothesis that S-CEC is synthesized by roots and transported to young leaves or, alternatively, that the leaves are synthesizing the compound and sending the excess into the phloem and downward. Labeling experiments are needed to answer this question.

The possibility that S-CEC is serving a function other than nitrogen storage deserves consideration. Although generally less toxic than alkaloids or cyanogenic glycosides (both of which are absent in *Calliandra* spp.), nonprotein amino acids are widely perceived as being part of the chemical defense of higher plants (Rosenthal and Bell, 1979). *Calliandra* seeds and new young leaves appear to have a double defense in the large quantities of both nonprotein sulfur amino acids and the imino acids they contain. In addition to S-CEC, at least four other nonprotein sulfur amino acids including djenkolic acid and S-(β-carboxyisopropyl)-cysteine are found in lesser but significant concentrations (Romeo and Swain, unpublished). Both S-CEC and djenkolic acid are known to be insecticidal from a number of reports (Rehr et al.,1973; Janzen et al., 1977; Navon and Bernays, 1978; Evans and Bell, 1979; Shea and Romeo, unpublished). Additionally, an array of nine rare imino acids are spread variously throughout members of the genus (Romeo et al., 1983). The imino acids are present in concentrations at which insecticidal activity has been demonstrated (Romeo, 1984a). Unlike the seeds, mature *Calliandra* leaves accumulate only the imino compounds. These high concentrations are a predictable feature, and the genetic basis for this accumulation has been established by populational sampling (Romeo, 1984b). New leaves, however, have not yet developed the additional physical attributes that one associates with chemical protection in mature leaves such as toughness, waxiness, and decreased water content. The presence of both types of defensive amino acids in the young leaves as well as the seeds may be

simply a reflection of the need to protect those parts which are most essential to survival and most vulnerable to herbivory.

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DEMONSTRATION OF AN ODOROUS INTRAMALE PRIMER EFFECT IN SHORT-TAILED VOLE, *Microtus agrestis* L.

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Abstract—Anal (proctodeal) glands of male *Microtus agrestis* housed in social isolation undergo severe atrophy. Their weight and volume is significantly lower than those of the stock control males. The atrophied glands can be revived by subjecting deprived voles to various social odors. Atrophied glands of isolated males do not respond to the odors of male and female urine, voided feces of females, and unvoided feces of males. Atrophied anal glands of males exposed to voided male feces (which have passed the orifice of the anal gland) and soiled bedding from adult males show strong recrudescence. The mean weight and volume of the glands and plasma testosterone level are significantly higher than of males maintained in complete social isolation, although they are significantly less than those of stock control males. Atrophied glands of socially deprived males strongly respond to the odor of ethereal extract of gland secretion. In males exposed daily to anal gland secretion extract, the weight and volume of the gland and plasma testosterone level increase and are not significantly different from those of stock controls. They enjoy higher plasma testosterone levels and consequently larger and more active anal glands than complete isolates.

Key Words—Vole, *Microtus agrestis*, anal gland, odor, urine, feces, testosterone, social deprivation.

INTRODUCTION

Although they appear to be widespread among mammals, little attention has been paid to anal (proctodeal) glands. In small rodents of the genus *Microtus*

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they occur at the distal end of the rectum, enveloped by the rectal sphincter muscles (Vrtis, 1929; Khan, 1984), but this envelopment is not universal among small mammals (Stoddart, unpublished). In his thorough study of the anal gland complex in the European vole *M. arvalis*, Vrtis (1929) observed that the gland of males was much larger than in females and that it underwent a cycle of development linked with the testicular cycle. Although he presented no behavioral data in substantiation, he suggested that its oily secretion was used as a sexual attractant or stimulant during the voles' breeding season.

More recently, and working with the yellow vole *Lagurus luteus*, Fan (1982) observed that fecal pellets coated in anal gland secretion were deposited densely around the breeding nest and along the colony's territorial boundaries, in such a way that it suggests the function of the glandular secretion is not directly for sexual purposes but for a more broadly based social effect.

This study was undertaken as part of a comprehensive examination of the structure and development of the anal glands of the short-tailed vole, *M. agrestis*, which investigated, in particular, the physiological and anatomical response of the gland to sex hormones (Khan, 1984). Since we knew the anal gland of *M. agrestis* to be highly sensitive to circulating levels of blood testosterone (Khan and Stoddart, in preparation), we designed a series of experiments to examine whether anal gland secretion, which is normally deposited passively on feces, is able to influence anal gland development in other males via a testosterone effect. At the same time we investigated whether urine odor was also able to influence plasma testosterone, and hence anal gland size, as has been shown to occur in rats.

METHODS AND MATERIALS

All experimental and control voles (other than controls taken from stock), were housed individually in shoe-box cages and fed with standard laboratory food and water ad libitum. Stock voles were housed in mixed sex groups of six to eight in rat cages in an animal house where the lighting regime was 16 hr light and 8 hr dark. Sociosexually isolated voles were removed from their mothers at weaning (24 days) and kept individually in shoe-box cages. Each cage was kept under the same light conditions in a separate room of the Zoology Department of King's College for eight weeks. At this time the isolated voles were subjected either to an experimental odor treatment for three weeks or to a further three-week isolation. Voles in the latter category are referred to as "complete isolates."

Urine and feces were separately collected in a glass metabolic chamber, removed daily, and stored in a deep freeze until needed. To prepare anal gland extracts, glands of mature stock males were cleared of any surrounding tissue or fat and macerated in ether until the ether became cloudy and little was left

of the sebaceous parenchyma. Supernatant extract was pipetted off and stored in a deep freeze until needed. For presentation to a test subject, a piece of extract-impregnated filter paper (2 × 1 cm) was laid daily on the sawdust in the subject's cage. Ether alone was used for controls. To expose a subject to the odor of urine, a small vial was filled with glass wool soaked in urine and attached to the side of the cage holding the experimental male. In the case of the controls, urine was replaced by distilled water.

Anal gland weight was obtained by weighing with a microgram balance following dissection and removal of the rectal sphincter musculature and adhering mesentery. Gland volume was measured by volumetric displacement, using a modified microvolumeter (Khan and Stoddart, in preparation). In control males, a linear relationship between body weight and gland weight and volume was observed. Accordingly, these parameters have been scaled up or down, as appropriate, to what their value would have been had they been taken from a vole of standard weight of 20 g (Khan, 1984). Plasma testosterone was established by radioimmunoassay (Collins et al., 1972). The number of subjects in each test is shown in the tables, but was never more than 10.

RESULTS

The results shown in Table 1 indicated that very substantial differences with standard parameters occur between the stock males and those subjected to complete sociosexual isolation. Isolated males exposed to female urine, male urine, female feces odor, and unvoided male feces show no significant differences in the standard parameters when compared to the complete isolates, but all show a substantial difference when compared to the stock males. Males exposed to the odor of voided male feces, in contrast, show considerable similarity with the stock males, and substantial differences from the complete isolates. It is noticeable, however, that anal gland weight and volume are significantly lower than those of stock males, but they are noticeably higher than those of subjects exposed to the other odorants. Table 2 examines the effect of voided and unvoided feces odor on isolated males in more detail. It is apparent that there is no difference in testes weight between classes of subjects but substantial differences in the other parameters. In Table 3 a comparison is made between males exposed to voided feces odor and soiled bedding odor taken from stock cages. This table indicates that there is no difference in the standard parameters between these groups with the exception of testes weight, which is significantly higher in the soiled bedding exposure trials than in the male voided feces trials.

As the only perceptible difference between voided and unvoided feces is the presence of or absence of the coating of anal gland secretion, experiments were designed in which isolated males were exposed to anal gland secretion. The results are shown in Table 4. It is evident that, when compared to complete

TABLE 1. COMPARISON OF STANDARD PARAMETERS OF MALES EXPOSED TO VARIOUS ODORANTS WITH STOCK MALES AND COMPLETE ISOLATES[a]

Standard parameters	Stock males ($N = 30$)	Isolated males exposed to female urine ($N = 10$)	Isolated males exposed to male urine ($N = 10$)	Isolated males exposed to female feces odor ($N = 10$)	Isolated males exposed to male unvoided feces odor ($N = 11$)	Isolated males exposed to male voided feces odor ($N = 15$)	Complete isolates ($N = 15$)
Body weight (g)	33.72^{ACD}_{C}	21.04^{A}	22.19^{A}	20.1^{D}	21.10^{C}	28.5_{C}	21.8_{C}
Gland weight (mg)	42.71^{CD}_{D}	5.47^{D}	7.70^{C}	6.6^{D}	8.65^{D}	19.91^{D}_{D}	6.29_{D}
Gland volume (ml)	0.040^{BD}_{D}	0.005^{D}	0.006^{B}	0.006^{D}	0.006^{D}	0.015^{D}_{D}	0.005_{D}
Weight of testes (mg)	264.00^{B}_{D}	147.98^{B}	88.66	152.6^{D}	192.98	251.27_{C}	158.22_{CD}
Plasma 'T' (pg/ml)	590.86^{BCD}_{D}	32.59^{D}	74.90^{D}	42.8^{B}	62.11^{C}	339.42_{C}	70.89_{CD}

[a] A = $P < 0.05$, >0.02; B = $P < 0.02$, >0.01; C = $P < 0.01$, >0.001; D = $P < 0.001$. Superscripts = comparisons (within horizontal rows) with stock males. Subscripts = comparisons (within horizontal rows) with complete isolates. No superscripts or subscripts denotes no significant difference.

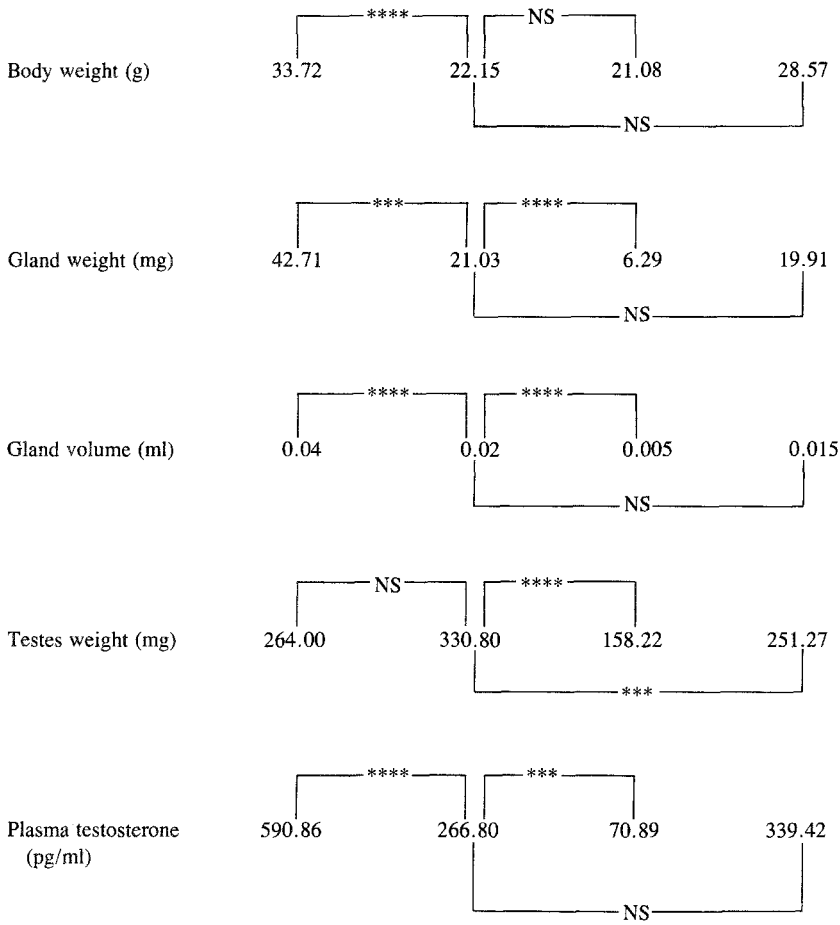
TABLE 2. COMPARISON OF STANDARD PARAMETERS OF ISOLATED MALES EXPOSED TO MALE VOIDED FECES ODOR WITH THOSE OF STOCK CONTROLS, COMPLETE ISOLATES, AND MALES EXPOSED TO UNVOIDED MALE FECES ODOR[a]

Standard parameters	Isolated males exposed to male voided feces odor (N = 15)	Complete isolates (N = 15)	Isolated males exposed to unvoided male feces odor (N = 10)
Body weight (g)	28.57	*** 21.08	21.08
		***	
Gland weight (mg)	19.91	**** 6.29	8.65
		**	
Gland volume (ml)	0.01	**** 0.005	0.006
		***	
Weight of testes (mg)	251.27	*** 158.22	192.98
		NS	
Plasma testosterone (pg/ml)	339.46	*** 70.89	62.11
		***	

[a]Levels of statistical significance: **** = $P < 0.001$; *** = $P < 0.01$, >0.001; ** = $P < 0.02$, >0.01; * = $P < 0.05$, >0.02; NS = not significant.

TABLE 3. COMPARISON OF STANDARD PARAMETERS OF MALES EXPOSED TO SOILED BEDDING ODOR FROM STOCK CAGES WITH THOSE OF STOCK CONTROLS, COMPLETE ISOLATES, AND MALES EXPOSED TO MALE VOIDED FECES ODOR[a]

Standard parameters	Stock males (N = 20)	Males exposed to soiled bedding odor (N = 10)	Complete isolates (N = 15)	Males exposed to male voided feces odor (N = 15)
Body weight (g)	33.72	22.15	21.08	28.57
Gland weight (mg)	42.71	21.03	6.29	19.91
Gland volume (ml)	0.04	0.02	0.005	0.015
Testes weight (mg)	264.00	330.80	158.22	251.27
Plasma testosterone (pg/ml)	590.86	266.80	70.89	339.42



[a] Levels of significance as in Table 2.

TABLE 4. COMPARISON OF STANDARD PARAMETERS OF MALES EXPOSED TO MALE ANAL GLAND SECRETION EXTRACT WITH THOSE OF STOCK CONTROLS AND COMPLETE ISOLATES[a]

Standard parameters	Stock males ($N = 16$)[b]	Males exposed to gland secretion extract ($N = 15$)	Complete isolates ($N = 15$)
Body weight (g)	23.97	NS 25.44	NS 21.08
Gland weight (mg)	23.96	NS 18.40	*** 6.29
Gland volume (ml)	0.021	NS 0.018	**** 0.005
Weight of testes (mg)	300.14	NS 244.73	* 158.22
Plasma testosterone (pg/ml)	220.15	NS 163.76	*** 70.89

[a] Levels of significance as in Table 2.
[b] Stock males for this experiment were exposed to ether-soaked filter paper with the same frequency as the experimental voles were subject to ethereal extract.

isolates, extract-exposed voles experience a development of the gland and a rise in blood testosterone, such that there is no difference between the experimental group and stock males. In Table 5 this group is compared with those exposed to voided male feces odor and soiled sawdust from stock cages. The most significant observation is that the plasma testosterone level of this group is significantly lower than that of either groups with which it is compared.

TABLE 5. COMPARISON OF STANDARD PARAMETERS OF MALES EXPOSED TO GLAND SECRETION EXTRACT WITH THOSE OF MALES EXPOSED TO MALE VOIDED FECES ODOR AND THOSE EXPOSED TO SOILED SAWDUST FROM STOCK CONTROL CAGES[a]

Standard parameters	Males exposed to male voided feces odor (N = 15)	Males exposed to gland secretion extract (N = 15)	Males exposed soiled sawdust from stock cages (N = 10)
Body weight (g)	28.57	25.44	22.15
	NS	NS	
		NS	
Gland weight (mg)	19.91	18.40	21.03
	NS	NS	
		NS	
Gland volume (ml)	0.015	0.018	0.02
	NS	NS	
		NS	
Weight of testes (mg)	251.27	244.73	330.80
	NS	*	
		*	
Plasma testosterone (pg/ml)	339.42	163.76	266.80
	**	**	
		*	

[a]Levels of significance as in Table 2.

DISCUSSION

We have demonstrated elsewhere that sociosexual isolation of newly weaned males *M. agrestis* for between 8 and 11 weeks results in severe atrophy of the anal gland. Testes and seminal vesicle weights are significantly lower than in controls of the same age and body weight, as are plasma testosterone levels (Khan and Stoddart, in preparation). The present experiments reveal that the odor of male or female urine, unvoided male feces and voided female feces have no effect on atrophied anal glands of deprived males. With respect to the effect of female urine odor, these data are contrary to those obtained by Purvis and Haynes (1978) on rats.

It was assumed from anatomical investigation that voided feces would be coated with anal gland secretion. Sociosexually deprived males showed a significant increase in the standard parameters when exposed to the odor of voided feces of stock males, although the glands fail to reach the weight and volume of the glands of stock males. Males exposed to voided feces odor also have significantly heavier bodies than complete isolates. Soiled sawdust from the stock cages, and presumably impregnated with odors other than just those of urine and feces, brought about a similar increase in gland size. An ethereal extract of anal gland secretion similarly returned anal gland size to that of the stock controls, but it is significant to note that, with the exception of testes weight, all the standard parameters of this group were lower than those of the stock controls used in other experiments. It would appear that ether odor has an inhibitory effect on the anal glands and plasma testosterone level in stock males.

Our observation that exposure of isolated males to voided male feces which are coated with anal gland secretion, or to soiled bedding from control cages of normal healthy animals, or to the extract of anal gland secretion itself for three weeks causes enlargement of the weight and volume of anal glands. A concomitant increase in plasma testosterone level suggests that the odor of anal gland secretion of *M. agrestis* modulates the endocrine status of males and may be regarded as an important component of the social environment. Our data refer only to exposure periods of three weeks' duration; we know nothing of any chronic effect of prolonged exposure or habituation arising therefrom.

We can tentatively conclude that anal gland secretion exerts an odorous effect on the anal gland of other males via its effect on plasma testosterone. It appears that odors of female urine, male urine, female feces, and unvoided male feces have no effect on male plasma testosterone levels and the anal gland. The biological significance of this intramale primer effect remains unclear.

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Sabulodes caberata GUENÉE (LEPIDOPTERA: GEOMETRIDAE) Components of Its Sex Pheromone Gland[1]

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Abstract—A 6,9-nonadecadiene, (Z)-9-nonadecene, and *n*-nonadecane were identified in extracts of sex pheromone glands of female *Sabulodes caberata* Guenée. Potential pheromonal activity of gland components was assessed by electroantennogram measurements of gas-chromatographic fractions of abdominal tip extracts. Chemical identification was based on gas chromatography and mass spectrometry of parent compounds, hydrogenation products, ozonolysis products, dimethyl disulfide adducts, and synthesis. The monoene was synthesized via coupling of alkyl and acetylenic intermediates. (Z,Z)-6,9-Nonadecadiene (Z6,Z9-19 : H) was synthesized from methyl linoleate via chain lengthening. Synthetic Z6,Z9-19 : H had the same retention times on polar and nonpolar capillary gas-chromatographic columns as the 6,9-nonadecadiene from the sex pheromone glands. In field tests Z6,Z9-19 : H alone produced trap catch of male *S. caberata* and addition of (Z)-9-nonadecene or *n*-nonadecane had no effect on trap catch.

Key Words—*Sabulodes caberata* Guenée, *Sabulodes aegrotata* Guenée, omnivorous looper, Lepidoptera, Geometridae, sex pheromone, (Z,Z)-6,9-nonadecadiene, (Z)-9-nonadecene, *n*-nonadecane

[1]This paper reports the results of research only. Mention of a commercial product in this paper does not constitute a recommendation by the U.S. Department of Agriculture.

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INTRODUCTION

Sabulodes caberata Guenée, the omnivorous looper, is a general feeder which is primarily an economic pest on avocados in California. It feeds on both foliage and fruit. As with other foliage feeders, higher populations can be tolerated before an economic threshold is reached than with pests that feed exclusively on the fruit. The identification of the sex pheromone of *S. caberata* was undertaken along with that of another fruit and foliage feeder of avocados, *Amorbia cuneana* (Walsingham) (McDonough et al., 1982; Hoffmann et al., 1983; Bailey et al., 1986), as part of a study to develop an integrated management program for these two insect species based on the use of *Trichogramma platneri* Nagarkatti parasites and pheromone monitoring traps.

Preliminary tests (Hoffmann et al., unpublished) with virgin female *S. caberata*-baited traps resulted in large captures of males, indicating the potential for using synthetic pheromone-baited traps to monitor this pest. Also *S. caberata* was shown to have an external sex pheromone gland (Smithhisler et al., 1985) in contrast to the internal gland reported for another species of Geometridae (Werner, 1977). The sex pheromone of *S. caberata* is chemically similar to the pheromones of other members of the geometrid family which have recently been reported.

METHODS AND MATERIALS

Insects

Pupae or larvae were collected from avocado groves in Ventura County, California, and the larvae were reared in a bioclimatic chamber at the University of California South Coast Field Station, Santa Ana, on artificial diet (Johnson and Federici, 1982). The pupae were sexed and sent to Yakima, Washington, where they were placed in a bed of moist vermiculite at 21 $\pm$ 1°C, relative humidity 68 $\pm$ 2%, and 14 hr light–10 hr dark. Emerging moths were collected daily. Males and virgin females were maintained separately on a diet of beer, sucrose, and ascorbic acid (Calkins and Sutter, 1976).

Collection of Pheromone

Female moths (2–4 days old) were collected 2–6 hr after the beginning of scotophase and placed in a refrigerator 10–60 min prior to dissection. Abdominal tips were excised at the 7th intersegmental membrane and extracted with dichloromethane for about 15 min. The sex pheromone gland (SPG) is epidermal tissue between the 8th and 9th abdominal segments (Smithhisler et al., 1985).

Gas Chromatography (GC)

The following analytical gas chromatography columns were used: (1) 60 m × 0.25 mm ID fused silica capillary column with methyl silicone bonded phase (DB-1, J and W Scientific, Inc., Rancho Cordova, California); and (2) 60 m × 0.25 mm ID glass capillary column with polyethylene glycol liquid phase (Carbowax 20 M®, Hewlett-Packard Corp., Bellevue, Washington). Both columns were operated in the splitless mode with the following program: 40 sec delay on inlet purge; 2 min at 80°C; 32°/min to various isothermal temperatures of 160–190°C for various runs; samples were used as heptane solutions.

Purification of SPG Components

Purification of SPG components from extracts was effected by collection from a gas chromatograph (Hewlett-Packard, model 5711, Avondale, Pennsylvania) equipped with a flame ionization detector and a metal, glass-lined, column-effluent splitter (Scientific Glass Engineering, Inc., Austin, Texas) (one part of effluent to detector and nine parts to collection trap). The column was 1.8 m × 2.3 mm OD silanized glass containing 3% methyl silicone (SE-30®) on 80–100 mesh Gas Chrom Q®. Eluting materials were collected with an efficiency of 70–90% in glass tubes cooled in Dry Ice–acetone.

Electroantennogram Measurements (EAG)

Natural and synthetic samples were bioassayed by the EAG procedure as previously described (Kamm and McDonough, 1980).

Ozonolysis

Ozonolysis of EAG active material, which had been purified by gas chromatography, was conducted in a mixture of dichloromethane and heptane at −70°C with an ultraviolet ozonizer (Orec Co., model 03VI, Phoenix, Arizona), and the ozonide was reduced with triply recrystallized triphenyl phosphine (Beroza and Bierl, 1967). The ozonized sample was injected on an analytical gas chromatographic column (1) at 35° for 6 min, programmed at 10°/min to 200°C and then held at that temperature. The retention times of eluting peaks were compared with *n*-alkanal standards.

Hydrogenation of Diene–Monoene Fraction

The purified EAG-active material was allowed to react with lithium aluminum hydride plus platinum catalyst by the procedure of Bierl-Leonhardt and DeVilbiss (1981). The hydrogenated material was then analyzed by gas chro-

matography–mass spectrometry (GC-MS) and compared with authentic hydrocarbons.

Dimethyl Disulfide (DMDS) Derivative of Monoene

The DMDS derivative was prepared by the procedure of Buser et al. (1983). A dichloromethane solution containing 30 ng of monoene and 570 ng of diene which had been isolated from SPG extract by gas chromatography was transferred to a 1-ml Reacti-vial® (Pierce Chemical Co., Rockford, Illinois) and evaporated just to dryness in a stream of nitrogen. The residue was dissolved in 50 μl heptane and 50 μl of DMDS, and 10 μl of a saturated solution of I_2 in diethyl ether were added. The vial was sealed and the mixture was heated overnight at 40°C in a convection oven. Then the solution was diluted with 200 μl heptane and excess I_2 was removed by adding 100 μl of 5% $Na_2S_2O_3$ solution and shaking. The heptane layer was removed with a syringe and concentrated to 20 μl; 2 μl was used for mass spectral analysis.

Gas Chromatography–Mass Spectrometry

Two different GC-MS instruments were used: (1) Finnegan Corp. (Sunnyvale, California) model 4000, quadrupole mass spectrometer with computerized data collection and a GC inlet consisting of a methyl silicone (SP-2100®) capillary column (30 m × 0.25 mm ID) with splitless injector; and (2) Hewlett-Packard (Avondale, Pennsylvania) model 5970 quadrupole mass selective detector with computerized data collection and a model 5790 gas chromatograph inlet equipped with fused silica capillary column (60 m × 0.25 mm ID) with splitless injection. The liquid phase was methyl silicone (DB-1®). Instrument 1 was used to characterize SPG components and their hydrogenation products. Instrument 2 was used to characterize SPG components, the DMDS derivative of the monoene, and the synthetic intermediates and products.

Synthesis

9-Nonadecyne. 1-Decyne (1.70 g, 12.3 mM) and lithium amide (1.1 g, 48 mM) in 50 ml dry dioxane were refluxed under nitrogen for 3.5 hr and cooled to room temperature. Then 1-bromononane (5.6 g, 27 mM) was added dropwise over a period of 30 min. The mixture was refluxed for 18 hr, cooled, and extracted with water–hexane. The hexane layer was washed successively with 2 N HCl and water and then dried with Na_2SO_4. The yield after liquid chromatographic purification (silica gel and hexane) by gas chromatographic analysis was 1.27 g (39%).

(Z)-9-Nonadecene (Z9-19H) and (E)-9-Nonadecene (E9-19:H). 9-Nonadecyne (0.2 g) in 40 ml ethanol containing four drops of quinoline and 0.4 g palladium (5%)–calcium carbonate catalyst was reduced with hydrogen for 5

min. The solution was filtered and partitioned into hexane and water, and the hexane layer was dried with sodium sulfate. Gas chromatographic analysis showed 86.1% *Z*9-, 13.9% *E*9–19:H, 3.0% *n*-nonadecane, and no unreacted 9-nonadecyne. GC-MS confirmed these products.

9-Nonadecyne (50 mg) was also reduced with "P-2 nickel" (Brown and Ahuja, 1973a,b) to give a product of 98% *Z*9- and 2% *E*9–19:H.

Linoleyl Alcohol. Methyl linoleate (25 g) was added dropwise to a stirred solution of 5 g lithium aluminum hydride in 300 ml ether. Excess hydride was destroyed with ethyl acetate, and the ether solution was extracted successively with ammonium chloride solution and water. The yield of linoleyl alcohol after liquid chromatography (silica gel, hexane–ether, 3:1) was 17.5 g (77%).

Linoleyl Bromide. Phosphorous tribromide (18.0 g) in 100 ml of dry dichloromethane was added dropwise over 1.5 hr to a stirred solution of 17.5 g of linoleyl alcohol in 100 ml dry dichloromethane cooled with an ice bath. Then water was added dropwise until the fuming stopped. The solution was washed with water and dried with sodium sulfate. The yield of product after liquid chromatography (silica gel, hexane) was 7.3 g (34%).

Linoleyl Iodide. A solution of linoleyl bromide (7.3 g, 22 mM) and sodium iodide (6.6 g, 44 mM) in 66 ml of acetone was stirred for 2.5 hr. Hexane (300 ml) was added, and the solution was filtered and washed with water. The yield after liquid chromatography (silica gel, hexane) was 6.6 g (80%).

(Z,Z)-6,9-Nonadecadiene (Z6,Z9–19:H). Methyl magnesium iodide (25 mM) from 3.5 g of methyl iodide and 0.66 g of magnesium in 30 ml of ether was added dropwise to a cooled (0–10°C) and stirred solution of 6.6 g (17.5 mM) linoleyl iodide, 0.074 g (1.75 mM) lithium chloride, and 0.12 g (0.88 mM) cupric chloride in 150 ml tetrahydrofuran (Fouquet and Schlosser, 1974). When the addition of methyl magnesium iodide was completed, the solution was allowed to warm to room temperature over an hour, and then partitioned into hexane–water. After work-up and liquid chromatography (silica gel, hexane), the yield was 4.5 g (98%).

Field Tests

Field tests were conducted in commercial avocado groves located in Ventura and Orange counties. Synthetic pheromone was tested after impregnation of dichloromethane solutions into rubber septa (West Co., Phoenixville, Pennsylvania) which were placed in Pherocon® 1C traps (Zoecon Corp., Palo Alto, California). Traps were hung from peripheral branches on mature avocado trees, 1–3 m above the ground, at a distance between traps of no less than 40 m. Each treatment was replicated four times and arranged in a randomized block design. Traps were rotated one position each time they were checked to minimize bias of location.

Traps were checked a number of times equal to an even multiple of the

number of treatments in the test. The Ventura county test was checked four times at three-day intervals. The Orange county test was checked 12 times at two-day intervals.

RESULTS

Structure. Figure 1 shows a GC trace (capillary, methyl silicone column) of the extract of the female SPG (0.4 female equivalent). When the extract from four females was injected into a GC with a methyl silicone-packed column and fractions corresponding to the volatility range encompassing zero time to the retention time of tetracosane were collected and their EAG activity determined, only the fraction containing peaks A and B of Figure 1 (which were not separated on the packed column) possessed strong EAG activity (3 mV vs. 1–1.5 mV for the other fractions). The retention times of peaks A and B relative to *n*-nonadecane on the methyl silicone column were 0.90 and 0.93, respectively,

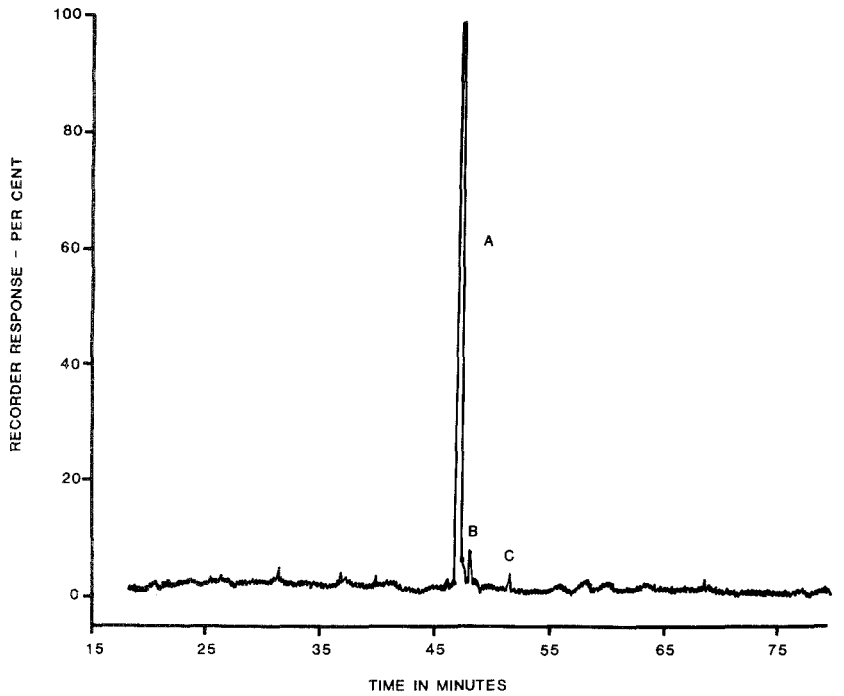

FIG. 1. Gas chromatogram of the extract of the female sex pheromone gland (0.4 female equivalent) of *S. caberata*. Components A and B were both in the EAG active fraction collected from the packed GC column. C is *n*-nonadecane. The amount of peak A represented by the GC trace was about 15 ng.

and on the Carbowax 20 M column were 1.11 and 1.04, respectively. The retention times suggest hydrocarbons with 19 or more carbon atoms and unsaturation. Mass spectra showed A to be a doubly unsaturated 19 carbon hydrocarbon (M+, m/z = 264) and B to be a singly unsaturated 19 carbon hydrocarbon (M+, m/z = 266). Peak C (Figure 1) was *n*-nonadecane. When the GC collected fraction containing A and B was hydrogenated, the only product was *n*-nonadecane based on retention time and mass spectrum. The only products detected from ozonolysis were hexanal and decanal, indicating a 6,9-nonadecadiene.

The addition of dimethyl disulfide (DMDS) to a monoene increases the GC retention index by about 600 units. Thus, when the EAG active fraction of 30 ng of nonadecene and 570 ng of 6,9-nonadecadiene was converted to the dimethyl disulfide derivative, only one GC peak was expected corresponding to mono addition. However, two peaks were found in that region. The second eluting peak was narrow and had the molecular weight (M+, m/z = 360, 30%) and principal peaks (m/z = 187, 100%; m/z = 173, 92%) corresponding to the DMDS adduct of 9-nonadecene. The first eluting peak was broad and had a molecular weight (M+, m/z = 358, 7%) and strong peaks (m/z = 131, 31%; m/z = 227, 16%; m/z = 171, 17%; m/z = 187, 38%) corresponding to a mixture of mono adducts to 6,9-nonadecadiene. The first eluting broad peak was of about the same intensity as the monoene adduct. Thus, the percent of mono adduct formation by the diene was about 5%.

Synthesis. 9-Nonadecene was synthesized using a catalyst which produces mainly *Z* but also a significant quantity of the *E* isomer. The 9-nonadecene from the SPG had the same retention time as the synthetic *Z* isomer. Synthesis with the more modern catalyst, P-2 nickel, gave a product of 98% *Z* isomer which was used for the field tests.

*Z*6,*Z*9–19 : H was conveniently synthesized from methyl linoleate. It had the same mass spectrum and the same retention times as 6,9-nonadecadiene from the SPG on both methyl silicone and Carbowax 20 M capillary columns.

Field Tests. Field tests were conducted during a period of low moth flight activity. The first field test was conducted to determine what combination of components was important for trap catch (Table 1). *Z*6,*Z*9–19 : H effected trap catch by itself and addition of *Z*9–19 : H or *n*-nonadecane in proportions in which they occurred in the SPG had no effect on trap catch. The second field test was conducted to determine the effect of dosage on trap catch (Table 2). Trap catch was constant for dosages of 3–30 mg; 1 mg was not quite as effective.

DISCUSSION

Although the configuration of 6,9–19 : H from the SPG has not been rigorously established, it is probably the *ZZ* isomer. Capillary columns separate *Z*

TABLE 1. TEST OF IDENTIFIED COMPONENTS FROM SPG OF FEMALE *S. caberata*[a]

Lure composition	Total catch, Significance[b]
1 mg Z6,Z9-19:H	54 a
1 mg Z6,Z9-19:H + 3% Z9-19:H	54 a
1 mg Z6,Z9-19:H + 3% Z9-19:H + 2.5% 19:H	59 a
Blank	0 b

[a] Proportions found in the gland were used. Four replicates and a 12-day test. December 1983, Ventura County.
[b] Duncan's new multiple-range test ($P = 0.05$).

and *E* isomers; retention times of *ZE* and *EZ* isomers might overlap, but *EE* and *ZZ* should have distinctive retention times. Thus, the coincidence of the Z6,Z9-19:H retention times on polar and nonpolar capillary columns with the SPG component and the demonstrated attractancy of Z6,Z9-19:H in field tests strongly suggests that the main active component of the SPG is Z6,Z9-19:H.

Z6,Z9-19:H has been previously reported as a sex pheromone component of another geometrid, *Bupalus piniarius* L. (Bestmann and Vostrowsky, 1982). Also, in combination with Z3,Z6,Z9-19:H, Z6,Z9-19:H has been found in field screening tests to be attractive to *Alsophila quadripunctata* Esp. (Szöcs et al., 1984).

The first reports of the structures of sex pheromones of species of Geometridae were made relatively recently. The sex pheromone of the winter moth, *Operophtera brumata* (L.), is (1,3Z,6Z,9Z)-1,3,6,9-nonadecatetraene (Roelofs et al., 1982; Bestmann et al., 1982). Becker et al. (1983) reported (Z,Z,Z)-3,6,9-nonadecatriene to be a component of the giant looper, *Boarmia* (*Ascotis*) *selenaria* Schiffermüller, and Wong et al. (1984) found the sex pheromone of

TABLE 2. EFFECT OF DOSAGE OF (Z,Z)-6,9-NONADECADIENE ON TRAP CATCH OF MALE *S. caberata*[a]

Dose (mg)	Catch/trap, significance[b]
1	28.8 b
3	42.3 a
10	47.3 a
30	44.0 a
Blank	0 c

[a] Test from May 14 to June 6, 1984; 4 Replicates; Orange County.
[b] Duncan's new multiple-range test ($P = 0.05$).

the fall cankerworm moth *Alsophila pometaria* (Harris) to be a ternary mixture of (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene, (*Z*,*Z*,*Z*,*E*)- and (*Z*,*Z*,*Z*,*Z*)-3,6,9,11-nonadecatetraene. Thus far the common theme of all sex pheromone components of the Geometridae is a 19-carbon straight chain with 6*Z* and 9*Z* double bonds. In other studies (Wong et al., 1985), a variety of C_{18}–C_{22} unsaturated hydrocarbons and unsaturated epoxides have been found attractive to other species of Geometridae in field trapping tests.

*Z*6,*Z*9–19:H is conveniently synthesized from methyl linoleate, methyl (9*Z*,12*Z*)-9,12-octadecadienoate, by functional group modification and chain lengthening. Methyl linoleate is commercially available and inexpensive. This type of synthesis has been used previously by Underhill et al. (1983) and Heath et al. (1983) to synthesize noctuid sex pheromones of similar structure. Thus (3*Z*,6*Z*,9*Z*)-3,6,9-eicosatriene and (3*Z*,6*Z*,9*Z*)-3,6,9-heneicosatriene were synthesized from methyl linolenate, i.e., methyl (*Z*9,*Z*12,*Z*15)-9,12,15-octadecatrienoate. These syntheses may mimic the methods used by the insects. Recent biosynthetic studies have shown that sex pheromone components are synthesized from fatty acid moieties (Wolf et al., 1981; Bjostad and Roelofs, 1984, and references cited therein) and that lepidopteran insects possess enzymes for both chain lengthening and shortening of 18-carbon acids.

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PINOSYLVIN AND PINOSYLVIN METHYL ETHER AS FEEDING DETERRENTS IN GREEN ALDER

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Abstract—Snowshoe hare (*Lepus americanus*) feeding preferences for Alaskan green alder (*Alnus crispa*) are governed by the concentrations of two deterrent secondary metabolites, pinosylvin and pinosylvin methyl ether. For instance, the preference of mature internodes over juvenile internodes was correlated with about a threefold increase in the levels of these compounds in the juvenile form. During the last year of the study, however, the levels of these compounds dropped below the threshold of avoidance in both types of internodes, resulting in nondiscriminatory use by hares even though the relative levels of these metabolites remained the same between the juvenile and mature form internodes. These conclusions are strongly supported with feeding bioassays using pure pinosylvin, pinosylvin methyl ether, and other less active secondary metabolites found in alder.

Key Words—Pinosylvin, pinosylvin methyl ether, *Alnus crispa*, snowshoe hare, herbivore, plant defense, feeding deterrence, alder.

INTRODUCTION

Crowns of adult Alaskan green alder (*A. crispa*) are readily available to snowshoe hares in winter because snow bends them to the ground in late fall. Even though snowshoe hares frequently reside within the subnivean space created by snowbent adult *A. crispa*, twigs and bark of *A. crispa* are relatively lightly used as winter food by free-ranging hares as compared to highly palatable early successional woody plants such as *Salix alaxensis* (Wolf and Zasada, 1979; Bryant and Kuropat, 1980). When winter-dormant twigs of adult *A. crispa* are used, hares reject essentially all buds and catkins and eat internodes pri-

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marily less than 4 mm diameter (Bryant et al., 1983a). Very little use of juvenile form *A. crispa* has been observed. These observations are interesting in that green alder is an abundant shrub which contains relatively high levels of protein (Chapin and Kedrowski, 1983), a nutrient traditionally associated with high palatability of browse to snowshoe hares (e.g., Sinclair et al., 1982).

Several years ago we reported that the low palatabilities of *A. crispa* buds and catkins to the snowshoe hare are the result of the concentrations of pinosylvin methyl ether found in these plant parts (Bryant et al., 1983a). However, it was also clear that the low palatability of *A. crispa* internodes could not be attributed to the effects of this substance since pinosylvin methyl ether was present in internodes only in very low concentrations relative to those found deterrent in bioassays. Consequently we initiated a more detailed study of the chemistry of *A. crispa*.

This study had three objectives. The first objective was to gain a more complete understanding of the chemical basis of snowshoe hare use of *A. crispa* parts and growth stages. Our strategy for realizing this goal was: (1) determine what phytochemicals influence hare use of *A. crispa*, (2) quantify the levels of these substances in winter-dormant *A. crispa* growth stages and parts, and (3) correlate concentrations of biologically active substances with snowshoe hare use of winter-dormant *A. crispa*. The second objective was to determine if subtle differences in the structures of active components could affect food choice by snowshoe hares. The ramifications of realizing this objective would be the need for future work to place more emphasis on specific compounds and less on "total phenolics," "percent resins," "vanillin reactives," etc. Finally, we wished to determine the cause of the overall unpalatability of *A. crispa* as compared to *S. alaxensis*, a highly preferred species that frequently precedes *A. crispa* during plant succession in Alaska.

METHODS AND MATERIALS

Sample Collection and Storage. Winter-dormant, small diameter (up to 4 mm) twigs of both physiologically adult and juvenile (Kozlowski, 1971; Kramer and Kozlowski, 1979) *A. crispa* plants were collected at five locations near Fairbanks, Alaska, over three consecutive winters (1982–1984). Collection sites were old fields where *A. crispa* and Alaska feltleaf willow (*S. alaxensis*) were dominant species in primary succession. Each collection of adult material or juvenile material from each of the five locations contained five individual twigs sampled at random from each of five randomly selected adult or juvenile plants. When juvenile and adult *A. crispa* were directly compared, the juvenile and adult plants sampled were nearest neighbors (less than 3 m separation). This sampling procedure minimized chemical variation caused by site differences. All collections were stored in tightly sealed plastic bags at $-40°C$ prior to use in feeding trials or chemical analysis.

Isolation of Secondary Metabolites. Because chemical analyses of resins from different alder parts (buds, catkins, and internodes) demonstrated them to be qualitatively similar and because catkins were the most convenient source of resin, secondary metabolites were isolated from catkin extracts. Alder catkins (200 g; fresh wt) were soaked in 2.0 liters of acetone overnight. The acetone was decanted and flash evaporated to yield a viscous oil which was dissolved in 300 ml of ether and washed with two 100-ml portions of a saturated sodium chloride solution. After drying the ether ($MgSO_4$) and subsequent removal of solvent, 12.0 g (6.0% of catkin mass) of oil remained.

A chromatographic column was prepared by the addition of 1.2 kg of silica gel (60–200 mesh) in a methylene chloride slurry to a column (7.6 × 70 cm). After loading the column with the above extract, the sample was eluted with methylene chloride. The progress of the separation was monitored by thin-layer chromatography (silica gel; methylene chloride). Four major components (pinosylvin, pinosylvin monomethyl ether, pinostrobin, and 2-phenethyl cinnamate; Figure 1) were identified by their melting points as well as proton nuclear magnetic resonance, infrared, and mass spectra.

The first 2.8 liters of eluant yielded 1.06 g (8.8% of extract) of unidentified fats (R_f = 0.72). Bioassays of this fraction demonstrated negligible deterrence and hence additional investigations of this fraction were abandoned.

2-Phenethyl cinnamate (0.76 g, 6.3% of extract; R_f = 0.48) was obtained in relatively pure form (as shown by [^{1}H]NMR) as an oil in the 2.8- to 3.5-liter fraction. Because additional purification was difficult, pure material (mp = 57–58°C; lit. 56–57°C, Asakawa, 1970) was obtained by standard synthetic methods from cinnamic acid and benzyl alcohol.

Pinostrobin (2.81 g, 23.4% of extract; R_f = 0.33) was isolated as yellowish crystals in the 3.5- to 7.8-liter fraction. Pure pinostrobin (mp = 96–97°C; lit. 99–100°C, Asakawa, 1970) was readily obtained by flash chromatography (Still et al., 1978) (silica gel; methylene chloride) followed by recrystallization from petroleum ether (30–60°C).

Pinosylvin monomethyl ether (2.79 g, 23.3% of extract; R_f = 0.17), contaminated with pinostrobin (TLC), was isolated as brownish crystals in the 7.8- to 12.2-liter fraction. Pure colorless crystals (mp = 120–121°C; lit. 119–120°C, Asakawa, 1971) were readily obtained by recrystallization from petroleum ether–methylene chloride (1 : 1) followed by sublimation (140°C; 0.05 mm Hg).

After all of the pinosylvin monomethyl ether had eluted, the solvent composition was gradually changed to 20% ether (ca. 2%/liter). Pinosylvin (2.49 g, 20.8% of extract; R_f = 0.03) was obtained after an additional 12 liters of solvent was eluted. It is important that the solvent composition is changed gradually, otherwise the pinosylvin is obtained as a tar that is very difficult to recrystallize. Given patience in the elution, however, slightly discolored crystals were obtained that easily provided pure pinosylvin (mp = 156–157°C; lit. 156–158°C, Asakawa, 1971) by recrystallization from petroleum ether–methylene chloride (1 : 1).

Quantification of Secondary Metabolites. Plant parts (1–4 g) to be analyzed for secondary plant metabolite concentrations were extracted overnight in ether (10 ml/g plant). After filtration and drying ($MgSO_4$), the ether was removed and the residue weighed. Dry wt/fresh wt ratios were used to calculate the dry mass concentrations. The ratios used were 0.50 for internodes and 0.65 for buds and catkins.

High-pressure liquid chromatography (HPLC) was the method best suited for most of the quantifications except that of pinostrobin. Because pinostrobin was determined to be essentially inactive as an herbivore deterrent, a routine quantification of it was not required. When quantification of pinostrobin was required, gas–liquid chromatography (GLC) was used. The advantage of HPLC was the ability to analyze for pinosylvin, a strongly deterrent substance which could not be directly analyzed by GLC. Both methods of analysis are described below.

The plant residue described above was dissolved in a carefully measured volume of acetonitrile (ca. 20 ml) containing an accurately weighed amount of resorcinol (ca. 2 mg/g plant part). Twenty microliters of the solution was injected onto a C-18 reverse phase analytical column (25 cm × 4 mm) and was eluted with 70% aqueous acetonitrile (flow rate = 1.0 ml/min). Individual components were detected with an ultraviolet detector (Hitachi, model 100-10) set at 285 nm. As mentioned previously, pinostrobin was not detected using those conditions. The retention times for resorcinol, pinosylvin, pinosylvin monomethyl ether, and 2-phenethyl cinnamate were 3.0, 3.7, 5.6, and 12 min, respectively. Peak areas were obtained by interfacing an automatic digital integrator to the detector. In a separate experiment using a standard mixture, the relative sensitivities of pinosylvin, pinosylvin monomethyl ether, and 2-phenethyl cinnamate were 6.19, 6.28, and 7.62 g/g resorcinol, respectively. Reproducibility was within 2%.

In order to determine pinostrobin levels, GLC was utilized. The residue described earlier was dissolved in ether and spiked with a measured quantity of pinosylvin dimethyl ether as an internal standard. Quantitative analysis was carried out on a 1.8-m × 2-mm OV-101 (2%) column using a temperature program (T_1 = 100°C 4 min, 8°C/min; T_2 = 200°C) and a flame ionization detector. Flow rate was 55 ml/min. The retention times of pinostrobin and pinosylvin dimethyl ether were 5.8 and 9.1 min, respectively.

Test for Alkaloids. Three parts of *A. crispa* (buds, juvenile internodes, and mature internodes) were individually tested for alkaloids using Dragendorff's reagent (Gordon and Ford, 1972). Plant parts (ca. 20 g) were extracted for about 30 min with boiling ethanol (10 ml), and the resulting solution was filtered. Five drops of the filtrate were placed on a clean filter paper followed by one drop of Dragendorff's reagent. An orange spot is a positive test for the presence of organic bases (i.e., alkaloids).

Free-Choice Feeding Trials. Captive hares were offered a choice of physiologically mature *S. alaxensis*, mature *A. crispa*, and juvenile *A. crispa* browse in ca. 75-, 50-, and 50-g bundles, respectively, in addition to chow (Quality Texture; Fisher Mills, Seattle, Washington) ad libitum. At the end of the 24-hr feeding period, the weight of each browse eaten was determined. The experiments were performed in April of 1982 and 1984 using 10 and 5 hares respectively.

Bioassay of A. crispa *Extracts.* Four feeding trials were performed to elucidate what substances deter snowshoe hare use of green alder. Captive hares maintained upon a combination of chow (Quality Texture), oatmeal, adult *S. alaxensis* browse, and adult and juvenile *A. crispa* browse were used in bioassays. In all experiments solvent-treated oatmeal was used as a control. Treated oatmeal and controls were offered in 5- to 20-g quantities in a random array of muffin pans. During all experiments except experiment 4, hares had access to chow and adult and juvenile *A. crispa* browse ad libitum. At the end of each feeding trial, all remaining oatmeal was removed and weighed. The number of hares used in each feeding trial ranged from 5 to 10. Hare preferences are expressed as a preference index (PI = % treated oatmeal eaten/% control oatmeal eaten). Probabilities that the PI values differ significantly from unity are reported as P values and are calculated according to a paired t test.

In the first experiment crude diethyl ether extracts were bioassayed for feeding deterrence at both 2% and 4% dry mass concentrations which were the approximate levels found in alder internodes (Table 1). Diethyl ether was allowed to evaporate from the oatmeal in a fume hood over 24 hr, and diethyl ether-treated oatmeal served as the control for resin-treated oatmeal. Additionally, β-sitosterol-treated oatmeal (4% dry mass) was offered to hares during these experiments. This experiment allowed the deterrence of crude *A. crispa* fractions to be compared with that of a secondary metabolite that is not deterrent to snowshoe hare feeding (Bryant et al., 1983a) and thus helped ensure the reliability of our bioassay.

In experiment 2, pinostrobin, pinosylvin, pinosylvin monomethyl ether, and pinosylvin dimethyl ether (synthesized from 3,5-dimethoxy benzaldehyde and benzyldiphenylphosphine oxide) were simultaneously bioassayed at the same concentration, 1.5% dry weight oatmeal. In addition to these substances, 2-phenethyl cinnamate was offered at 1.0 and 2.0% dry weight concentrations. All five compounds were applied as a diethyl ether solution. Diethyl ether-treated oatmeal served as the control for this experiment.

In experiment 3, secondary metabolites that were strongly deterrent in experiment 2 (pinosylvin and pinosylvin monomethyl ether) were offered to hares at decreased concentrations (0.5% and 0.1% dry mass). Conversely, secondary metabolites that proved moderately deterrent (pinostrobin and pinosylvin dimethyl ether) or nondeterrent (2-phenethyl cinnamate) were offered at a higher

concentration (4% dry mass). In this experiment, each metabolite was offered singly with its respective control (diethyl ether treated oatmeal).

In experiment 4, hares were offered only pinosylvin-treated and pinosylvin monomethyl ether-treated oatmeal (10 g each) for 24 hr. Both substances were applied to oatmeal at a concentration of 1.5% dry mass. The hares had no access to alternative foods, e.g., chow, oatmeal, or browse, in this experiment.

RESULTS

Free-Choice Feeding Trials with Fresh Browse. Figure 1 presents data for two winters of snowshoe hare use of fresh browse. When offered fresh mature *S. alaxensis*, mature *A. crispa* and juvenile *A. crispa* twigs during the winter of 1981–1982 captive snowshoe hares preferred twigs of adult *A. crispa* over twigs of juvenile *A. crispa* ($P < 0.001$; t test). During the winter of 1983–1984, however, hares no longer differentiated between juvenile and adult *A. crispa* ($P > 0.25$; t test). Throughout both years, a large preference for *S. alaxensis* over alder was evident ($P < 0.001$; t test).

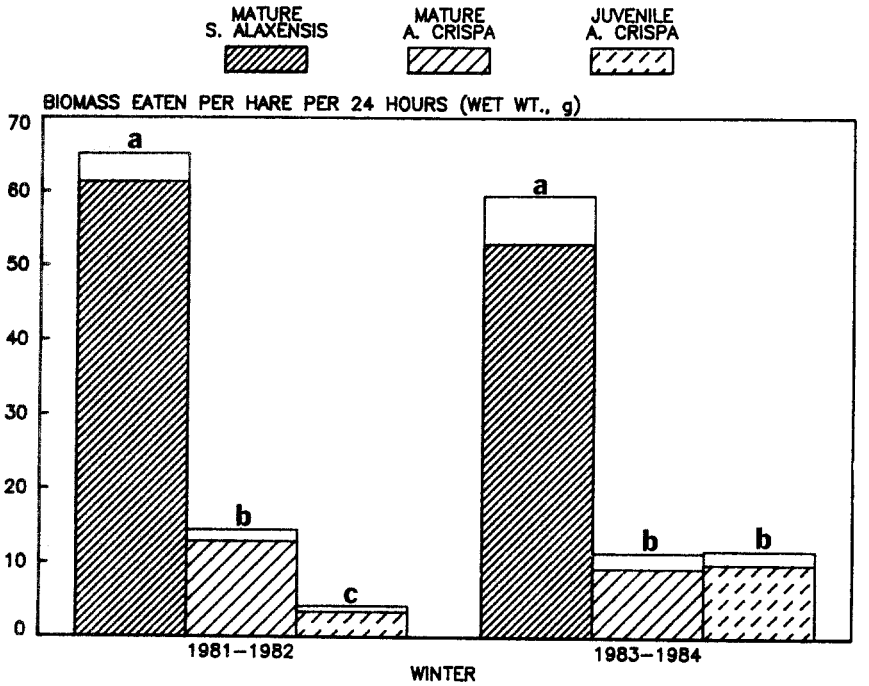

FIG. 1. Use of mature *S. alaxensis* and *A. crispa* growth stages by captive snowshoe hares. Bars with same superscripts do not differ at $P \leq 0.05$. Unshaded region represents mean plus one standard error.

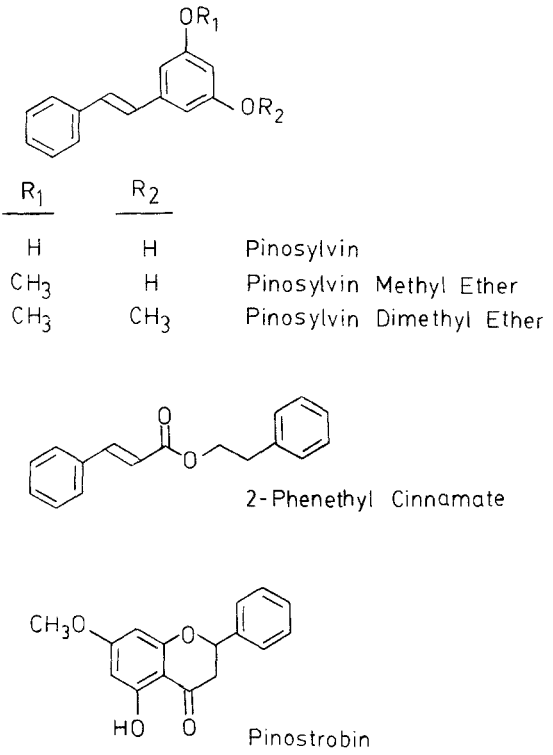

FIG. 2. Structures of the major secondary metabolites of green alder and of pinosylvin dimethyl ether.

Bioassay of A. crispa *Extracts.* Fractionation of the ether extract by column chromatography gave four principle components: pinosylvin, pinosylvin methyl ether, 2-phenethyl cinnamate, and pinostrobin (Figure 2). When offered simultaneously at 1.5% dry mass concentration (experiment 2), pinosylvin and pinosylvin monomethyl ether were very deterrent ($P < 0.0001$); pinostrobin and pinosylvin dimethyl ether were moderately deterrent ($P < 0.001$). 2-Phenethyl cinnamate demonstrated a slight deterrence at 2% concentration ($P < 0.05$). Decreasing the concentrations of pinosylvin and pinosylvin monomethyl ether decreased deterrence. At 0.10% dry mass pinosylvin was still deterrent ($P < 0.001$), but pinosylvin monomethyl ether was not ($P > 0.10$). Increasing the concentrations of pinostrobin, pinosylvin dimethyl ether, or 2-phenethyl cinnamate to 4% did not significantly increase deterrence. These results are summarized in Figure 3.

When hares only had access to pinosylvin and pinosylvin methyl ether-treated oatmeal (1.5% dry wt; experiment 4) they ate only 2.4 $\pm$ 0.6 g/hare/24 hr. Normal consumption over a 24-hr period is 50–100 g/hare.

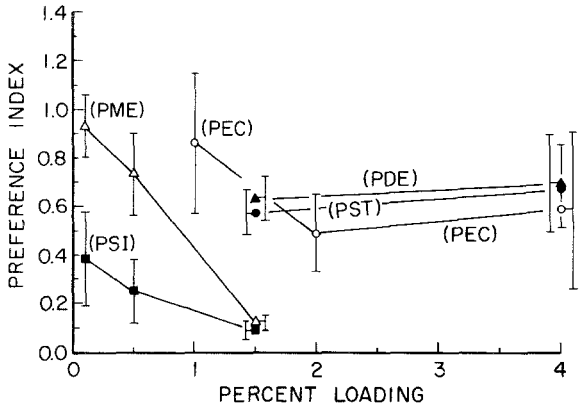

FIG. 3. Effects of alder metabolites on snowshoe hare feeding (experiments 2 and 3). Error bars represent plus and minus one standard error. PI = % treatment eaten ÷ % control eaten.

Chemical Analysis of A. crispa. All plant parts tested had less pinosylvin and pinosylvin methyl ether during the winter of 1983–1984 than in previous winters (Table 1; $P < 0.001$; t test). For convenience, the plant parts are listed in Table 1 in order of increasing palatability to hares (Bryant et al., 1983a). The levels of pinostrobin and 2-phenethyl cinnamate are presented only for the first winter because they are not important as feeding deterrents at levels present in alder (compare the data of Table 1 with those of Figure 3). No detectable (0.0001%) levels of pinosylvin dimethyl ether, a secondary metabolite found in another alder, *A. sieboldiana* (Asakawa, 1971), could be found in any sample nor was any alkaloid detected by Dragendorff's test.

DISCUSSION

From the bioassay data in Figure 3, it is apparent that hares are sensitive to specific compounds. For example, at typical concentrations found in *A. crispa* buds (1.5%), both pinosylvin and pinosylvin monomethyl ether are highly repellent (PI = ca. 0.1) while the corresponding dimethyl ether is only marginally active (PI = 0.63). Even though it can be argued that the latter compound does not occur in *A. crispa* (and hence is not recognized by hares) or has no phenolic functionality, pinostrobin similarly is essentially inactive (PI = 0.57; 1.5%) and is a phenol found in green alder. While the data presented here for *A. crispa* generally support the hypothesis (Bryant and Kuropat, 1980) that resinous plants or plant parts are unpalatable to herbivores (buds and catkins versus internodes), it is also clear that the hares' discrimination between (relatively nonresinous) juvenile and mature stages of internodes is based upon levels of specific com-

TABLE 1. PERCENT DRY MASS COMPOSITION OF ALDER PARTS OVER 3 CONSECUTIVE WINTERS (MEANS AND STANDARD ERRORS)

Winter	Compound	*N*	Bud	Catkin	Juvenile plant internode	Mature plant internode
1981–1982	Pinosylvin	1,2[a,c]	2.9, 3.2	0.74	0.11, 0.088	0.035
	Pinosylvin methyl ether	3[b]	2.3 ± 0.2	1.4 ± 0.2	0.52 ± 0.01	0.041 ± 0.012
	Pinostrobin	3[b]	1.7 ± 0.2	0.74 ± 0.03	0.028 ± 0.001	0.012 ± 0.002
	Phenethyl cinnamate	3[b]	2.3 ± 0.1	1.1 ± 0.1	0.019 ± 0.003	0.050 ± 0.003
	Resin	3	34.3 ± 2.8	25.2 ± 2.3	4.4 ± 0.2	4.9 ± 0.5
1982–1983	Pinosylvin	4[a]	4.0 ± 0.9			0.052 ± 0.036
	Pinosylvin methyl ether	4[a]	3.4 ± 0.6			0.044 ± 0.030
	Resin	4	23.7 ± 1.2			3.3 ± 0.2
1983–1984	Pinosylvin	9[a]	1.4 ± 0.4		0.042 ± 0.012	0.013 ± 0.008
	Pinosylvin methyl ether	9[a]	1.7 ± 0.5		0.032 ± 0.008	0.0065 ± 0.0070
	Resin	9	28.0 ± 1.4		2.8 ± 0.2	2.6 ± 0.5

[a] Determined by HPLC analysis.
[b] Determined by GC analysis.
[c] Range of values presented.

pounds rather than total resins. Hence it is important that correlations of "total phenolics" or "percent resins" with palatability be treated with skepticism.

The levels of pinosylvin methyl ether in the buds and catkins are sufficient to account for their rejection by snowshoe hares (Bryant et al., 1983a). The addition of similar levels of pinosylvin provides additional support for our earlier thesis that use of green alder by snowshoe hares is governed in part by its secondary chemistry. It is notable that the slight preference of catkins over buds (Bryant, personal observation) is also reflected by lower levels of these two active chemicals in catkins. Although we have not tested whether hares can detect such variation in concentrations of pinosylvin and its monomethyl ether at these high levels, the circumstantial evidence is compelling.

The internodes of alder, however, contain much lower levels of all the secondary metabolites measured; and, in order to correlate plant chemistry with hare use, levels of both pinosylvin and pinosylvin methyl ether must be considered. For simplicity, other compounds will not be discussed here due to their negligible repellency at concentrations typically found in internodes.

Several major considerations emerge when attempting to predict palatability of internodes based on our bioassay data. First, synergistic relationships have not been tested. The possibility exists that while it is apparent that hares readily detect and avoid pinosylvin and pinosylvin monomethyl ether, certain mixtures of all four major secondary metabolites (and other minor substances) might be more recognizable by hares and more repellent to them. The possibility of a synergism becomes appealing upon realizing that the repellency of resin in Table 2 cannot be totally accounted for by the repellency of its individual components at their respective concentrations. For example, although resin demonstrates similar deterrency at 2% loading (Table 2) as both pinosylvin and pinosylvin methyl ether do at 1.5% loading (Figure 3), the maximum levels of pinosylvin and pinosylvin methyl ether in the internode resins are only 2.3% and 11.8%, respectively (Table 1; winter 1981–1982). Thus the bioassay using 2.0% resin involves as an upper estimate, about 0.04% and 0.24% loading of pinosylvin and pinosylvin methyl ether, respectively. To justify the observed repellency of resin, a small but significant synergistic relationship seems reasonable.

A second problem is that our bioassays are based on treated oatmeal. Because the food value of oatmeal is much higher than alder internodes, hares may respond differently to secondary metabolites present in internodes versus those placed on oatmeal. Indeed, in the case of retarding wood decay, Hart and Shrimpton (1979) have shown that the activities of pinosylvin and its methyl ether are greater in agar solutions than in the heartwood of several evergreens. Presumably the cause of this decline in activity results from a portion of the pinosylvins being chemically bound in the heartwood. Since the pinosylvin and pinosylvin methyl ether levels measured in our study were unbound, a similar decline in activity in alder is unlikely. Still, caution must be exercised when

TABLE 2. EFFECTS OF ALDER RESIN ON SNOWSHOE HARE FEEDING (EXPERIMENT 1)

Sample	Loading % dry mass	N^a	Treated oatmeal offered (g); treated oatmeal eaten (g)	Untreated oatmeal offered (g); untreated oatmeal eaten (g)	PI[b]	P[c]
Ether	ca. 15	10	15.0; 14.8 ± 0.4	15.0; 14.0 ± 0.9	1.1 ± 0.1	>0.25
Resin	2.0	10	5.0; 0.50 ± 0.15	15.0; 14.2 ± 0.4	0.10 ± 0.03	<0.0001
	4.0	10	5.0; 0.038 ± 0.023	15.0; 14.2 ± 0.4	0.0080 ± 0.0050	<0.0001
β-Sitosterol	4.0	10	20.0; 16.0 ± 1.0	20.0; 16.3 ± 1.2	1.02 ± 0.10	>0.25

[a] Number of hares used in trial.
[b] PI = % treatment consumed ÷ % control consumed.
[c] One tailed t test H_0: PI = 1.00.

attempting to predict the absolute palatabilities of alder parts based upon the palatabilities of alder's secondary metabolites placed on oatmeal.

Regardless, we feel that our bioassays suggest that as long as the combined levels of pinosylvin and pinosylvin monomethyl ether in internodes are not below the "threshold of avoidance" by hares, internodes that contain the lower inclusive amounts will be the preferred food choice. That Table 1 confirms the predicted inverse relationship between palatability of internodes and chemical composition is adamantine evidence that hare use of *A. crispa* parts is governed by variable levels of both pinosylvin and its methyl ether. For example, the threefold preference of mature over juvenile growth in the winter of 1981–1982 (Figure 1) correlates with a significant decrease (Table 1) in the amounts of both of these deterrent phytochemicals in the preferred mature browse. It is also apparent from the bioassay data in Figure 3 that the amount of pinosylvin present in the juvenile internodes (0.10%; winter 1981–1982) is above the threshold of detection by hares (PI = 0.38; 0.1%; $P < 0.01$, t test). Thus the "two-level" defense we suggested earlier (Bryant et al., 1983a) merely reflects a chemical defense of internodes based upon the levels of two substances while the unpalatability of buds and catkins compared to internodes can be explained by levels of either of the two substances.

During the winter of 1983–1984, however, hares no longer discriminated between juvenile and mature green alder internodes (Figure 1). While there still remains about a threefold increase in the levels of both stilbenes in the juvenile internodes, we maintain that the overall drop in these levels from those of previous years (Table 1) has been sufficient to pass below the threshold of avoidance by hares. The resultant increase in juvenile green alder palatability during the winter of 1983–1984 suggests that environmental factors may have a subtle but real effect on hare use of woody plants. The specific factors that can have such impacts on *A. crispa* chemistry have not been ascertained, but we believe one possibility is drought, which was experienced during the spring of 1983 in the Fairbanks area.

These results indicate that allocation of secondary metabolites among plant parts by species such as *A. crispa* strongly influences hare use of woody plant parts and growth stages. This conclusion has precedent in several other recent studies of the woody plant–hare interaction in winter (e.g., Barikmo, 1976; Klein, 1977; Bryant, 1981a,b; Pehrson, 1980, 1981, 1983, 1984; Bryant et al., 1983a,b; Sulkinoja, 1983; Reichardt et al., 1984; Tahvanainen et al., 1985). At this point the accumulated evidence on the effects of secondary plant metabolites on palatability is sufficient to suggest that optimal foraging hypotheses must include allocation of plant resources to toxic and/or digestion-inhibiting chemical defense in order to explain the foraging behavior of boreal forest vertebrate herbivores such as snowshoe hares.

Finally we point out an unexpected result of this study that is germane to

theories of plant–herbivore interactions that attempt to provide a chemical rationale for selective use of plant species by generalist herbivores such as snowshoe hares (e.g., Feeny, 1976; Rhoades and Cates, 1976; Bryant and Kuropat, 1980; Bryant et al., 1983a, 1984; Coley, 1983). If snowshoe hares were not deterred by the combined levels of pinosylvin and pinosylvin methyl ether in the juvenile internodes of the last year of study (0.074% ± 0.014%), then it is extremely unlikely that they were repelled by the combined levels of these compounds present in the mature internodes during the winter of 1981–1982 (0.076% ± 0.026). This prediction is confirmed by the data in Figure 1 that demonstrate hares' use of mature alder internodes did not change significantly between the winters of 1981–1982 and 1983–1984 even though threefold and sixfold decreases in the levels of pinosylvin and pinosylvin methyl ether, respectively, were recorded (Table 1). Consequently snowshoe hare preference for *A. crispa* parts and growth stages can be adequately explained by varying concentrations of two specific secondary metabolites, but these same compounds cannot explain the low palatability of mature *A. crispa* relative to *S. alaxensis* (Figure 1).

This result indicates that the "gestalt" that hares use to distinguish between parts and growth stages within a woody plant species differs from that which enables hares to distinguish between different plant species. Although we have not determined why *A. crispa* is less palatable than *S. alaxensis* (Bryant et al., 1985), Bryant et al. (1983a,b) indicate it is not caused by a lack of nutritional chemicals, e.g., protein or soluble carbohydrate, in *A. crispa* as compared to *S. alaxensis*. Disregarding the possibility of a dominant synergistic interaction, low-molecular-weight, ether-soluble secondary metabolites are unlikely explanations of the low palatability of *A. crispa* as compared to *S. alaxensis*. Only fractions of *A. crispa* containing pinosylvin or pinosylvin methyl ether demonstrated any large activity, and a negative Dragendorff's test precludes the possibility of significant amounts of alkaloids. Similarly, there is no large difference in the levels of structural carbohydrate or lignin in *A. crispa* and *S. alaxensis* winter dormant twigs (Bryant et al., 1983a,b; 1985). We also cannot detect any obvious physical difference between winter dormant twigs of these species.

In short, there is now substantial evidence that levels of specific secondary metabolites strongly influence (if not determine) hares' selective use of woody plant parts and growth stages within a browse species (e.g., Bryant, 1981a,b; Bryant et al., 1983a,b, 1984, 1985; Tahvanainen et al., 1985). The basis for hare selective use of woody plant species as winter food is, however, still an open question.

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PREDICTION OF RELEASE RATIOS OF MULTICOMPONENT PHEROMONES FROM RUBBER SEPTA[1]

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Abstract—A method has been developed to predict the release ratio of the components of blends of alcohols, acetates, and/or aldehydes from rubber septa. The calculations of predicted release ratios are based on the relative vapor pressures of the components. The relative vapor pressures of the compounds were calculated from their retention indices on a liquid crystal capillary gas chromatographic column. The correlation between the theoretically predicted and experimentally determined ratios was very good. Thus, formulations can be prepared that will release a desired ratio of the components of a multicomponent pheromone blend.

Key Words—Pheromone blends, formulation on rubber septa, relative vapor pressure, liquid crystals.

INTRODUCTION

The sex pheromones of many lepidopteran insect species have been identified, and the practical value of several of these has been demonstrated in programs designed to monitor or suppress pest insect populations. Additionally, extensive studies have been conducted to analyze and evaluate insect behavior in the laboratory and in the field. Several materials have been adapted or developed to release pheromones at controlled rates to increase the effectiveness of these chemicals in monitoring or control programs. Chemical analyses of pheromone gland constituents and volatiles collected from calling females, in addition to

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

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behavioral studies and field trapping experiments, have established that most lepidopteran pheromones consist of blends of two or more compounds. Usually a precise ratio of the components of a blend is required to elicit optimum response. However, thus far there has been very little research to develop an accurate method to predict and control the precise ratio and rate at which a pheromone blend is released from a formulation.

There have been several investigations in which the ratio of components in a pheromone blend loaded onto a controlled release substrate was empirically adjusted to give optimum trap captures. Additionally, the release rate of individual pheromonal compounds from different substrates has been measured. Butler and McDonough (1979, 1981) and McDonough and Butler (1983) determined the half-lives of several n-alkyl and alkenyl acetates and alcohols loaded individually on rubber septa by quantitating the amount of a compound remaining on a septum after various periods of time. Similarly, Leonhardt and Moreno (1982) measured release rates of several pheromones and pheromone blends from laminated Hercon® dispensers by gas–liquid chromatographic (GLC) analysis of quantities of the materials remaining in the dispensers after they were aged varying amounts of time in a greenhouse and in the field. Baker et al. (1980) collected and quantified Oriental fruit moth pheromone deposited on the wall of a glass flask and determined that (*Z*)-8-dodecen-1-ol (*Z*8–12 : OH) was emitted three times faster than (*Z*)-8-dodecenyl acetate (*Z*8–12 : Ac) from rubber septa.

Using an improved version of Regnault's (1845) gas saturation technique, Hiroka and Suwanai (1978) measured the vapor pressures of 12 alkyl compounds including alkanes, acetates, and an alcohol. Their investigation of a binary mixture of lauryl and myristyl acetate demonstrated that a binary sex pheromone system behaves approximately according to Raoult's Law, i.e., the mole fraction of a compound in the vapor above a binary liquid mixture correlated closely with the mole fraction of the compound in the liquid mixture and the relative vapor pressures of the two compounds. Thus, two compounds with equal vapor pressure would occur in the same ratio in the vapor as in the liquid. Other references pertaining to pheromone release rates from various substrates include the work of Weatherston et al. (1982) and several chapters in books edited by Mitchell (1981) and Kydonieus and Beroza (1982).

The information developed so far allows the determination of the average release rate of a pheromone over a period of days and, in some cases, may allow the approximate average ratio to be determined and controlled. Unfortunately, there is as yet no information that will allow one to predict and control with a reasonable degree of accuracy the precise ratio and rate at which the components of a pheromone blend will be released during a period of 1 hr or less under a given set of conditions. Our interest in analyzing the behavioral responses of males of several species of *Heliothis*, *Spodoptera*, and other Lep-

idoptera to their respective pheromone blends requires a method to predict and control release rates and ratios for blends of compounds that might vary considerably in chain length and functionality.

In a previous paper, we demonstrated that the retention time of a compound on a liquid crystal capillary column could be correlated with the compound's reported vapor pressure and/or reported half-life (Heath and Tumlinson, 1986). The correlation of the retention time expressed in equivalent chain length units (ECLU) of seven acetates and the natural logarithm of their reported vapor pressures (Olsson et al., 1983), resulted in a coefficient of determination (r^2) of 0.999. Attempts to correlate the retention times of the seven acetates on capillary columns coated with isotropic phases (apolar OV-101® and polar CW-20) with their vapor pressures resulted in drastically reduced coefficients of determination of 0.724 and 0.446, respectively. Correlation of the retention time (in ECLU) on the liquid crystal column with the reported half-lives (Butler and McDonough, 1979, 1981; McDonough and Butler, 1983) of five saturated acetates (10–14 carbons), four monounsaturated acetates, and four monounsaturated alcohols (12–16 carbons), resulted in a high degree of correlation (r^2 of 0.995). Based on these findings, we proceeded on the hypothesis that relative vapor pressure and evaporation rates of many compounds could be approximated based on the compounds' retention times on a liquid crystal column. This information could then be used to predict the release ratios of components in pheromone blends.

We report in this paper the results of our investigation into the prediction of the release ratios of various combinations of acetates, aldehydes, and alcohols from rubber septa based on retention indices of the compounds on a liquid crystal column.

METHODS AND MATERIALS

Chemicals used for this investigation (Table 1) were obtained from Chemical Samples Co. (Columbus, Ohio). In several instances synthetic modification of the functional group was required to give the desired compound. All compounds were purified by $AgNO_3$ liquid chromatography or recrystallized from pentane, distilled in a short-path distillation apparatus, and analyzed on three different capillary columns to ensure a high degree of purity. Capillary columns were drawn and coated in our laboratory by previously described techniques (Heath et al., 1980, 1981). The three columns used were: 46 m × 0.25 mm ID cholesteryl-*p*-chlorocinnamate (a cholesteric liquid crystal phase); 46 m × 0.25 mm ID OV-101; and 42 m × 0.25 mm ID SP-2340®. Columns were operated in either the split or splitless mode with helium as the carrier gas at a linear flow of 18 cm/sec. The columns were installed in a Varian® 3700 gas

TABLE 1. RELATIVE VAPOR PRESSURE AND HALF-LIVES CALCULATED FROM EQUIVALENT CHAIN LENGTH (ECLU) OF COMPOUNDS CHROMATOGRAPHED ON LIQUID CRYSTAL CAPILLARY GLC COLUMN

Compound[a]	ECLU[b]	Relative vapor pressure[c]	Relative half-lives (days)
Sat-12:Ac	1200	0.4633	38.1
Z9-14:Al	1213	0.4062	43.4
Sat-14:Al	1250	0.2804	63.1
Z9-14:OH	1296	0.1769	100.4
Sat-13:Ac	1300	0.1700	104.6
Z11-14:OH	1306	0.1601	111.1
Z9-14:Ac	1366	0.0877	203.7
Sat-14:Ac	1400	0.0624	287.1
Z11-16:Al	1413	0.0549	327.4
Sat-16:Al	1450	0.0379	475.8
Z11-16:OH	1447	0.0390	461.0
Sat-15:Ac	1500	0.0229	788.4
Z11-16:Ac	1562	0.0123	1474.7
Sat-16:Ac	1600	0.0086	2164.6

[a] Example: (Z)-9-tetradecenal = (Z9-14:Al), (Z)-9-tetradecen-1-ol = (Z9-14:OH), (Z)-9-tetradecenyl acetate = (Z9-14:Ac), (Z)-11-hexedecenal = (Z11-16:Al), and (Z)-11-hexadecenyl acetate = (Z11-16:Ac).
[b] Established with saturated acetates.
[c] Calculated from equations 1 and 2 in text.

chromatograph equipped with flame ionization detectors with a lower limit of detection of ca. 0.2 ng for hexadecane.

Rubber septa (Cat. No. 8753-D22, A.H. Thomas Co., Philadelphia, Pennsylvania) were exhaustively extracted with CH_2Cl_2 for 24 hr and air dried a minimum of three days before use. A septum was loaded by depositing the desired blend, dissolved in 100 μl of hexane, into the cup of the large end. The quantity of pheromone loaded varied somewhat, depending on the number of blend components but was maintained between the limits of 400 μg and 600 μg per septum.

The collection apparatus is an adaptation of a Grob and Zücher (1976) design and includes modifications by P.S. Beevor and coworkers (Tropical Products Institute, London, England) to collect pheromones from insects. We have adapted and further modified this method (Tumlinson et al., 1982). Briefly, it consists of a small charcoal trap prepared by sealing 3–5 mg of charcoal (Bender and Hobein AG, Zurich, Switzerland) between two 325-mesh stainless-steel frits in a 6-mm OD, 3.7-mm ID Pyrex® tube. Air is delivered through the silanized glass aeration apparatus at a flow rate of 0.5–1 liter/min. The septa are placed ca. 2 cm upwind from the charcoal trap and aerated for 1–2 hr. When

aeration is complete, the filter is rinsed with six aliquots (15–20 μl each) of distilled dichloromethane, the aliquots are combined, an internal standard is added, and the solution is concentrated to about 5–10 μl by gently warming. Isooctane or another solvent of choice is added for analysis by capillary GLC with splitless injection.

In a preliminary experiment, a backup charcoal trap was connected to the outlet of the primary trap. Extraction and analysis of the secondary trap resulted in no detectable amount of material. Additionally, recoverability of compounds from the charcoal trap was determined by depositing a known amount of the compound (usually as a blend with three other compounds) onto the charcoal directly in 20- to 50-ng amounts. The compounds were extracted after solvent evaporation and analyzed as previously described. Before each experiment, the aeration chamber was cleaned by rinsing with hexane, acetone, water, acetone, and hexane. The chambers were dried overnight at ca. 100°C. The charcoal traps were cleaned by rinsing with ca. 4 ml CH_2Cl_2 and CS_2. The traps were then dried using a flow of nitrogen.

Determination of relative vapor pressure and half-lives of compounds used in this investigation are based on the previously determined equations (Heath and Tumlinson, 1986). The equation for the relative vapor pressure of a compound is

$$(\ln)\ \text{vapor pressure} = (\text{ECLU} - 1123)/-99.9 \tag{1}$$

and the equation for a compound's half-life is

$$(\ln)\ \text{half-life} = -8.48 + 0.0101 \cdot \text{ECLU} \tag{2}$$

where ECLU is equal to the retention time of a compound on a cholesteryl-*p*-chlorocinnamate capillary column relative to saturated acetates (Swoboda, 1962).

To derive a formula for determining the release ratio from rubber septa of components in pheromone blends, several assumptions were made. We assumed that the relative vapor pressure of the solute in rubber septa could be approximated by using ECLU values obtained by chromatography on a liquid crystal stationary phase (isotopic phases showed no correlation) (Heath and Tumlinson, 1986). Thus in this assumption, the proportionality constant as required by Henry's Law is empirically determined, and the rate of evaporation of a compound from rubber septa is also correlated with the retention time of the compound in the liquid crystal phase. The ratio of a component in the vapor from a multicomponent blend is the percent of the compound in the liquid times its evaporation rate, divided by the sum of each of the components (in %) in the liquid multiplied by their respective evaporation rates. It is well documented that the evaporation rates of pheromones from rubber septa can be determined by measuring their half-lives and is a first-order equation (Butler and McDonough, 1979, 1981; McDonough and Butler, 1983). If we assume that the

evaporation of one component will not affect the evaporation rate of another component, our formula for release ratio is the same as that using relative vapor pressure. However, in this case evaporation rate is in days, and we must convert all half-lives to their reciprocals for calculation of release ratios using equation (3).

The calculation of the percent release of a compound when formulated in a blend on rubber septa is based on use of the calculated relative vapor pressure of the compound or calculated half-life of the compound. The equation for calculating percent release of a compound is

$$R_1 = \frac{L_1 P_1^r}{L_1 P_1^r + L_2 P_2^r + L_n P_n^r} \tag{3}$$

where L is the percent load of the compound and P^r is the reciprocal of the relative half-life (evaporation rate) or the relative vapor pressure of the compound.

RESULTS AND DISCUSSION

The equivalent chain length, calculated relative vapor pressures, and calculated relative half-lives for all compounds included in this study are presented in Table 1. Various blends were prepared and the theoretical release ratio of each component was determined using equation 3. The volatiles released by the blend when formulated on rubber septa were trapped and analyzed.

Prior to analysis of charcoal-trapped volatile blends, the aeration apparatus and charcoal traps were evaluated to determine the amount of material lost due to adsorption on glass surfaces or irreversible adsorption on the trap. The percent of each compound recovered when blends containing ca. 50 ng of each component were deposited directly on the trap and then reextracted without aeration is given in Table 2. The recoveries ranged from 74 to 94%, and data obtained in subsequent experiments were corrected to account for the losses on the trap. Analysis of the solvent rinses of the internal glass surfaces of the apparatus after experiments indicated that no measurable material was adsorbed on the silanized glass surfaces. This is probably because the septa were placed within 2 cm of the trap, and thus there was very little glass surface downwind of the septum for the volatiles to contact. Analysis of backup traps connected in series to the primary traps indicated no significant breakthrough of compounds from the first trap occurred.

The various blends of compounds that were formulated and aerated are listed in Table 3. Different ratios of the same blend of compounds were analyzed to test the hypothesis that this method would be valid over a wide range of ratios. Volatiles from the septa were analyzed over a seven-day interval. Analyses were obtained on 1- to 2-, 3- to 4-, and 5- to 7-day-old septa (after

TABLE 2. PERCENT RECOVERY OF COMPOUNDS FROM CHARCOAL FILTER

Compound[a]	Recovered (%)	Standard deviation	Replicate
Sat-12:Ac	91	8.5	6
Z9-14:Al	78	14.19	6
Sat-14:Al	76	9.5	6
Z9-14:OH	76	8.7	6
Sat-13:Ac	94	8.4	6
Z11-14:OH	80	11.0	8
Z9-14:Ac	86	7.6	6
Sat-14:Ac	87	8.2	6
Z11-16:Al	76	7.0	6
Sat-16:Al	74	16.2	6
Z11-16:OH	79	9.4	10
Z11-16:Ac	85	7.6	6
Sat-15:Ac	91	10.2	10
Sat-16:Ac	87	7.8	6

[a] Example, (Z)-9-tetradecenal = Z9-14:Al, (Z)-9-tetradecen-1-ol = Z9-14:OH, (Z)-9-tetradecenyl acetate = Z9-14:Ac), (Z)-11-hexadecenal = Z11-16:Al, and (Z)-11-hexadecenyl acetate = Z11-16:Ac.

the three-day aging process). The analysis of each blend at a given time interval was replicated at least three times. Because there was no significant change in the measured ratio during the seven-day interval, the data from all analyses were averaged and compared to the calculated theoretical release ratio. For blends that contained compounds for which the half-lives had been reported by Butler and McDonough (1979, 1981) and McDonough and Butler (1983), we calculated the predicted release ratio based on reported half-lives and compared the data with the predicted release ratios based on relative vapor pressure and with the measured release ratios (Table 3).

An attempt was made to analyze a representative number of different combinations of molecular weights and/or functional groups to correlate as closely as possible with actual pheromone blends found in a variety of insects.

The results obtained when C_{12}–C_{14} saturated acetates were formulated as three-component blends in approximately equal amounts showed that the release ratio of the acetates could be predicted with less than 4% error (Table 3). When the 16-carbon acetate was formulated with other saturated acetates, a large difference was found between percent recovered and percent theoretical. McDonough and Butler (1979) found similar discrepancies with the half-life of the 16-carbon acetate when compared to shorter chain length acetates and suggested that the deviation was due to the inability of the larger acetate molecules to penetrate the rubber matrix. Another possible explanation is that the 16-

TABLE 3. COMPARISON OF PERCENT LOAD, PERCENT RATIO CALCULATED, MEAN PERCENT FOUND AND STANDARD DEVIATION

Blend[a]	Load (%)	% Calculated using vapor pressure or calculated half life	Reported half-life[b]	Found (%)	
				Mean[c]	SD
Sat-12:Ac	41.1	73.8	74.4	70.1	2.2
Sat-13:Ac	28.8	18.9	19.8	21.5	1.4
Sat-14:Ac	30.1	7.2	5.6	8.5	0.9
Sat-13:Ac	37.7	70.6	77.5	70.2	1.5
Sat-14:Ac	31.1	21.5	17.8	21.4	1.6
Sat-15:Ac	31.1	7.9	4.6	8.5	1.9
Sat-14:Ac	36.9	69.2		54.8	5.9
Sat-15:Ac	33.2	23.2		19.4	2.2
Sat-16:Ac	29.3	7.6		26.6	8.0
Z9-14:Al	27.3	55.1		51.9	3.2
Sat-14:Al	23.7	33.1		34.7	1.3
Z11-16:Al	29.5	8.1		8.0	2.1
Sat-16:Al	19.6	3.7		5.0	1.8
Z9-14:Al	19.0	49.9		45.1	2.9
Sat-14:Al	17.0	30.7		31.4	1.5
Z11-16:Al	37.0	12.9		10.1	2.0
Sat-16:Al	27.0	6.5		10.5	3.1
Z9-14:Al	6.0	29.2		28.7	4.0
Sat-14:Al	6.0	20.2		23.0	3.5
Z11-16:Al	52.0	34.2		28.2	3.8
Sat-16:Al	36.0	16.4		20.1	2.9
Z9-14:OH	23.8	38.5	34.4	38.6	3.8
Sat-13:Ac	31.9	49:6	55.2	49.9	3.2
Z11-16:OH	18.3	6.5	7.2	4.7	4.2
Sat-15:Ac	25.9	5.4	3.2	6.7	1.2
Z9-14:OH	14.7	33.2	30.3	33.6	3.6
Sat-13:Ac	19.1	41.5	47.0	42.2	1.9
Z11-16:OH	28.8	14.3	16.1	10.6	2.9
Sat-16:Ac	37.4	11.0	6.7	13.6	2.1
Z9-14:OH	5.2	19.6	18.5	20.5	1.8
Sat-13:Ac	6.5	23.6	27.6	24.7	1.6
Z11-16:OH	41.4	34.3	39.8	26.2	4.1
Sat-15:Ac	45.9	22.4	14.1	28.7	2.6
Z9-14:Al	26.3	53.1		50.9	2.4
Sat-13:Ac	25.6	35.7		32.8	3.2
Z11-16:Al	25.3	6.9		12.2	6.1
Sat-15:Ac	22.7	4.3		4.1	2.2

TABLE 3. Continued

Blend[a]	Load (%)	% Calculated using vapor pressure or calculated half life	Reported half-life[b]	Found (%)	
				Mean[c]	SD
Z9-14:Al	15.2	45.0		48.2	3.8
Sat-13:Ac	15.0	30.8		26.8	4.8
Z11-16:Al	39.7	15.9		16	3.1
Sat-15:Ac	29.9	8.3		9	2.0
Z9-14:Al	2.7	16.5		17.3 (13.6)[d]	6.2
Z9-14:Ac	66.4	76.8		76.7 (76.9)	6.9
Z11-16:Al	3.6	2.6		3.1 (6.7)	1.1
Z11-16:Ac	25.7	4.1		2.9 (3.4)	0.7
Z9-14:Al	7.4	36.6		32.4 (32.0)	4.1
Z9-14:Ac	58.8	55.2		60.6 (60.3)	3.8
Z11-16:Al	8.4	4.9		4.3 (5.8)	0.6
Z11-16:Ac	25.4	3.3		2.6 (1.9)	1.1

[a] Example: (Z)-9-tetradecenal = Z9-14:Al, (Z)-9-tetradecen-1-ol = Z9-14:OH, (Z)-9-tetradecenyl acetate = Z9-14:Ac, (Z)-11-hexedecenal = Z11-16:Al, and (Z)-11-hexadecenyl acetate = Z11-16:Ac.

[b] Calculated from reported half-lives (Butler and McDonough, 1979, 1981; McDonough and Butler, 1983).

[c] Mean is calculated from the average obtained from the 1- to 2-, 3- to 4-, and 5- to 6-day-old samples. Each mean represents a minimum of nine analysis.

[d] Analysis obtained on samples left in the field in parentheses.

carbon acetate is a solid at room temperature, and our inability to predict the release rate may be due to the establishment of a solid–liquid phase equilibrium.

The relative vapor pressure data obtained for the aldehydes using the retention time data obtained on the liquid crystal column enables us to predict relative release rates for the aldehyde blends with less than 6% error for any given component in the three blends investigated (Table 3). Greater variability occurred between calculated and measured released ratios when blends were composed of acetates and alcohols or acetates and aldehydes (Table 3). There are at least two possible explanations for this. The aldehydes and the alcohols are adsorbed more strongly by the charcoal and glass surfaces, and thus small but immeasurable differences could contribute to greater error when analyzing blends composed of compounds with two or more types of functional groups. Also, solutions of compounds with more than one type of functional group may differ more in physical chemical properties than do compounds with one type of functionality. Further investigation of this phenomenon is required to determine the actual reason for these discrepancies. Nonetheless, as a first approximation, the experimental results are very close to the calculated values, and this

is the most accurate method developed thus far for predicting release ratios of components in pheromone blends.

A greater disparity between the predicted release ratio and measured release ratio was obtained when reported half-lives (Butler and McDonough, 1979, 1981; McDonough and Butler, 1983) were used in the calculations (Table 3). It should be noted that the error is significantly reduced (equal to that observed using relative vapor pressure values) if the values for the half-lives are determined using equation 2.

As an example of the practical application of this study, we formulated blends containing two aldehydes and two acetates (Z)-9-tetradecenal (Z9–14:Al), (Z)-9-tetradecenyl acetate (Z9–14:Ac), (Z)-11-hexedecenal (Z11–16:Al), and (Z)-11-hexadecenyl acetate (Z11–16:Ac) in two different ratios. All septa were analyzed in the laboratory on the first or second day after loading. Then one batch was placed in traps in the field for four days while an identical batch was aged in the laboratory during the same period and analyzed again at the end of three to four days. At five to seven days after loading, release ratios of the blends were again measured from both lab- and field-aged septa. The result of this study (Table 3) indicated that the placement of septa in the field for a five-day period did not increase significantly the variation of the release ratio of the compounds when compared to the lab septa.

CONCLUSIONS

The purpose of this investigation was to explore the hypothesis that relative release rates (half-lives) and relative vapor pressure of individual compounds could be used to calculate the approximate release ratios of the compounds formulated as a blend in rubber septa. Our results indicate that, at least to a first approximation, our hypotheses enabled us to determine release ratios. Certain refinements in measurement techniques or more accurate correction factors for recoveries of alcohols and aldehydes may yield more accurate data. Additionally, our investigation was based on an empirical approach and does not provide fundamental reasons for the existence of the correlation of the retention time of a compound on a liquid crystal column and its predicted release ratio when formulated in a blend on rubber septa. Thus there exists a need to further define the mechanism of evaporation of pheromones from rubber septa. However, the use of the method described should greatly facilitate the determination of the percent load required to provide the desired release ratio of components in pheromone blends.

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