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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).

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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-1-01 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,

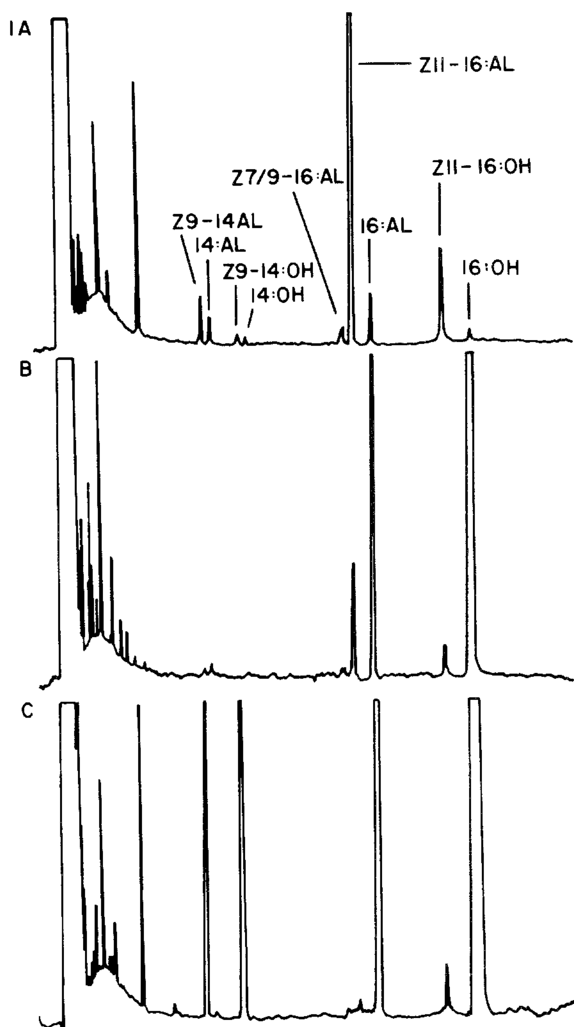

FIG. 1. Chromatograms of gland extracts of *H. virescens* on the SPB1 column. (A) Extract of untreated gland. (B) Extract of gland treated with 1 μg of 16:OH in DMSO. (C) Extract treated with a 3:1 ratio of 16:OH and Z9–14:OH.

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)

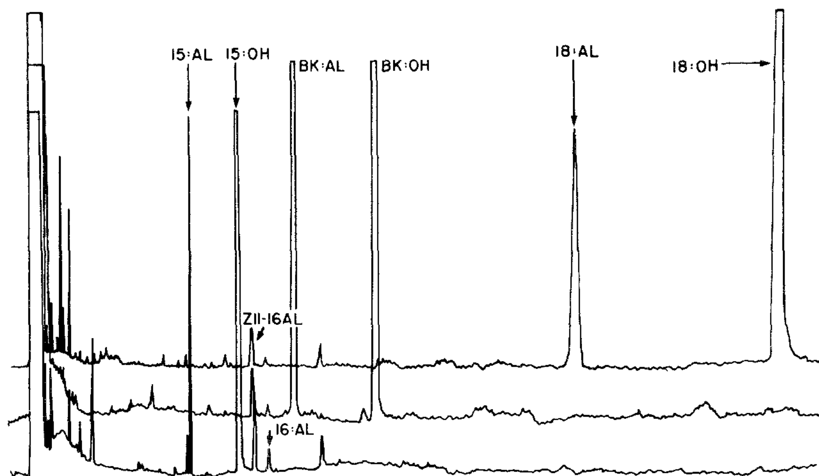

FIG. 2. Chromatograms of extracts of glands treated with octadecanol, pentadecanol and bombykol on the SPB1 column. Octodecanal = 18:AL, octadecanol = 18:OH, pentadecanal = 15:AL, pentadecanol = 15:OH, BKAL = bombykal, BKOH = bombykol.

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 Z9–14: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 Z9–14: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 E11–14: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;

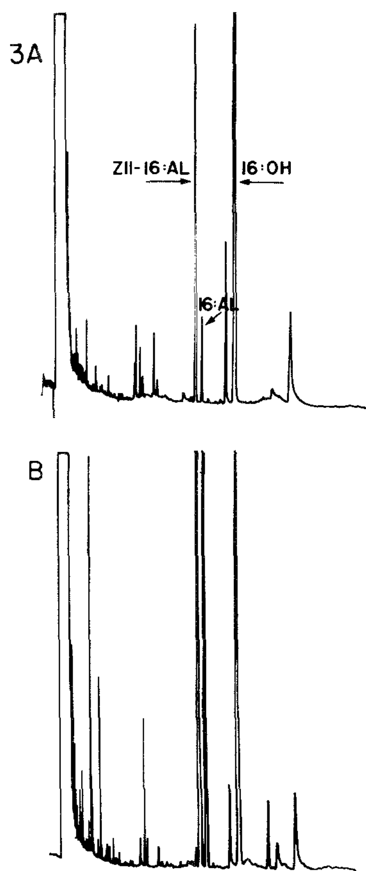

FIG. 3. Chromatograms of glands of calling *H. virescens* on the SPB1 column treated with 100 ng of 16:OH in DMSO and maintained (A) under N_2 for 30 min, and (B) under N_2 for 15 min and then air for 15 min.

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

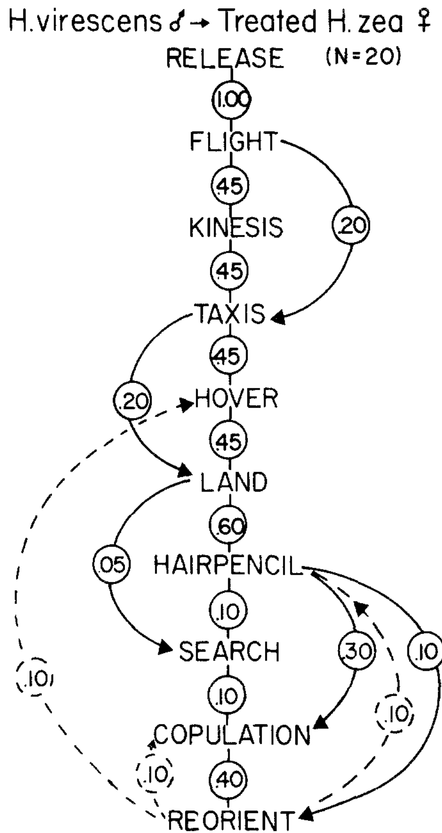

FIG. 4. Ethogram of the responses of *H. virescens* males ($N = 20$) to *H. zea* females treated with 20 ng of Z9–14 : OH and 14 : OH and placed in the upwind end of the 2-m-long flight tunnel. Numbers indicate the probabilities that each transition will occur.

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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