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STUDIES ON THE BIOSYNTHESIS OF (E)-11-TETRADECENYL ACETATE AND (Z)-11-TETRADECENYL ACETATE IN PHEROMONE GLANDS OF PLATYNOTA STULTANA (LEPIDOPTERA:TORTRICIDAE)

presented by
JONATHAN JEWELL NEAL

has been accepted towards fulfillment
of the requirements for

M.S. degree in ENTOMOLOGY

George Nyen
Major professor

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# STUDIES ON THE BIOSYNTHESIS OF (E)-11-TETRADECENYL ACETATE AND (Z)-11-TETRADECENYL ACETATE IN PHEROMONE GLANDS OF PLATYNOTA STULTANA (LEPIDOPTERA:TORTRICIDAE)

By

Jonathan Jewell Neal

A THESIS

Submitted to
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1980

## ABSTRACT

STUDIES ON THE BIOSYNTHESIS OF (E)-11-TETRADECENYL ACETATE AND (Z)-11-TETRADECENYL ACETATE IN PHEROMONE GLANDS OF *PLATYNOTA STULTANA* (LEPIDOPTERA:TORTRICIDAE)

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Potential precursors of (E)-11-tetradecenyl acetate were injected into hemocaels of fifth instar, pupae, and adult *Platynota stultana*, or topically applied to the pheromone glands of adult moths. Glands were analyzed for (E)-11-tetradecenyl acetate content and specific activity of $^{14}C$ or for percentage (E) and (Z) composition of the 11-tetradecenyl acetates.

1-$^{14}C$-myristic acid applied topically to adult moths was incorporated into (E)-11-tetradecenyl acetate. Evidence concerning 1-$^{14}C$-acetate or 1-$^{14}C$-myristyl acetate was inconclusive. Application of (E)-11-tetradecenol or (Z)-11-tetradecenol to moths did not alter the naturally occurring ratio (88:12) of (E)-11-tetradecenyl acetate to (Z)-11-tetradecenyl acetate.

Myristic acid was discovered to be retained by a 3% OV-1 column and liberated upon subsequent injections.

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Some samples of (E)-11-tetradecenyl acetate from moths treated with $^{14}C$-myristic acid were ozonized and chromatographed. $^{14}C$ appeared in the 11-ethoxyundecanal collected but not in the 11-tetradecenyl acetate fraction, strong evidence that $^{14}C$ from myristic acid is incorporated into (E)-11-tetradecenyl acetate.

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# INTRODUCTION

The importance of pheromones as an integral part of insect communication systems has been established for many insect species. Pheromones are in some cases essential to species recognition and reproduction and have been implicated as important agents in speciation, caste determination, tracking and defensive behavior (Birch, 1974). Undoubtedly as more is learned about these complex systems, novel strategies will evolve to make use of this knowledge.

Although much attention has been focused on identification of the chemical components of pheromone systems, there are still many unanswered questions regarding other aspects of the chemical communication system. Pheromone biosynthesis is one such aspect. An extensive literature search yields only a few papers concerning this topic.

Three basic techniques have been used to obtain information in previous studies of pheromone biosynthesis. These include labeling with radioactive isotopes, use of isomeric precursors to alter the ratio of isomeric pheromone components produced, and analysis of the contents of the pheromone-producing tissue. Jones and Berger (1978) showed that $^{14}C$ acetate was incorporated into cis-7-dodecenyl acetate, the pheromone of Trichoplusia ni. Schmidt and

Monroe (1976) demonstrated incorporation of $^{14}C$ oleic acid into nonanal and undecanal, pheromone components of Galleria mellonella. They also obtained small amounts of incorporation with $^{14}C$ acetate and $^{14}C$ propionate. Thompson and Mitlin (1978) showed incorporation of tritium labeled nerol and geraniol into 4 monoterpene compounds comprising male boll weevil pheromone. Kasang, et al. (1974) found large amounts of (Z)-2-methyl-7-octadecene in Porthetria dispar pheromone glands and used tritium labeled material to show its incorporation into the pheromone cis-7,8-epoxy-2-methyloctadecane. Crewe and Ross (1975) used $^{14}C$ and $^{35}S$ labeling to show incorporation of the $CH_3$-S-moity from methionine into dimethyl disulphide and dimethyl trisulphide (the status of these compounds as pheromones is unproven). Renwick et al. (1976) showed that the relative amounts of cis and trans verbenol produced by Ips paraconfusis could be controlled by the relative amounts of (-) and (+) α-pinene in the diet.

Platynota stultana, the omnivorous leaf roller (OLR) has several traits which makes it a good test insect for studying pheromone biosynthesis. It can be reared on an artificial diet, has a relatively short period of development, is large enough to be easily injected and has a pheromone that can be obtained in relatively pure form in quantities large enough for analysis. Pheromone production appears to be confined to an eversible gland on the dorsal side of the abdomen. Its pheromone, identified by Hill and Roelofs (1975), consists of a mixture of (E)- and (Z)-11-

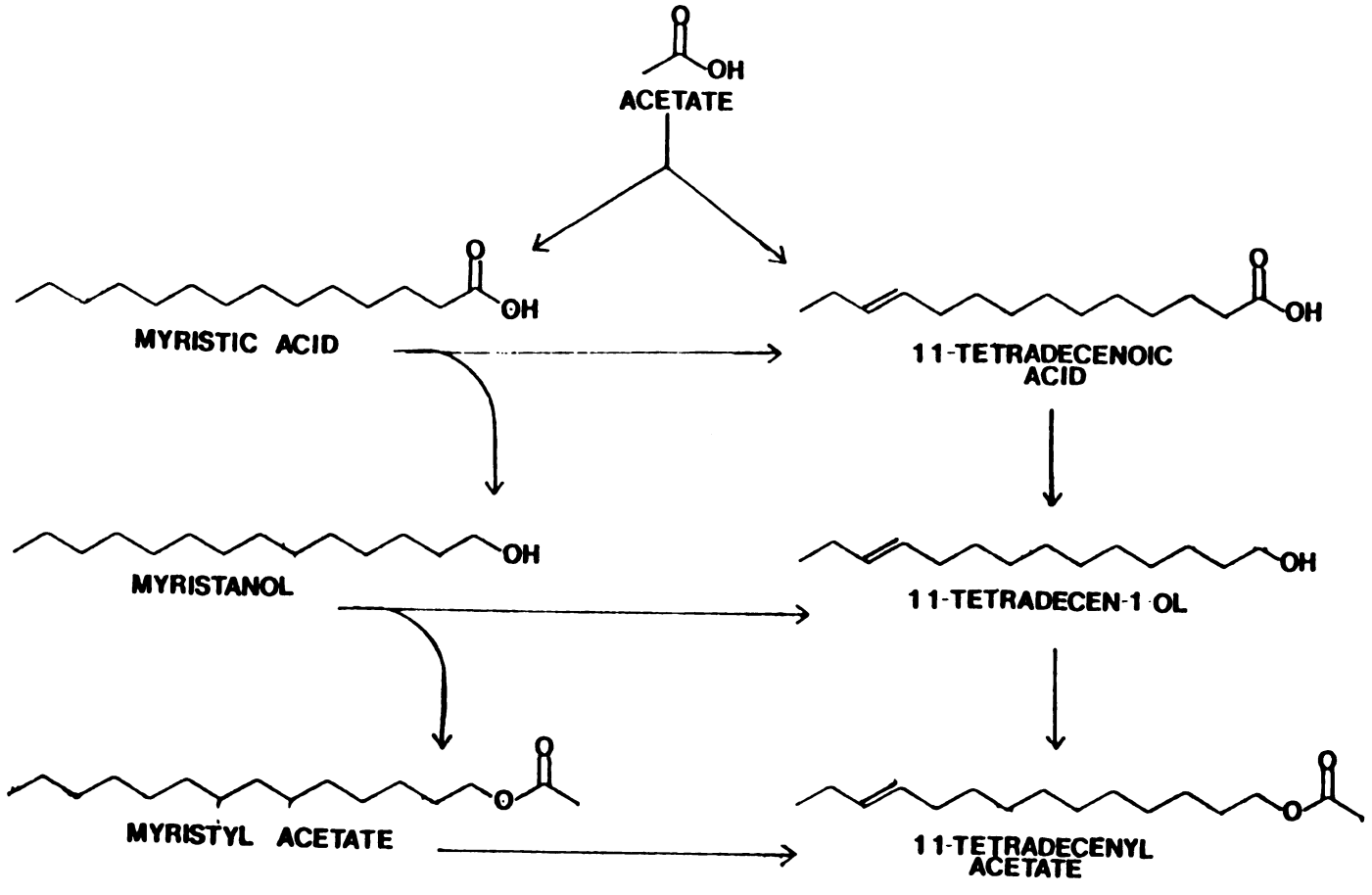
tetradecenyl acetate in a ratio of 88 to 12. No papers concerning pheromone biosynthesis of this species have yet been published.

As the framework for a working hypothesis, several closely related possible biosynthetic pathways are proposed (Figure 1). This hypothesis assumes that pheromone biosynthesis starts with acetate and proceeds through normal fatty acid biosynthesis to either myristic acid (tetradecanoic acid) or 11-tetradecenoic acid. The acid would undergo subsequent reduction to the corresponding alcohol and esterification to an acetate. If myristic acid were involved, the introduction of the double bond could occur at any of the steps shown.

The double bond is of prime interest because of its constant cis/trans ratio and because the same acetates ((E)- and (Z)-11-tetradecenyl acetate) in different ratios are used as the pheromone in several closely related species of leaf rollers (Klun et al., 1974; Roelofs et al., 1974; Hill et al., 1974). It is important that female moths maintain precise control of this ratio in order to be attractive to male moths of only the same species (Baker et al., 1975). Insights into regulation of this aspect of pheromone biosynthesis may shed light on aspects of species evolution as well as provide insight into the possibility of developing resistence to control strategies utilizing pheromones. In addition to these insights such knowledge might lead to novel pheromone based control strategies.

Figure 1. Several hypothetical pathways for the biosynthesis of (E)-11-tetradecenyl acetate.

FIGURE 1


OH
ACETATE
OH
MYRISTIC ACID
OH
11-TETRADECENOIC ACID
OH
MYRISTANOL
OH
11-TETRADECEN-1-OL
O
MYRISTYL ACETATE
O
11-TETRADECENYL ACETATE

## MATERIALS AND METHODS

### Rearing

Adult Platynota stultana were allowed to mate in and deposit egg masses on the sides of 8 liter polyethylene bags. Egg masses were surface-sterilized with .05% sodium hypochlorite solution, rinsed with distilled water, dried, and allowed to hatch in glass shell vials with cotton plugs. Approximately 50 first instar larvae were transferred with a camel hair brush to waxed 16 oz. paper cups containing modified pinto bean diet (Shorey & Hale, 1965) and kept in a constant environment chamber (photoperiod = 16L:8D, temperature = 24°C, humidity = 65%-85%). After 21 days, pupae were removed from the diet, sexed, and allowed to emerge in 1 liter polyethylene bags containing paper toweling to absorb molting fluid. Adults were transferred every 24 hours to 1 liter polyethylene bags containing a wetted cotton wick.

### Gas Chromatography

Gas liquid chromatographic (GLC) analysis was done on a Packard Series 7300 Gas Chromatograph. GLC columns (glass, 2 m x 4 mm) were packed with either 3% OV-1 on 100-120 mesh Gas Chrom Q or 10% GEXF-1150 on 100-120 mesh

AW-DMCS treated Gas Chrom W. Flame ionization detection was used for quantification. When used for purification, the entire effluvia from the column was diverted through glass capillary tubes sandwiched between slabs of dry ice. Samples were recovered by rinsing the capillary tubes with ca. 0.5 ml $CS_2$. Fraction cuts were made in relation to the retention time of standards run immediately prior to sample injection.

## Radioassays

Quantitation of $^{14}C$ was done on a Searle Isocap/300 Liquid Scintillation System with high background reject, or a (refrigerated) Packard Tricarb. Samples were placed in glass scintillation vials containing 15 ml of a fluor mixture (0.67 l toluene, 0.33 l triton X100, 4 g PPO, 0.5 g dimethyl POPOP).

Background counts were determined by counting blank vials prepared identically to those containing samples (blank vials contained 15 ml of cocktail plus amount of solvent used for dissolving sample). Channels ratio in conjunction with $^{14}C$ quench series was used for converting counts to disintegrations and was checked against an external standard. Both methods gave good agreement. Samples and blanks were counted for consecutive 10 min intervals until the level of significance reached 90% (Seelye, 1975).

## Chemicals

(Z)-11-tetradecenyl acetate, (E)-11-tetradecenyl acetate, (Z)-11-tetradecen-1-ol and (E)-11-tetradecen-1-ol were purchased from Farchan Chemicals Inc., 1-$^{14}$C acetic acid and 1-$^{14}$C- stearic acid from ICN Pharmaceuticals Inc. and 1-$^{14}$C tetradecanoic acid and 1-$^{14}$C tetradecanol from DHOM Products Inc. 1-$^{14}$C tetradecyl acetate was synthesized by addition of excess AR grade acetyl chloride (Mallinckrodt Chemical Inc.) to 1-$^{14}$C tetradecanol. The reaction was monitored at 10 minute intervals by GLC (3% OV-1 at 160°) until complete and purified by collection from the same system. Purity was confirmed by TLC (Silica gel G/Benzene: ether-9:1) and by GLC (3% OV-1 at 160° and 10% GEXF-1150 at 150°C). All solvents used were distilled prior to use.

## Introduction of Test Chemicals to Moths

All chemicals were administered by means of injection or topical application using an ISCO model M microapplicator. Needles were made by pulling 2 mm glass tubing and inserting the blunt end into a half hole rubber septum. The septum assembly was fitted onto a calibrated 25 µl fixed needle syringe with the needle shortened to 1 cm. Injections were made intersegmentally into the abdominal cavity. Topical applications were made by pressing the abdomen to evert the pheromone gland and placing the tip of the needle directly on the gland.

## Pheromone Extraction, Purification and Quantitation

Pheromone glands were dissected by pressing the abdomen to evert the gland, and excising the everted tissue with micro-dissecting scissors. Glands were immediately placed in 1 dram vials containing 0.5 ml methylene chloride and extracted for 24 hr at -18°C. Extracts were then filtered through glass wool and reduced to ca. 5 µl for GLC injection. Samples were collected from the 3% OV-1 column which was used to separate 14 carbon alcohols from their corresponding acetates. The fraction from ±1 minute of the center of the (E)-11-tetradecenyl acetate peak was reduced to approximately 5 µl and injected onto the 10% GEXF-1150 column. Collections from -1.5 to -0.5 min (tetradecyl acetate peak) and -0.5 to +0.5 min of the center of the (E)-11-tetradecenyl acetate peak were made.

The fraction containing (E)-11-tetradecenyl acetate was diluted to 5ml with $CS_2$. Palmitoleic acid methyl ester was added as an internal standard. Two 0.5 ml aliquots were placed in scintillation vials for $^{14}C$ analysis and two 0.5 ml aliquots were reduced to ca. 5 µl and analyzed for (E)-11-tetradecenyl acetate on a 10% GEXF-1150 column. The fraction from -1.5 min to -0.5 from the center of the (E)-11-tetradecenyl acetate peak was submitted to liquid scintillation for $^{14}C$ analysis.

Ozonolysis of (E)-11-tetradecenyl acetate

Samples containing (E)-11-tetradecenyl acetate in $CS_2$ were placed in a dry ice-acetone bath. Ozone was bubbled through the mixture until a distinct blue color appeared, indicating saturation. Nitrogen was then bubbled through the mixture to drive off excess oxygen and ca. 2 mg triphenylphosphine was added and allowed to react for 30 min. The solvent was then reduced to ca. 5 µl for GLC. The 11-ethoxy-undecanal was purified by collection from a 3% OV-1 column and analyzed for $^{14}C$ content. The propanal produced during ozonolysis was lost during the solvent reduction step.

## RESULTS

### Topical Application of (E) and (Z) 11-tetradecenol to Pheromone Glands

Table 1 shows the effects of topical application of 0.05 µl of both (E)- or (Z)-11-tetradecenol on 11-tetradecenyl acetate production in adult female OLR's. Listed in the table are the date the replicate was treated, the (E)- and (Z)-percentages of the moth produced 11-tetradecenyl acetate and the amount of (E)-11-tetradecenyl acetate per tip.

The data show that with the exception of the 3/28/78 experiment, (Z)-11-tetradecenol treated moths had 11-tetradecenyl acetate compositions very close to the 11% (Z) isomer - 89% (E) isomer blend found in the control moths. The replicate from 3/28/78 is thought to have been accidently contaminated during analysis, but was included for completeness. The other three samples are all within the limit of experimental variation expected.

Moths treated with (E)-11-tetradecenol, with the exception of the 2/23/78 replicate, have 11-tetradecenyl acetate composition close to that of the control moths. The 2/23/78 experiment is again thought to have been accidently contaminated during analysis but was included for completeness.

Table 1.--The effect of topical application of (Z) or (E) 11-tetradecen-1-OL on the ratio of (E) to (Z) isomers of 11-tetradecenyl acetate.[a]

| Treatment | Date | # of Tips | % (Z) 11-tetra decenyl acetate | % (E) 11-tetra decenyl acetate | (E)-11-tetra-decenyl acetate Per Tip (ng) |
|---|---|---|---|---|---|
| (Z)-11-tetra-decenol | 2/23/78 | 50 | 14 | 86 | ND[b] |
| | 3/28/78 | 50 | 64 | 36 | ND |
| | 6/6/78 | 17 | 14 | 86 | 7.4 |
| | 6/14/78 | 21 | 8 | 92 | 7.5 |
| | 6/14/78 | 21 | 16 | 84 | 3.3 |
| (E)-11-tetra decenol | 2/23/78 | 50 | 48(35)[c] | 52(65) | ND |
| | 3/28/78 | 50 | 10 | 90 | ND |
| | 6/6/78 | 15 | 10 | 90 | 5.2 |
| | 6/14/78 | 19 | 10 | 90 | 12 |
| Control (No Treatment) | 3/28/78 | 50 | 10 | 90 | ND |
| | 6/06/78 | 14 | 11 | 89 | ND |

[a]Female OLR's received 0.1 µl of a 50% ether solution of either (Z)-11-tetradecenol or (E)-11-tetradecenol or were untreated (control). Twenty-four hours after treatment, pheromone glands were extracted in methylene chloride, 11-tetradecenyl acetate purified by trapping from 3% OV-1, and the extract analyzed for the (E) and (Z) percentages of 11-tetradecenyl acetate and the total amount of (E)-11-tetradecenyl acetate as given.

[b]ND = Not Determined.

[c]Results in parenthesis were obtained first. Sample was then resubmitted onto OV-1 for further purification to give results listed.

## Injection of 4 $^{14}C$ Compounds as a Function of Life Stage

Female OLR's were injected with four $^{14}C$ labeled compounds at different life stages and at different times within those life stages as shown in Table 2. Listed are the date the replicate was injected, the amount of moth produced (E)-11-tetradecenyl acetate per aliquot tested, the disintegrations per minute in that aliquot and the ratio of the moles of $^{14}C$ to the moles of (E)-11-tetradecenyl acetate. Groups of 50 females were used; each moth received at least 50,000 dpm.

The data show that injection of the long chain compounds (myristic acid, myristanol, and myristyl acetate) into female OLR's did not result in $^{14}C$ incorporation into (E)-11-tetradecenyl acetate. Injected $^{14}C$ acetate, however, resulted in activity in the (E)-11-tetradecenyl acetate fraction. These activities showed sizable variation but in general were about the same for all study periods. Experiments done in 1977 show activity, while those done after 1977 do not. There were no differences between procedures used before and after the end of 1977.

## Injection vs. Topical Application of 4 $^{14}C$ Compounds to Moths

Table 3 compares the $^{14}C$ activity in the (E)-11-tetradecenyl acetate peak resulting from both injection and topical application of four $^{14}C$ labeled compounds. The table shows the date the label was applied, the amount of (E)-11-

Table 2.--The effect of injection timing on the $^{14}C$ activity in the (E)-11-tetradecenyl acetate fraction[a] from Platynota stultana treated with four different compounds.

| Date | 1-$^{14}C$ Acetate | | | 1-$^{14}C$-Myristic Acid | | | 1-$^{14}C$-Myristanol | | | 1-$^{14}C$-Myristyl Acetate | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ng/sample[b] | dpm | ratio[c] | ng/sample | dpm | ratio | ng/sample | dpm | ratio | ng/sample | dpm | ratio |
| **5th Instar** | | | | | | | | | | | | |
| 9/8/77 | 156 | 15 | .0002 | | | | | | | | | |
| 11/10/77 | 120 | 12 | .0002 | | | | | | | | | |
| **0-24 Hour Pupae** | | | | | | | | | | | | |
| 9/8/77 | 48 | 30 | .0007 | | | | | | | | | |
| 11/10/77 | 52 | 13 | .0005 | | | | | | | | | |
| **48-72 Hour Pupae** | | | | | | | | | | | | |
| 8/3/78 | 31 | B[d] | - | 241 | B | - | 53 | B | - | 51 | B | - |
| 8/4/78 | 16 | B | - | 213 | B | - | 47 | B | - | 25 | B | - |
| 8/5/78 | 21 | B | - | 51 | B | - | 86 | B | - | 30 | B | - |
| **120-144 Hour Pupae** | | | | | | | | | | | | |
| 9/18/77 | 29 | 14 | .0007 | | | | | | | | | |
| 11/10/77 | 40 | 14 | .0006 | | | | | | | | | |
| **0-24 Hour Adults** | | | | | | | | | | | | |
| 9/8/77 | 52 | 28 | .0009 | | | | | | | | | |
| 11/10/77 | 104 | 45 | .0006 | | | | | | | | | |
| 11/24/77 | 48 | 20 | .0007 | 28 | 16 | .0011 | | | | | | |
| 8/7/78 | 42 | B | - | 178 | B | - | 119 | B | - | 59 | B | - |
| 8/8/78 | 63 | B | - | 78 | B | - | 79 | B | - | 152 | B | - |
| 8/8/78 | 75 | B | - | 98 | B | - | 176 | B | - | 99 | B | - |
| 9/24/79 | | | | - | B | - | | | | | | |

[a](E)-11-tetradecenyl acetate was collected from 2 GLC columns -3% OV-1 then 10% GEXF-1150.

[b]ng/sample of (E)-11-tetradecenyl acetate.

[c]Ratio of the moles of $^{14}C$ to the moles of (E)-11-tetradecenyl acetate.

[d]B = Background.

Table 3.--The effect of topical application and injection of four $^{14}C$ labeled compounds to moths on the $^{14}C$ activity in the (E)-11-tetradecenyl acetate fraction.

| Date | 1-$^{14}C$-Acetic Acid | | | 1-$^{14}C$ Myristic Acid | | | 1-$^{14}C$-Myristanol | | | 1-$^{14}C$-Myristyl Acetate | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ng/sample[b] | dpm | ratio[c] | ng/sample | dpm | ratio | ng/sample | dpm | ratio | ng/sample | dpm | ratio |
| **Injection** | | | | | | | | | | | | |
| 9/8/77 | 52 | 28 | .0009 | | | | | | | | | |
| 11/10/77 | 109 | 45 | .0006 | | | | | | | | | |
| 11/24/77 | 48 | 20 | .0007 | 28 | 16 | .0011 | | | | | | |
| 8/7/78 | 42 | B[d] | - | 177 | BB | - | 119 | B | - | 59 | B | - |
| 8/8/78 | 63 | B | - | 78 | BB | - | 79 | B | - | 152 | B | - |
| 8/8/78 | 75 | B | - | 96 | BB | - | 176 | B | - | 99 | B | - |
| 9/24/79 | | | | - | BB | | | | | | | |
| **Topical Application** | | | | | | | | | | | | |
| 11/24/77 | 31 | 24 | .0014 | 12 | 109 | .0112 | | | | | | |
| 1/20/78 | 9 | B | - | 21 | 32 | .0018 | 6 | 3 | .0006 | 11 | 60 | .0062 |
| 2/25/78 | 89 | 13 | .0003 | 69 | 17 | .0005 | 79 | 12 | .0002 | 19 | 16 | .0006 |
| 5/7/78 | | | | 70 | 39 | .0012 | | | | | | |
| 9/22/78 | | | | 86 | 250 | .0055 | | | | | | |
| 9/22/78 | | | | 148 | 37 | .0005 | | | | | | |
| 1/1/79 | 103 | 17 | .0003 | 84 | 50 | .0012 | | | | | | |
| 6/12/79 | 12 | B | - | 7 | B | - | 36 | B | - | 6 | B | - |
| 8/20/79 | - | B | - | | | | | | | | | |
| 9/24/79 | | | | 54 | B | - | | | | | | |

[a] (E)-11-tetradecenyl acetate was purified by trapping from GLC, first trapping from 3% OV-1 than 10% GEXF-1150.

[b] ng/sample of (E)-11-tetradecenyl acetate.

[c] Ratio = Total $^{14}C$ (Moles) divided by total (E)-11-tetradecenyl acetate.

[d] B = Background.

tetradecenyl acetate per aliquot, the disintgegration rate per aliquot, and the ratio of the moles of $^{14}C$ to the moles of (E)-11-tetradecenyl acetate for each of 4 compounds applied. Groups of 50 females were used and each moth received at least 50,000 dpm.

The data show that the method of application for $^{14}C$ acetate had little effect on the amount of $^{14}C$ in the (E)-11-tetradecenyl acetate fraction. Both methods produced only low levels of activity with much variation.

Moths treated topically with $^{14}C$ myristic acid showed much higher activity in the (E)-11-tetradecenyl acetate fraction than moths treated by injection. This treatment produced the greatest activity and highest incorporation ratios of any combination of possible precursor and application method tested. The results show much variation and there is no linear relationship between quantity of (E)-11-tetradecenyl acetate and $^{14}C$ activity.

There is little evidence to suggest that myristanol is incorporated into (E)-11-tetradecenyl acetate. $^{14}C$ activity in the pheromone fraction occurred only once where moths were treated by topical application, and not at all where treatment was by injection.

Treatment of female moths with $^{14}C$-myristyl acetate by injection produced no $^{14}C$ activity in the tetradecenyl acetate fraction and gave variable activity when applied topically. On the 10% GEXF-1150 column tetradecyl acetate elutes 1 min prior to the (E)-11-tetradecenyl acetate peak

under the conditions of these experiments. Because of the possibility of $^{14}C$ myristyl acetate contamination, the fraction 1 min prior to the (E)-11-tetradecenyl acetate fraction was trapped and analyzed for $^{14}C$ content. In no case did this fraction produce activity above background, indicating that tetradecyl acetate was not interfering with analysis.

## Topical Application of 1-$^{14}C$-Myristic Acid to 1 and 2 Day Old Moths

Table 4 compares the activity appearing in the (E)-11-tetradecenyl acetate fractions from moths treated as 0-24 hr old adults vs. those treated as 24-48 hr old adults. Fifty thousand dpm myristic acid was used as the $^{14}C$ label and glands were dissected 24 hr after treatment. The data show no difference between the 2 treatments.

Table 4.--Effect of timing of topical application of 1-$^{14}$C-myristic acid on the $^{14}$C activity appearing in the (E)-11-tetradecenyl acetate fraction.[a]

| Date | 0-24 Hour Adults | | | 24-48 Hour Adults | | |
|---|---|---|---|---|---|---|
| | ng/sample[b] | dpm | ratio[c] | ng/sample | dpm | ratio |
| 1/24/77 | 12 | 109 | .0112 | | | |
| 1/20/78 | 21 | 32 | .0018 | | | |
| 2/25/78 | 69 | 17 | .0005 | | | |
| 5/7/78 | 70 | 39 | .0012 | | | |
| 9/22/78 | 86 | 250 | .0055 | 135 | 97 | .0014 |
| 9/22/78 | 148 | 37 | .0005 | 333 | 61 | .0003 |
| 1/1/79 | 84 | 50 | .0012 | | | |
| 6/12/79 | 7 | B[d] | - | | | |
| 8/24/79 | 54 | B[d] | - | | | |

[a](E)-11-tetradecenyl acetate was collected first from a 3% OV-1 GLC column, then from 10% GEXF-1150.

[b]ng/sample of (E)-11-tetradecenyl acetate.

[c]Ratio of the moles of $^{14}$C to the moles of (E)-11-tetradecenyl acetate.

[d]B = Background.

# DISCUSSION

## Variability of the Data Collected

The results from $^{14}C$ tracer studies are quite variable. Isolated results taken one at a time both support and refute $^{14}C$ incorporation and the results from the experiments described previously must be viewed as a whole if reasonable conclusions are to be derived.

The large variations in the data, particularly those of the ratio of $^{14}C$ activity to the quantity of (E)-11-tetradecenyl acetate argue against incorporation. A large amount of $^{14}C$ in one replicate and no measurable $^{14}C$ in another without diminished (E)-11-tetradecenyl acetate content is suggestive of some underlying difference in technique or unidentified methodological artifact. Great care, however, was taken to insure that experimental conditions were consistent. Method and amount of injection, timing of the injections, timing of the dissections, and methods of analysis were unchanged throughout. Rearing of insects went unchanged and age of the insects in each group was carefully determined. Compounds tested were kept cold and in the dark when not in use. Standards were always gas chromatographed prior to submission of moth extracts. GC columns

were evaluated for performance, and when below standard, were repacked with packing from the same batch to enhance consistency. GC traces were free of significant visable contamination. The main scintillation counter used was equipped with high background reject such that any sample containing high energy counts greater than 1% of the total counts in the $^{14}C$ channel would be rejected. When a second instrument was used, a portion counted was often counted as well in the primary instrument. In all cases the two instruments agreed well.

## Gas Chromatographic Analysis of Materials Used in Labeling Experiments

The 4 compounds used in the $^{14}C$ labeling experiments were chromatographed to check for contaminants and the possibility of starting material ending up in the final sample.

Using column conditions identical to those used for isolating (E)-11-tetradecenyl acetate, acetic acid, myristic acid, myristanol and myristyl acetate were injected onto a 3% OV-1 column. No peaks which could be construed as contaminants were observed. Myristyl acetate appears to be the only compound capable of interfering with future analysis. On a 10% GEXF-1150 column, myristyl acetate and (E)-11-tetradecenyl acetate have retention times about 1 min apart (not quite baseline separation). Because myristyl acetate is also produced by the pheromone gland the fraction 1 min prior to the (E)-11-tetradecenyl acetate peak was collected

and analyzed for $^{14}C$ in all studies. In no case was $^{14}C$ activity observed in this fraction. This points against the possibility of contaminants from a tailing myristyl acetate peak.

It is interesting to note that $^{14}C$ myristyl acetate is not present in moths treated with $^{14}C$ myristyl acetate only 24 hr prior to extraction. This suggests that myristyl acetate is either degraded very rapidly, transported elsewhere within the moth or released from the pheromone gland during that interval.

Other experiments in this series give a different picture. In these studies the $^{14}C$ labeled myristanol, myristic acid, myristyl acetate and acetate used in labeling experiments were injected onto GC columns and trapped to see if $^{14}C$ contaminants in them could interfere with analysis. These were treated identically to moth samples; they were first trapped from 3% OV-1 and subsequently trapped from 10% GEXF-1150. Approximately 1μCi, the amount that would have been applied to samples of 50 moths, was used. Results are shown in Table 5. The data indicate that clearly it is possible for contaminants to appear in the fraction which would contain (E)-11-tetradecenyl acetate. The results are quite variable and further experimentation indicated that this resulted from the past history of the column rather than from compounds with long retention times. For example, the myristic acid from 12/16/79 was trapped from a different GEXF-1150 column than 12/18/79 and 12/20/79 samples

Table 5.--Contaminating $^{14}C$ activity in the (E)-11-tetradecenyl acetate fraction contributed by various $^{14}C$ labeling materials.[a]

| Date and Sample | | Labeling Material | | | |
|---|---|---|---|---|---|
| | | Myristanol dpm | Myristic Acid dpm | Acetate dpm | Myristyl Acetate dpm |
| 12/16/79 | I | 4 | 41 | | |
| 12/16/79 | II | 203 | 281 | | |
| 12/18/79 | I | | B[b] | | |
| 12/18/79 | II | | B[b] | | |
| 12/20/79 | I | | B[b] | | |
| 12/20/79 | II | | B[b] | | |
| 1/22/80 | | | | B[b] | 15 |

[a]Materials used to label female moths were trapped from a 3% OV-1 column and a 10% GEXF-1150 column. 1μCi, the amount of label applied to 50 female moths in other studies was used.

[b]B = Background.

with different results. Another demonstration of the effects of column history is seen in Table 6. Injection of $^{14}C$ myristic acid onto 3% OV-1 gave 1029 counts in the (E)-11-tetradecenyl acetate region. The following shot of unlabeled myristic acid had 10,826 counts in the (E)-11-tetradecenyl acetate region. Apparently, introduction of cold material can release $^{14}C$ labeled material that has hung up on the column. From this information alone, one could conclude that contamination is responsible for all the measured $^{14}C$ activity. This would also account for the large amount of variation as well as the nonlinearity between the amount of tetradecenyl acetate recovered and the measured $^{14}C$ content.

## Ozonolysis of Moth Produced (E)-11-tetradecenyl Acetate

In order to gain more information, samples from $^{14}C$ myristic acid treated moths showing relatively high $^{14}C$ activity and still having an adequate amount of sample left were subjected to ozonolysis. These samples were from fractions that originally were collected from 3% OV-1 and 10% GEXF-1150 columns and correspond to the (E)-11-tetradecenyl acetate areas. The GC traces of the samples on the GEXF-1150 column were free of visual contaminants with a detection limit of somewhat less than 1 ng. In no case did the fraction 1 min prior to the (E)-11-tetradecenyl acetate fraction show any potentially contaminating $^{14}C$ activity.

Table 6.--The effects of GC column history on $^{14}C$ activity in myristic acid samples collected from a 3% OV-1 GLC column.

| Sample | Markers | 12/18/79 | | 12/20/79 | | |
|---|---|---|---|---|---|---|
| | | A(dpm)[a] | B(dpm)[b] | A(dpm)[c] | B(dpm)[d] | C(dpm)[e] |
| Retention Time (Min) | | | | | | |
| 0-4 | | 20 | 565 | 4 | 49 | 38 |
| 4-7 | (E)-11-tetradecenol | 33 | 125 | B[f] | 30 | 1 |
| 7-9 | | 39 | 370 | 5 | 27 | 64 |
| 9-12 | (E)-11-tetradecenyl Acetate | 1253 | 23546 | 1004 | 10801 | 16 |
| | | | | | 283 | 12 |
| 12-15 | | 1173 | 6717 | 116 | | |
| | | | | | 41 | 29 |
| 15-30 | | 2855 | 9136 | 359 | | |
| 30-45 | | 1352 | 3884 | 166 | | |
| 45-60 | | 999 | 1194 | 156 | | |
| 60-75 | | | | 105 | | |
| 75-90 | | | | 129 | | |

[a] 12/18/79 A. 1μCi of 1-$^{14}C$ myristic acid was injected into the column at time = 0, after column was unused for several days.

[b] 12/18/79 B. 1μCi of 1-$^{14}C$ myristic acid was injected onto the column at time = 0, 60 min after the 12/18/79 A injection.

[c] 12/20/79 A. 1μCi of $^{14}C$ myristic acid was injected onto the column at time = 0, after column went unused for 2 da.

[d] 12/20/79 B. 10ng of unlabeled myristic acid were injected onto the column at time = 0, 90 min after the 12/20/79 A injection.

[e] 12/20/79 C. 1μCi of $^{14}C$ myristic acid was injected onto the column at time = 0, 30 min after 12/20.79 B was injected.

[f] B = Background.

Half of each sample was counted directly, the other half that underwent ozonolysis, was collected from 3% OV-1 and analyzed for $^{14}C$ content. Table 7 gives the experiment from which the sample came, the amount of $^{14}C$ in the half sample counted directly, the $^{14}C$ found in each fraction collected, the markers used and their corresponding retention times. The data are not complete due to high energy background present in some samples. In the 1/3/79 experiment, the $^{14}C$ activity appears to be spread throughout all the fractions.

In the 9/22/78 ozonized samples, the $^{14}C$ activity has been shifted from the fraction containing (E)-11-tetradecenyl acetate to the fraction containing the 13-carbon ozonolysis fragment. This is reasonable proof that that $^{14}C$ activity in those samples is in fact incorporated into (E)-11-tetradecenyl acetate. Unfortunately, it was not possible to analyze all samples in this manner.

## Summary

In view of the evidence presented, it can be concluded that $^{14}C$ labeled myristic acid, at least in some instances, is incorporated into (E)-11-tetradecenyl acetate. It is not known whether the myristic acid is incorporated directly, or if first is broken down--perhaps to acetate--and if so whether the label appears throughout the chain or in the acetate portion of the molecule. The fact that samples from 1-$^{14}C$-acetate labeled moths had lower ratios of $^{14}C$ to (E)-11-tetradecenyl acetate weights against

Table 7.--The effect of ozonolysis of (E)-11-tetradecenyl acetate samples from three groups of 1-$^{14}$C myristic acid treated moths on the $^{14}$C activity distribution in those samples.[a]

1/1/79 Study[a]

| Retention Time (Min)[b] | Markers | dpm |
|---|---|---|
| 0-5.2 | Propanal | 31 |
| 5.2-8.0 | 13 Carbon Ozonolysis Fragment | 22 |
| 8.0-12.0 | (E)-11-tetradecenyl acetate | 36 |
| | Directly Counted Half | 90 |

9/22/78 Study[c]

| Retention Time (Min)[b] | Markers | dpm | |
|---|---|---|---|
| | | 24-48I | 24-48II |
| 0-2 | Propanal | B[d] | 4 |
| 2-4.5 | | 3 | 3 |
| 4.5-6.7 | 13 Carbon Ozonolysis fragment | 36 | 15 |
| 6.7-7.2 | | 5 | 1 |
| 7.2-9.0 | (E)-11-tetradecenyl acetate | 4 | HB[e] |
| 9.0-12.0 | | HB[e] | 0 |
| 12.0-15.0 | | 7 | HB[e] |
| | Directly Counted Half | 53 | 28 |

[a]This is the same sample listed in Table 3 under myristic acid for 1/1/79.

[b]Retention time on a 3% OV-1 column (150°) from which ozonized samples were collected.

[c]These are the same samples listed in Table 4 under 24-48 hour adults.

[d]B = Background.

[e]HB = Rejected because of high background.

incorporation through breakdown to acetate. Also, 1-$^{14}$C-stearic acid which would be expected to behave similarly to myristic acid in any breakdown to acetate, failed to produce any $^{14}$C labeled (E)-11-tetradecenyl acetate. (These experiments were done on 1/20/78 and 2/25/78 but not listed in Table 3.)

Evidence that $^{14}$C acetate or myristyl acetate is incorporated into (E)-11-tetradecenyl acetate is inconclusive. It is doubtful that myristanol is incorporated into (E)-11-tetradecenyl acetate because of the low $^{14}$C content measured in samples from $^{14}$C myristanol treated moths. For the long chain compounds, topical application gives much higher $^{14}$C counts in the final sample than injection and may therefore be a better technique than injection in certain instances.

Relative to tetradecenyl acetate biosynthesis in Platynota stultana, myristic acid incorporation shows that the lipid pool is involved, but no particular pathway can be pinpointed. The large experimental variation may mean that myristic acid is not a direct precusor of (E)-11-tetradecenyl acetate. However, if myristic acid is incorporated directly, the lack of incorporation of myristanol would indicate that the next step in the pathway is not reduction to myristanol. This of course assumes myristanol penetrated the pheromone gland and was available for subsequent biosynthesis. The lack of effect of (E) or (Z) 11-tetradecenol on the percentage composition of (E) and (Z)-

11-tetradecenyl acetates would imply that regulation of the pheromone blend would occur after alcohol formation or that the alcohol itself is not directly in the line of synthesis. This again assumes alcohol penetration into the gland. Large amounts of 11-tetradecenol can still be found in extracts of the gland 24 hours after treatment with (E) or (Z) 11-tetradecenol. Hill and Roelofs have found that 14 carbon alcohols found in the pheromone gland have the same isomeric ratio as that of acetates. Since the alcohol is found in higher quantities in the glands of older moths, it is possible that the alcohol results from the breakdown of the corresponding acetate. If this is the case, then the biosynthetic pathway for (E)-11-tetradecenyl acetate is more complicated than the scheme originally hypothesized.

It is evident from this study that pheromone biosynthesis occurs in adult moths. Production occurs at least on both the first and second days after eclosion and possibly throughout the life of the moth.

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