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Aldehyde Pheromones in Lepidoptera: Evidence for an Acetate Ester Precursor in *Choristoneura fumiferana*

Abstract. *Labeling studies of the eastern spruce budworm in vivo indicate that* trans-*11-tetradecenyl acetate is synthesized specifically in the pheromone-producing gland and is degraded in concert with pheromone release; hence it may be a precursor to the* trans-*11-tetradecenal pheromone. Radioactivity from exogenously added labeled fatty acids did not appear to be directly incorporated into the ester, suggesting that de novo biosynthesis from acetate is the major route of ester biosynthesis. Conversion of the acetate ester to alcohol and aldehyde functional groups may be the principal method of regulating pheromone specificity between species of* Choristoneura.

Most lepidopteran sex attractants are characterized by a long, monounsaturated carbon chain and an oxygen-containing functional group that can be an aldehyde, an alcohol, or an acetate ester of the alcohol. Although a mechanism involving Δ^{11} desaturation and chain shortening was recently proposed to explain biosynthesis of monounsaturated acyl chains (*1*), little attention has been focused on the functional groups.

In the genus *Choristoneura* (order Lepidoptera, family Tortricidae), pheromones from different species appear to be distinguished principally by different functional groups, as the pheromones all contain Δ^{11-14} carbon chains (*2*). The pheromone of *C. occidentalis*, for example, is a mixture of roughly equal amounts of aldehyde, alcohol, and acetate ester (*3*), whereas in *C. fumiferana* the pheromone is 99 percent aldehyde (*4*) and the alcohol and acetate esters are inhibitory to the attraction of male insects (*5*). The presence of 11-tetradecen-1-ol acetate (TDA) in the gland of *C. fumiferana* first suggested a precursor role for the acetate ester (*4*). Although the subsequent identification of pheromone gland enzymes capable of producing the aldehyde pheromone (ratio of *trans*- to *cis*-11-tetradecenal, 96 to 4) from TDA (*6*) support this hypothesis, a precursor role of TDA has not yet been clearly demonstrated in the spruce budworm.

We report here that TDA in *C. fumiferana* is specifically synthesized de novo only in the pheromone gland. During the period of pheromone release (*7*) there was a large decrease in the amount of radioactivity associated with labeled TDA, suggesting that this lipid is a precursor of the pheromone. Storage of the aldehyde pheromone as a less-reactive precursor is an attractive hypothesis, since it allows the biosynthetic steps that require large expenditures of energy to occur continuously and not simply during the period of pheromone production. The hypothesis also raises the possibility that a similar metabolic relation exists between the aldehyde, alcohol, and acetate ester pheromones of other lepidopteran species (*2*).

Figure 1A (lanes 2 to 5) shows the time course of incorporation of radioactivity from [1-^{14}C]acetate into lipids by the pheromone-producing gland in vivo. In these experiments, ~1 nmol of the labeled acetate in dimethyl sulfoxide (DMSO) was topically applied to the gland or another body component of the living insect (*8*). The tissue was subsequently extracted with hexane and analyzed by thin-layer chromatography (TLC) on silica gel. After a 2-hour labeling period (lane 4), a substantial amount of radioactivity comigrated with an unlabeled TDA standard. Radioactivity was not incorporated into this lipid when labeled acetate was topically applied to the abdomen or other body parts, even if a large excess of the sample was analyzed, whereas all other lipids were still synthesized (lane 1 in Fig. 1A).

To confirm the chemical identity of the gland-specific lipid as TDA, we extracted the labeled lipid from the silica gel and subjected it to further analysis. The ester nature of the functional group is indicated by the finding that the long-chain saponification product (*9*) comigrated with TDA (Fig. 1B). Between 75 and 80 percent of the radioactivity was recovered as the fatty alcohol, indicating that synthesis of the alcohol was occurring de novo in the gland and that the radioactivity was not incorporated simply by acetylation of preexisting fatty alcohol.

The lipid migrated in a reversed-phase TLC system with a TDA standard (Fig. 1C), confirming that the chain length was consistent with an acetate ester of a

Table 1. Incorporation of radioactively labeled fatty acids into TDA. Each labeled fatty acid, dissolved in dimethyl sulfoxide, was topically applied to the glands of groups of female insects 4 to 6 days old (*8*). At least two groups of 15 to 20 insects each were used per treatment, and approximately the same amount of material was applied to each gland. Incorporation was measured by scintillation counting of TDA cut from the silica gel after visualization of the radiolabeled lipids by autoradiography. All incubation periods were 2 hours long.

Precursor	Specific activity (mCi/mmol)	Amount applied (nanomoles per insect)	Incorporation into TDA (percent)
[1-^{14}C]acetate	56	0.8	0.23 ± 0.01
[^{3}H]acetate	2800	0.7	0.15 ± 0.03
[1-^{14}C]laurate	36	0.8	0.38 ± 0.03
[1-^{14}C]myristate	31	1.7	0.39 ± 0.09
[1-^{14}C]palmitate	56	0.5	0.30 ± 0.01
[9,10-^{3}H]myristate	31	1.6	0.08 ± 0.04

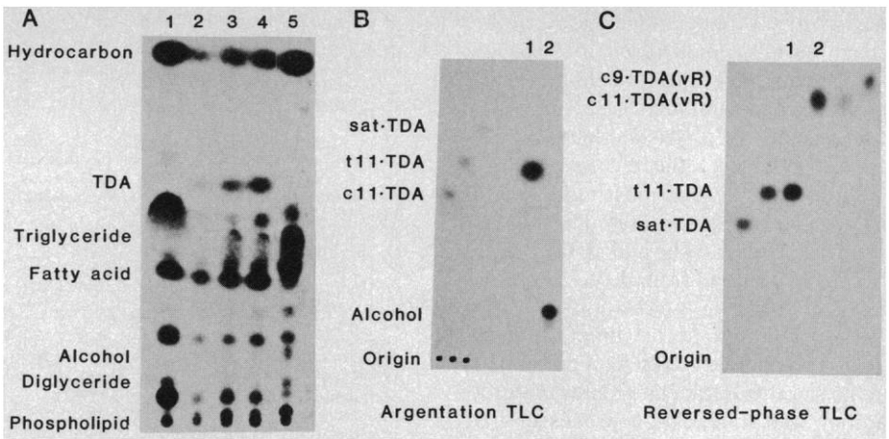

Fig. 1. De novo synthesis and analysis of gland-specific lipid. (A) Groups of eight glands were extracted for 20 to 30 minutes with 100 μl of hexane containing 10 percent acetone and 0.005 percent butylated hydroxytoluene as an antioxidant. Chromatography of this hexane extract was performed on Machery Nagel Sil N-UV_{254} TLC plates, heat-activated for 60 minutes at 110°C, and developed for 90 minutes in hexane, ether, and acetic acid (90:10:2). Shown is an autoradiogram of the glandular lipids incorporating radioactivity from [1-^{14}C]acetate after labeling periods of 15 minutes, 1 hour, 2 hours, and 20 hours (lanes 2 to 5, respectively). Incorporation of radioactivity by abdominal tissue is shown for a 2-hour labeling period in vivo (lane 1). Autoradiography was performed by exposing the TLC plate to Kodak X-AR OMAT film for 3 to 7 days at −70°C. Unlabeled standards, run in parallel, were visualized with I_2 vapor. In this system, hydrocarbons run with the solvent front while phospholipids remain at the origin. The gland-specific lipid (TDA) was extracted from the silica gel with diethyl ether and used for further analysis. (B) Autoradiograph of an argentation TLC plate, on which was run the ether-extracted TDA sample (lane 1) and the saponification product (*9*) of the ether-extracted TDA (lane 2). Argentation chromatography was performed on silica gel TLC plates that were heat-activated for 60 minutes at 110°C, sprayed with a 7.5 percent solution of $AgNO_3$ in 90 percent ethanol, and heated for a further 60 minutes. The plates were developed in the same solvent as the silica gel plates. The standards on the left show the excellent separation of saturated, *trans*-11-, and *cis*-11-TDA isomers. (C) Autoradiograph of a reversed-phase TLC plate on which was run the ether-extracted TDA sample (lane 1) and the von Rudloff oxidation product (*10*) of the ether-extracted TDA (lane 2). Reversed-phase chromatography was performed on Whatman KC18-F TLC plates, which were used without heating and developed for 20 minutes in CH_3CN, tetrahydrofuran, and methanol (65:1:10). The standards on the left show that the TDA sample has a chain length consistent with that of an unsaturated TDA isomer, while the standards on the right show the difference in migration between Δ^9- and Δ^{11}-TDA carried through the von Rudloff oxidation procedure.

monounsaturated 14-carbon alcohol. The major component of the lipid comigrated in the argentation TLC system with *trans*-11-TDA (Fig. 1B), showing that it contained a single site of unsaturation in the *trans* configuration. A small amount of the *cis* isomer was also detected, showing that this lipid had a *trans-cis* isomeric composition similar to that of the aldehyde pheromone. The position of the double bond was identified as Δ^{11} by measuring the chain length of the permanganate-periodate oxidation product (*10*) in the reversed-phase TLC system (Fig. 1C).

The amount of radioactivity incorporated into the classes of lipid common to the gland and abdomen either did not change or increased if the lipids were extracted after the nightly period of pheromone release. In contrast, the amount of radioactivity associated with the gland-specific lipid (TDA) decreased sharply (lane 5 in Fig. 1A), indicating that it was turned over or degraded during this period. This result suggests that there is a physiological relation between this lipid and the budworm pheromone, and is consistent with this lipid functioning as a precursor storage form of the aldehyde pheromone. Because topical applications of DMSO solutions to the gland surface interfere with pheromone release (*6*), we injected [^{14}C]acetate into the insect (*11*) to study the release of labeled aldehyde. Despite a lower incorporation of radioactivity into TDA, both trapped aldehyde and glandular ester levels could be measured, and, as shown in Fig. 2, the release of labeled aldehyde was well correlated with disappearance of the ester.

The pathway of TDA biosynthesis is of considerable interest, since acetate esters are used directly as pheromones by other lepidopteran species. During the normal route of fatty acid biosynthesis through malonyl–coenzyme A (malonyl-CoA), hydrogen is lost from acetyl-CoA, and thus [^{14}C]acetate and [^{3}H]acetate are differentially incorporated (Table 1). In addition, the observed ratio of ^{14}C in the alcohol and acetate moieties of TDA (~4:1) is also consistent with the normal route of fatty acid biosynthesis, since a decrease in acetyl-CoA–specific radioactivity would occur on passage through a malonyl-CoA pool before incorporation into the 14-carbon chain alcohol.

To determine whether chain shortening and Δ^{11} desaturation was involved in TDA biosynthesis in the budworm gland, as has been demonstrated in other insects (*1*), we topically applied a series of labeled fatty acids to the gland. It was expected that little radioactivity would be incorporated into TDA from [1-^{14}C]palmitate if chain shortening was the major route of synthesis, as the 1-^{14}C would be lost after partial β-oxidation. In addition, if direct desaturation of myristate were occurring, then a high incorporation of [1-^{14}C]myristate into TDA should have been observed. As shown in Table 1, the results of incorporation of radioactivity into TDA from laurate, myristate, and palmitate were very similar and were comparable to those for acetate, suggesting that all three fatty acids were first β-oxidized to acetyl-CoA and then resynthesized into TDA.

To confirm that myristate was converted into TDA through acetyl-CoA, we compared the incorporation into TDA from [1-^{14}C]myristate to that from [9,10-^{3}H]myristate (Table 1). The difference in incorporation of the two shows that the

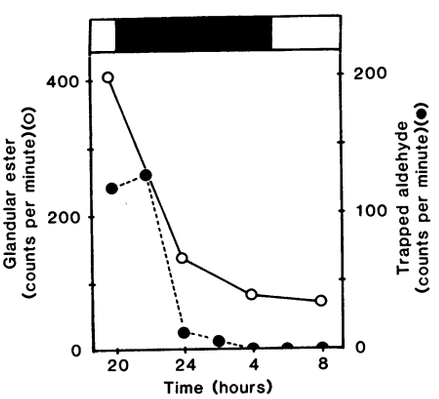

Fig. 2. Temporal relation between aldehyde release and ester degradation. In these experiments, ~18 nmol of labeled acetate in 1 μl of 0.05*M* phosphate buffer (*p*H 7.0) was injected into the abdomen of a living insect (*11*) between 1600 and 1700 hours; trapping began at 1800 hours. The ester levels for groups of 20 insects were measured by scintillation counting of TDA after silica gel TLC of the gland extracts, as described in the legend to Fig. 1. Labeled aldehyde from a group of 20 insects was trapped on Porapak Q for several successive 2-hour periods (*7*) and was purified by silica gel TLC before scintillation counting. The shaded rectangle indicates the dark phase of the daily light-dark cycle.

acyl chains were degraded and resynthesized, as this would result in a specific loss of tritium. Tritium was not lost by a direct mechanism such as Δ^9 desaturation since [1-^{14}C]myristate-labeled TDA consisted only of the Δ^{11} isomer. The findings indicate that most radioactivity is not directly incorporated into TDA from an exogenously added fatty acid, but results instead from de novo synthesis after complete β-oxidation.

The inability to detect direct desaturation of myristate could reflect differences in the rates or intracellular sites of desaturation and degradation processes that greatly favor β-oxidation. Alternatively, the CoA derivatives used in β-oxidation and as substrates for mammalian Δ^9-desaturases (*12*) may not be the preferred form for desaturation in insects. The possibility that desaturation occurs during fatty acid biosynthesis, as in bacterial systems (*13*), cannot be ruled out, although this process has not yet been observed in eukaryotic cells. It is of interest, in this regard, that the only Δ^{11}-desaturases reported have been in bacteria (*14*) and in insect tissue (*1*). The observation of direct Δ^{11}-desaturase activity in the bacterial system was facilitated by the lack of de novo fatty acid biosynthesis, and it may be necessary to reduce the level of biosynthesis or degradation of fatty acids before direct Δ^{11}-desaturation can be demonstrated in the spruce budworm.

Although it has not been possible to identify the exact route of TDA biosynthesis, our findings suggest that this compound is synthesized de novo from acetate even in the presence of exogenously added fatty acids. This observation may be relevant to the biosynthesis of the many other lepidopteran sex pheromones that contain acetate ester functional groups. In the budworm, however, the acetate ester appears to act as a pheromone precursor, as it is synthesized specifically in the pheromone gland and is degraded concurrently with pheromone release. The conversion of an acetate ester precursor into an alcohol or aldehyde pheromone could easily have arisen in evolution to provide an increase in sex pheromone specificity within a group of closely related species. The increase in specificity provided by modification of the functional group can be achieved without causing a major change in the biosynthesis or regulation of pheromone production.

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8. This technique is based on that described by L. B. Bjostad and W. L. Roelofs [*J. Biol. Chem.* **256**, 7936 (1981)], except that a 1-μl solution containing 20 nmol of labeled compound in DMSO was applied to 20 female glands. No excess DMSO was left on the gland surface.
9. Saponification was performed by dissolving the lipid in 0.5 ml of 0.5*N* KOH in 90 percent methanol, followed by a 60-minute incubation at 60°C. Neutral lipids were extracted from the saponification mixture with 2 × 1.0 ml of hexane after addition of 0.5 ml of water. In control experiments with tetradecanyl acetate labeled in the tetradecanol and acetate moieties, respectively, 98 percent of the alcohol and less than 1 percent of the acetate was extracted after saponification.
10. The procedure used was modified from E. von Rudloff [*Can. J. Chem.* **34**, 1413 (1956)]. Only 1 μg of labeled lipid was redissolved in 0.5 ml of *t*-butanol, to which was added 0.5 ml of oxidant (97.5 m*M* $NaIO_4$ and 2.5 m*M* $KMnO_4$) and 0.5 ml of 25 m*M* $KHCO_3$. After a 60-minute reaction at room temperature, the mixture was acidified with one to two drops of concentrated H_2SO_4 and extracted with 2 × 1.0 ml of hexane.
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Facilitated Sexual Behavior Reversed and Serotonin Restored by Raphe Nuclei Transplanted into Denervated Hypothalamus

Abstract. *Fetal raphe cells transplanted into the hypothalamus reversed facilitation of feminine sexual behavior in rats with brain lesions induced by 5,7-dihydroxytryptamine. Immunocytochemical and chemical analyses of serotonin indicate that reinnervation of the ventromedial nucleus of the hypothalamus by the transplants is associated with behavioral recovery. The findings suggest that transplanted fetal tissue can exert functional regulation over an innate, complex, hormone-dependent behavior in adult rats.*

The hypothalamus is an obligatory site for the mediation of gonadal hormone–dependent sexual behavior in the female rat. Within the hypothalamus, the ventromedial nucleus is believed to be critical for transmitting signals that regulate neuroendocrine circuits for reproductive behavior, since hormonal stimulation of this nucleus alone is sufficient to activate such behavior (*1*). Serotonergic systems are believed to play a major role in the hormonal regulation of sexual behavior through tonic inhibitory inputs to the hypothalamus (*2*). Consistent with this hypothesis, selective lesioning of serotonin-containing fibers in the hypothalamus by treatment with 5,7-dihydroxytryptamine (5,7-DHT) results in enhanced sexual behavior after administration of estradiol (*3*). Facilitated behavior is shown for up to 8 weeks and disappears coincident with reinnervation of the hypothalamus by serotonin-containing fibers 9 to 10 weeks after 5,7-DHT treatment (*4*).

Previous studies established that fetal brain cells can survive transplantation into neonatal, adult, and aged brains (*5*). Such transplants reverse stereotypical drug-induced behaviors seen after specific chemical lesioning of the adult host brain (*6*) and ameliorate age-related motor and learning deficits in the aged host (*7*). In addition, transplantation of preoptic tissue from a neonatal rat into the preoptic area of another neonatal rat increases the sexual behavior of the hosts in adulthood (*8*). We now report the ability of fetal cells transplanted into an adult brain to regulate an innate, complex, hormonally dependent behavior of the host. We transplanted fetal tissue rich in serotonergic raphe cell bodies into the hypothalamus of females with 5,7-DHT–induced lesions. The raphe transplants caused a rapid reversal of 5,7-DHT–facilitated sexual behavior and increased serotonin concentrations in the ventromedial nucleus.

One week after ovariectomy adult female rats (Charles River) received desmethylimipramine followed by bilateral stereotaxic application of 5,7-DHT creatinine sulfate (5 μg in 400 nl of ascorbic acid and saline) into the hypothalamus just dorsal to the ventromedial nuclei (*3*). Other females (group S) received sham injections of the ascorbic acid vehicle. One week later fetal tissue was stereotaxically placed into the hypothalamic