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The Integral Role of Triacyl Glycerols in the Biosynthesis of the Aldehydic Sex Pheromones of *Manduca sexta* (L.)

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Abstract—In a gland located near the tip of their abdomens, *Manduca sexta* females produce a pheromone blend comprised of hexadecanal, (*Z*)-9-hexadecenal, (*Z*)- and (*E*)-11-hexadecenal, (*E,Z*)- and (*E,E*)-10,12-hexadecadienal, and (*E,E,Z*)- and (*E,E,E*)-10,12,14-hexadecatrienal. These aldehydes are produced and released by evaporation from the surface of the gland only during a discrete period of the night. They are not stored in the gland and are found there only in very small amounts, if at all, during other times of the photoperiod. However, fatty acyl analogues of the pheromone aldehydes are present in the gland in relatively large amounts, primarily as components of triacyl glycerols, continuously from eclosion of the adults until death. The unsaturated components are produced from hexadecanoate, which is desaturated to the monoenes. Then, (*Z*)-11-hexadecenoate is desaturated and isomerized to form the conjugated dienes and ultimately the conjugated trienes. The fatty acyl precursors of the pheromones, stored as components of triacyl glycerols, are converted into aldehydes by a process triggered by a pheromone biosynthesis activating neuropeptide (PBAN), produced in the brain-subesophageal complex. It is not yet clear whether this conversion involves direct reduction of the acyl groups to aldehydes or reduction to alcohols followed by oxidation to aldehydes.
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Introduction

The tobacco hornworm moth, *Manduca sexta* (L.) (Lepidoptera: Sphingidae), is found throughout the greater part of the U.S., the West Indies, Mexico, Central America and parts of South America. Its larvae, which eat the leaves of a wide range of solanaceous plants including tobacco, tomato, eggplant, Jerusalem cherry and potato,[1] have a rapid rate of development and can attain a weight of 9 g or more by the fifth instar.[2] Furthermore, they can be reared easily on artificial diet, making this species readily available for a variety of research studies. Thus, it has become the experimental insect of choice for many physiological and biochemical studies, including investigations of olfactory neurophysiology,[3–5] lipid metabolism,[6,7] and endocrinology.[8]

Female *M. sexta* produce and release a sex pheromone which attracts conspecific males for the purpose of mating. In moths sex pheromones typically are synthesized in a gland located near the tip of the abdomen and are composed of blends of long-chain aliphatic hydrocarbons, alcohols, aldehydes, epoxides and/or esters. The general scheme of pheromone biosynthesis in most Lepidoptera involves fatty acid biosynthesis, desaturation, chain shortening or elongation, reduction, acetylation and possibly oxidation.[9–11] Different combinations of these reactions are employed by various species to achieve their own unique pheromone blends. The most prevalent enzymes used by these insects to introduce unsaturated bonds into the fatty acid chains are the Δ11-desaturases, and these, acting in concert with other desaturases, allow a variety of olefinic systems to be produced. For example, in the production of the major component of its pheromone blend, *Spodoptera littoralis* converts tetradecanoic acid, by the sequential action of a specific (*E*)-11-desaturase and then a (*Z*)-9-desaturase, into the (*Z,E*)-9,11-tetradecadienoate.[12]

In many insect species pheromone biosynthesis is induced by a pheromone biosynthesis activating neuropeptide (PBAN).[12–17] PBAN was discovered in the brain-subesophageal ganglion of females of the corn earworm moth, *Helicoverpa* (=*Heliothis*) *zea* and isolated and identified by Raina and colleagues.[18,19] In Lepidoptera it may function differently in various species to stimulate the production of pheromones. In *H. zea* the production of sex pheromone by females follows a circadian rhythm with the peak period of production being between the third and fifth h of the scotophase.[20] Little or no pheromone is present during the photophase,[20,21] although females can be induced to produce pheromone at any time by injection of PBAN.[21] In contrast, pheromone biosynthesis in the sex pheromone gland of females of the cabbage looper moth, *Trichoplusia ni*, is continuous[22] and not dependent on the presence of PBAN,[17] although pheromone release occurs only during the dark phase of the photoperiod. In the redbanded leafroller moth, *Argyrotaenia velutinana*, pheromone titer also remains high and does not cycle diurnally, but PBAN injected

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into decapitated moths stimulates pheromone biosynthesis by activating synthesis of octadecanoyl and hexadecanoyl intermediates, thus increasing the substrate supply for fatty acid synthesis.[17] In this species PBAN does not control other enzymes in the pheromone biosynthesis pathway. Also, studies by Jurenka et al.,[15] in which ^{14}C labeled acetates and deuterium labeled 16-carbon fatty acids were used to monitor pheromone biosynthesis, indicated that PBAN controls pheromone biosynthesis in *H. zea* females by regulating a step in or prior to fatty acid biosynthesis. However, in *Chrysodeixis chalcites*[13] it has been suggested that PBAN regulates Δ11-desaturase, while in *S. littoralis* the reduction of fatty acids to alcohols appears to be affected by PBAN.[12] Also, a study with *Bombyx mori* suggests that PBAN regulates the reduction of the acyl moiety to the alcohol in that species.[23]

Initially, we isolated and identified the components of the sex pheromone of *M. sexta* because of the importance of this species to both agriculture and science. The unique structures of some of the components led us to explore the biosynthetic mechanisms by which the pheromone blend characteristic of this species is produced. Here we present the results of a series of investigations and our current state of knowledge of pheromone biosynthesis in this species.

Results and Discussion

Identification of pheromone aldehydes and fatty acyl analogues in pheromone gland

The sex pheromone produced and released by *M. sexta* females was identified as a blend of eight 16-carbon aldehydes (Table 1).[24] The aldehydes are produced in a gland located ventrolaterally in the intersegmental membrane (ISM) between the eighth and ninth segments of the female abdomen (Fig. 1).[25-28] Two of the compounds found in the hexane extracts of the glands, *E*10,*Z*12–16:AL[‡] and *E*10,*E*12,*Z*14–16:AL, proved to be essential for biological activity in laboratory wind-tunnel bioassays. Electrophysiological studies showed that *E*10,*Z*12–16:AL selectively stimulates one of the two pheromone receptor cells in each trichoid sensillum on the antennae of *M. sexta* males. In most of these sensilla the second receptor responds to *E*10,*E*12,*Z*14–16:AL, but in some sensilla the second receptor responds to the *EEE* isomer.[29] Furthermore, Christensen et al.[30] showed that all the 16-carbon aldehydes found in the pheromone gland rinses elicit some form of response in olfactory interneurons in the brains of males, but *E*10,*Z*12–16:AL, *E*10,*E*12,*Z*14–16:AL and *E*10,*E*12,*E*14–16:AL evoke the greatest responses. Field tests confirmed the biological activity of a blend of all eight 16-carbon aldehydes.[31]

M. sexta store practically no sex pheromone aldehydes in the gland, biosynthesizing the pheromone only during a 3–5 h pheromone release period in the scotophase and immediately evaporating it from the surface of the gland. This raises the question of whether immediate precursors are stored in the gland and converted to aldehydes just prior to pheromone release or the pheromonal aldehydes are synthesized de novo when needed. Thus, as a first step in elucidating the pheromone biosynthetic process we analyzed the fatty acid methyl esters (FAME) produced by methanolysis of $CHCl_3$/MeOH extracts of the pheromone glands. The extracts were obtained at various times and under various conditions. This analysis revealed that fatty acyl analogues of the 16-carbon pheromone aldehydes are

[‡]A standard shorthand notation for pheromone molecules and analogues will be used in this paper. For example, 16:AL = hexadecanal, *E*11–16:FA = (*E*)-11-hexadecenoic acid, *E*10,*Z*12–16:AL = (*E*,*Z*)-10,12-hexadecadienal and D_3-*Z*11–16:FA = [16,16,16-$^2H_3$] (*Z*)-11-hexadecenoic acid.

Table 1. Aldehydes and fatty acids identified in hexane and $CHCl_3$:MeOH extracts, respectively, of female *M. sexta* pheromone glands

Aldehydes		ng/FGE[a]	Fatty acids		ng/FGE[a] (Bound)
(*Z*)-9-Hexadecenal	(*Z*9–16:AL)	0.8	(*Z*)-9-Hexadecenoic	(*Z*9–16:FA)	333
(*Z*)-11-Hexadecenal	(*Z*11–16:AL)	13.4	(*Z*)-11-Hexadecenoic	(*Z*11–16:FA)	3514
(*E*)-11-Hexadecenal	(*E*11–16:AL)	6.8	(*E*)-11-Hexadecenoic	(*E*11–16:FA)	214
Hexadecanal	(16:AL)	15.7	Hexadecanoic	(16:FA)	1651
(*E*,*Z*)-10,12-Hexadecadienal	(*E*10,*Z*12–16:AL)	23.8	(*E*,*Z*)-10,12-Hexadecadienoic	(*E*10,*Z*12–16:FA)	574
(*E*,*E*)-10,12-Hexadecadienal	(*E*10,*E*12–16:AL)	3.9	(*E*,*E*)-10,12-Hexadecadienoic	(*E*10,*E*12–16:FA)	144
(*E*,*E*,*Z*)-10,12,14-Hexadecatrienal	(*E*10,*E*12,*Z*14–16:AL)	11.3	(*E*,*E*,*Z*)-10,12,14-Hexadecatrienoic	(*E*10,*E*12,*Z*14–16:FA)	N[b]
(*E*,*E*,*E*)-10,12,14-Hexadecatrienal	(*E*10,*E*12,*E*14–16:AL)	1.2	(*E*,*E*,*E*)-10,12,14-Hexadecatrienoic	(*E*10,*E*12,*E*14–16:FA)	N
(*Z*)-11-Octadecenal	(*Z*11–18:AL)	6.2	(*Z*)-11-Octadecenoic	(*Z*11–18:FA)	N
(*Z*)-13-Octadecenal	(*Z*13–18:AL)	2.2	(*Z*)-13-Octadecenoic	(*Z*13–18:FA)	N
Octadecanal	(18:AL)	4.8	Octadecanoic	(18:FA)	N
(*Z*,*Z*)-11,13-Octadecadienal	(*Z*11,*Z*13–18:AL)	1.4	Not detected		
			(*Z*)-9-Octadecenoic	(*Z*9–18:FA)	N
			(*Z*,*Z*)-9,12-Octadecadienoic	(*Z*9,*Z*12;18:FA)	N
			(*Z*,*Z*,*Z*)-9,12,15-Octadecatrienoic	(*Z*9,*Z*12,*Z*15–18:FA)	N

[a]FGE = Female gland equivalent.
[b]N = not quantitated.

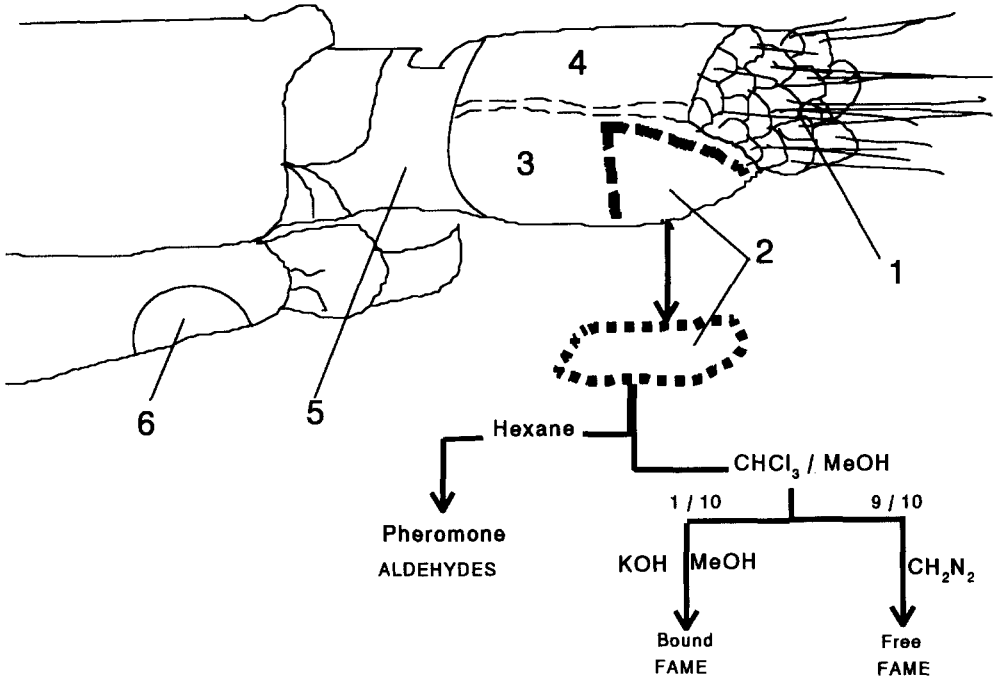

Figure 1. Drawing of the abdominal tip of the female tobacco hornworm moth, after Eaton.[26] 1, papillae anales; 2, pheromone gland; 3, ventral intersegmental membrane (ISM) anterior to the gland; 4, dorsal ISM above the gland; 5, 8th abdominal segment; 6, ISM between the 6th and 7th abdominal segments.

present continuously in relatively large quantities (1–6 μg/gland) in the pheromone gland from about 3 days before adults emerge from the pupal stage until the adults are 8 days old.[28] While common fatty acyl groups like hexadecanoate, (*Z*)-9-hexadecenoate, octadecanoate, (*Z*)-9-octadecenoate, (*Z,Z*)-9,12-octadecadienoate and (*Z,Z,Z*)-9,12,15-octadecatrienoate were detected in all the body tissues of females that we examined, uncommon fatty acid pheromone analogues [(*Z*)- and (*E*)-11-hexadecenoate, (*E,Z*)- and (*E,E*)-10,12-hexadecadienoate, and (*E,E,Z*)- and (*E,E,E*)-10,12,14-hexadecatrienoate] were not found in significant amounts in tissues other than the epidermal cells of the pheromone gland (Fig. 1).

Analysis of the methyl esters formed by reaction of diazomethane with the free fatty acids in the $CHCl_3$/MeOH extracts of the ISM gave very similar results to those obtained for the bound fatty acids. The primary difference was that the free fatty acid analogues of the pheromones were present in much smaller quantities (about 1%) than the bound fatty acid analogues in the ISM.[28]

Biosynthesis of olefinic systems

The presence of Δ10,12- and Δ10,12,14-, as well as Δ11-unsaturated compounds in its pheromone blend raises the question of how *M. sexta* produces the conjugated olefinic compounds. This system is similar to that of the silkworm moth which has both Δ11- and Δ10,12-unsaturated fatty acids in its gland. In the latter moth evidence suggests that the Δ10,12-system is produced from the Δ11-fatty acid via an isomerase.[32,33]

To determine the biosynthetic pathway by which *M. sexta* produces the conjugated dienes and trienes of its pheromone blend, we applied deuterium-labeled putative fatty acyl precursors to the pheromone gland and, after inducing pheromone biosynthesis by injection of PBAN into the abdomen of the moth, extracted and analyzed the labeled products by GC–MS.[34]

When glands were incubated in vivo with D_3-16:FA for 24 h, with PBAN injected into the female abdomens after 23 h, the three deuterium atoms were incorporated into seven 16-carbon aldehydes, 16:Al, *Z*11–16:Al, *E*11–16:Al, *E*10,*Z*12–16:Al, *E*10,*E*12–16:Al, *E*10,*E*12,*Z*14–16:Al and *E*10,*E*12,*E*14–16:Al. Thus, hexadecanoate is the precursor of all the 16-carbon aldehydes produced in the pheromone gland, except possibly for *Z*9–16:Al, which is present in such small amounts naturally that it would not be detected in the labeled form. Similarly, the deuterium label was found in the hexadecanoate, (*E*)- and (*Z*)-11-hexadecenoate, (*E,E*)- and (*E,Z*)-10,12-hexadecadienoate methyl esters (Table 1). Analysis of the diazomethane esterified free fatty acid methyl esters in these same extracts revealed that the same acids were labeled in the free form as in the bound form. Labeled hexadecatrienoates were not found in these extracts in either the free or the bound form, but this is not surprising since the natural forms of these compounds are present in very small quantities and are extremely labile and thus difficult to detect as FAME by capillary GC.[28]

Incubation of glands with D_3-*Z*11–16:FA resulted in incorporation of the label into not only the two diene

aldehydes and their fatty acyl precursors, but also into the triene aldehydes and fatty acids. Since D_3-*E*11–16:FA was present as an impurity (ca. 2%) in the D_3-*Z*11–16:FA it was impossible to determine whether or not isomerization of the (*Z*) isomer to the (*E*) isomer occurred. However, no D_3-16:FA or D_3-16:Al was detected. When the glands were incubated with D_3-*E*11–16:FA the corresponding labeled aldehyde was produced as before, but none of the labeled (*Z*) isomer, 10,12-dienes or trienes were detected. These results appear to rule out the existence of (*E*/*Z*) isomerases in the gland and, in conjunction with the evidence that label from hexadecanoic acid was incorporated into both (*Z*)- and (*E*)-11-monoenes, suggest that two specific Δ-11-desaturase enzymatic systems are involved in the biosynthesis of monoenes from hexadecanoic acid.[34] Then, the conjugated trienes are formed by further desaturation and isomerization of *Z*11–16:FA, probably via the *E*10,*Z*12–16:FA, although we do not have evidence for the latter (Fig. 2).

When the gland was incubated with [13,13,14,14,15,15,16,16,16,-$^2H_9$] (*E*)-11-hexadecenoic acid (D_9-*E*11–16:FA) the only labeled aldehyde detected was D_9-*E*11–16:Al, with an [M+1] ion at (*m*/*z*) 248 . In contrast to the (*E*) isomer, incubation of the glands with D_9-*Z*11–16:FA resulted in incorporation of the label into *Z*11–16:Al and, with the loss of one deuterium atom, into D_8-*E*10,*Z*12–16:Al and D_8-*E*10,*E*12–16:Al, and the analogous fatty acyl precursors. These results suggest that the mechanism for biosynthesis of dienes in *M. sexta* is similar to that employed by the silkworm moth, *B. mori*, to produce the 10,12-hexadecadienyl system[32] and by the codling moth, *Cydia pomonella*, to produce (*E*,*E*)-8,10-dodecadienol from the (*E*)-9-dodecenyl precursor.[35] In particular, the mechanism appears similar to that of the codling moth, in which [11,11,12,12,12-$^2H_5$](*Z*)-9-dodecenoic acid was converted to the 8,10-diene with the loss of one deuterium atom. In this case Lofstedt and Bengtsson[35] proposed that the key steps involved the oxidation of one of the α-positions next to the double bond, followed by a 1,4 elimination of water.

Neither labeled 16:Al nor *E*11–16:Al nor their labeled fatty acyl precursors were found in the extracts of glands incubated with D_9-*Z*11–16:FA. Furthermore, no labeled triene aldehydes or fatty acids could be detected by MS. Even when the gland was induced to incorporate more of this compound by the use of different solvents, which resulted in more of the labeled diene aldehydes being produced, labeled trienes could not be detected in the gland extracts. Since glands incubated with D_3-*Z*11–16:FA produce D_3-labeled trienes as well as dienes, an isotope effect apparently hinders the conversion of D_9-*Z*11–16:FA to the conjugated triene aldehydes.

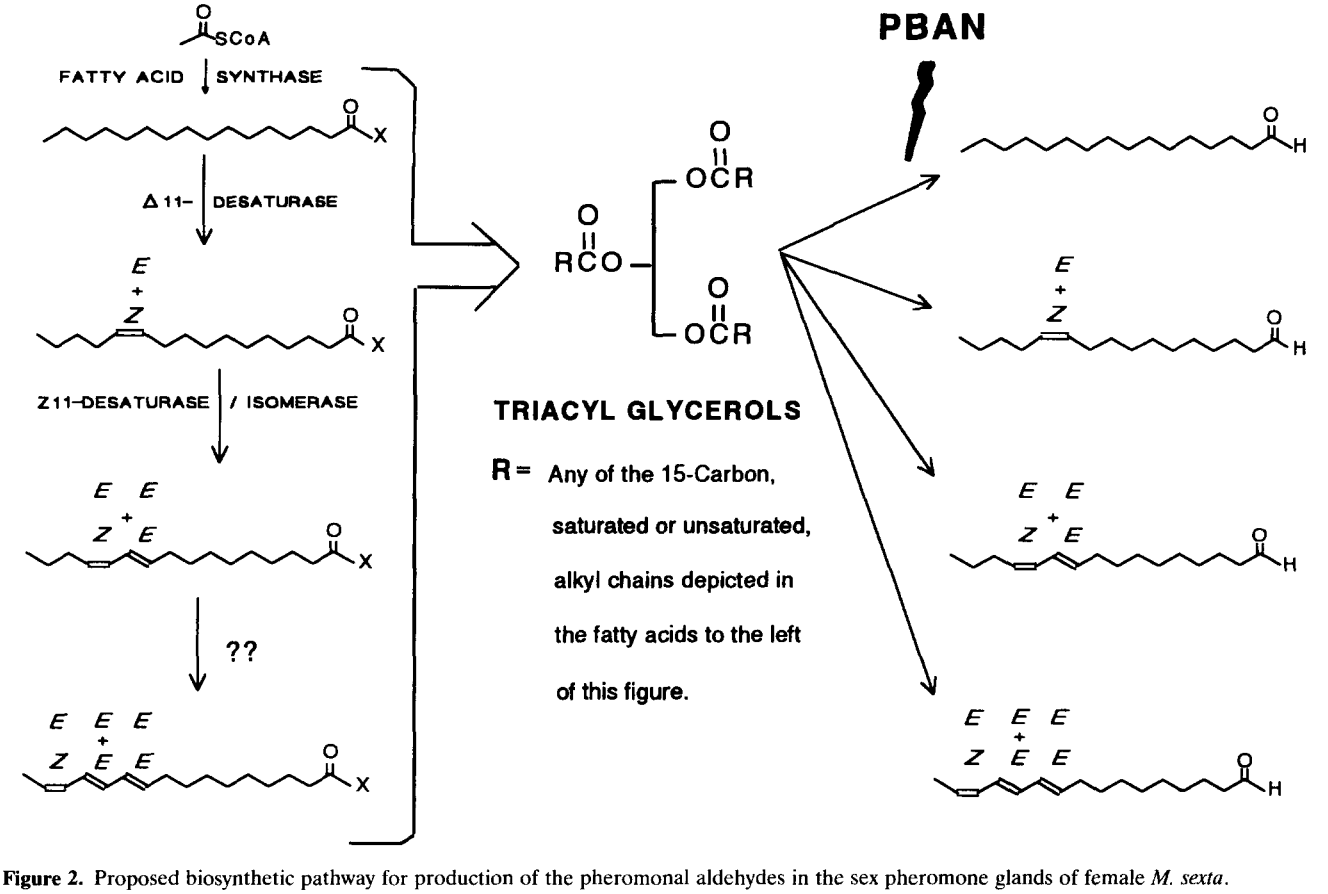

Figure 2. Proposed biosynthetic pathway for production of the pheromonal aldehydes in the sex pheromone glands of female *M. sexta*.

Triacyl glycerols in pheromone gland

The relatively large amount of fatty acyl analogues of pheromone bound to lipids in the gland during the lifetime of the moth suggests that pheromone precursors are stored as lipid derivatives.[28] TLC and HPLC analyses of extracts of sex pheromone glands of *M. sexta* showed that triacylglycerols are the most abundant lipid class in the gland and conjugated olefinic acyl analogues of major active pheromone components occur principally in the triacyl glycerols (TG). Analysis of gland extracts, to which *E*10,*Z*12–16:OH had been added as an internal standard, by HPLC on a 5 μm silica gel column eluted with hexane:ether:acetic acid (780:220:2) and monitored by an RI detector, indicated that one gland contained ca. 11 μg of TG and 1.5 μg of diacyl glycerols (DG). With a UV detector set at 230 nm or 267 nm, conjugated diene (ca. 0.9 μg) and triene (ca. 0.3 μg) acyl moieties were detected in the TG of one gland, but were not measurable in DG.[36] In comparison, hexane extracts of glands contain about 28 ng of the diene aldehydes and 77 ng of all the 16-carbon aldehydes combined.[24] Thus, the reservoir of fatty acyl pheromone analogues stored as components of TG in the glands is large relative to the amounts of pheromone aldehydes produced.

Since the majority of lipids found in glands were TG when extracts were analyzed by HPLC with the solvent system indicated above, the TG were examined further by HPLC on the same column with the less polar solvent hexane:acetonitrile (1000:6.5). With this solvent system the very complicated composition of TG in the gland was revealed and more than 10 peaks with conjugated diene acyl moieties were found when the eluant was monitored at 230 nm (Fig. 3). The pattern of triene TG found by HPLC was similar to that of the diene TG. However, the trienes are less stable and present in smaller quantities and thus the triene TG have not been investigated further.

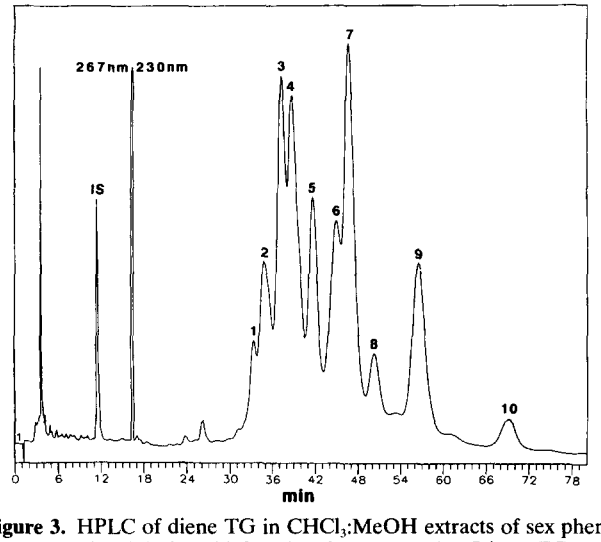

Figure 3. HPLC of diene TG in $CHCl_3$:MeOH extracts of sex pheromone glands of 2 day-old female *M. sexta* moths. Diene TG were separated on a 0.64 × 25 cm silica column with hexane:acetonitrile (1000:6.5) as the mobile phase. Eluting components were detected with a UV monitor set at 267 nm for detection of the internal standard (IS, methyl (*E*) cinnamate, 20 ng/gland) and at 230 nm for detection of diene TG.

GLC analysis of base methanolysis products of each HPLC peak indicated that the TG in HPLC peaks 2, 3 and 4 contained one conjugated C-16-diene and two other acyl moieties. The TG components of peak 7 were comprised of two conjugated C-16-dienes and one other acyl moiety. All three acyl moieties associated with the TG of peak 9 were conjugated C-16-dienes. Also, only conjugated C-16-diene acyl moieties were detected in the methanolysis products of peak 10. However, peak 10 had a longer retention volume than peak 9 on silica gel HPLC. On the basis of the HPLC retention volume and the fact that a relatively small amount of C-16-diene FAME was detected by GLC analysis, we hypothesize that the TG in peak 10 may be comprised of conjugated diene and triene acyl moieties. The triene acyl moieties were not detectable as FAME by capillary GLC with splitless injection because of their instability.

Correlation between pheromone production and glandular content of diene TG

The effect of pheromone release for 4 h during the natural signaling period was to significantly lower the level of the diene TG in the glands (Fig. 4).[36] To confirm this correlation between pheromone production and content of diene TG, females that had completed signaling were immediately treated with multiple injections of PBAN or H_2O (control). The pheromone aldehydes, extracted with hexane, were analyzed by GLC and the diene TG, extracted with $CHCl_3$/MeOH from the same glands, were analyzed by HPLC. Amounts of pheromone aldehydes in extracts of glands of females treated with PBAN twice (4 h incubation after natural signaling, P2–4h) were slightly higher than in extracts of females injected with PBAN and incubated for 1 h (P1–1h) during the photophase. In contrast, in the same two treatments the quantities of diene TG were significantly lower in extracts of females that had received two injections of PBAN (Fig. 4). Extension of PBAN treatments to include a third injection and a 7 h incubation (P3–7h) resulted in a further decline in the quantity of diene TG in the gland. In fact, after 4 h of natural pheromone release plus 7 h of PBAN-induced pheromone biosynthesis glands contained ca. 70% less diene TG than those of females sampled 10 min after they began to release pheromone naturally. This indicates that TG are involved in the biosynthesis of the pheromone aldehydes, and may serve as precursor donors for pheromone components.

It was intriguing that amounts of diene TG in extracts of glands from moths treated with three injections of PBAN and incubated for 10 h (P3–10h) were higher than in extracts from moths receiving the same treatment, but only 7 h incubation (P3–7h). The same amount of aldehydes was found in extracts of glands from both treatments. It is possible that the increase in

quantity of diene TG may have resulted from recycling of acyl moieties which were converted from pheromone aldehydes, because the glands of moths were never protruded to release the aldehydes into the air during the 10 h incubation. However, a more likely explanation for the increase in diene TG is that glands of

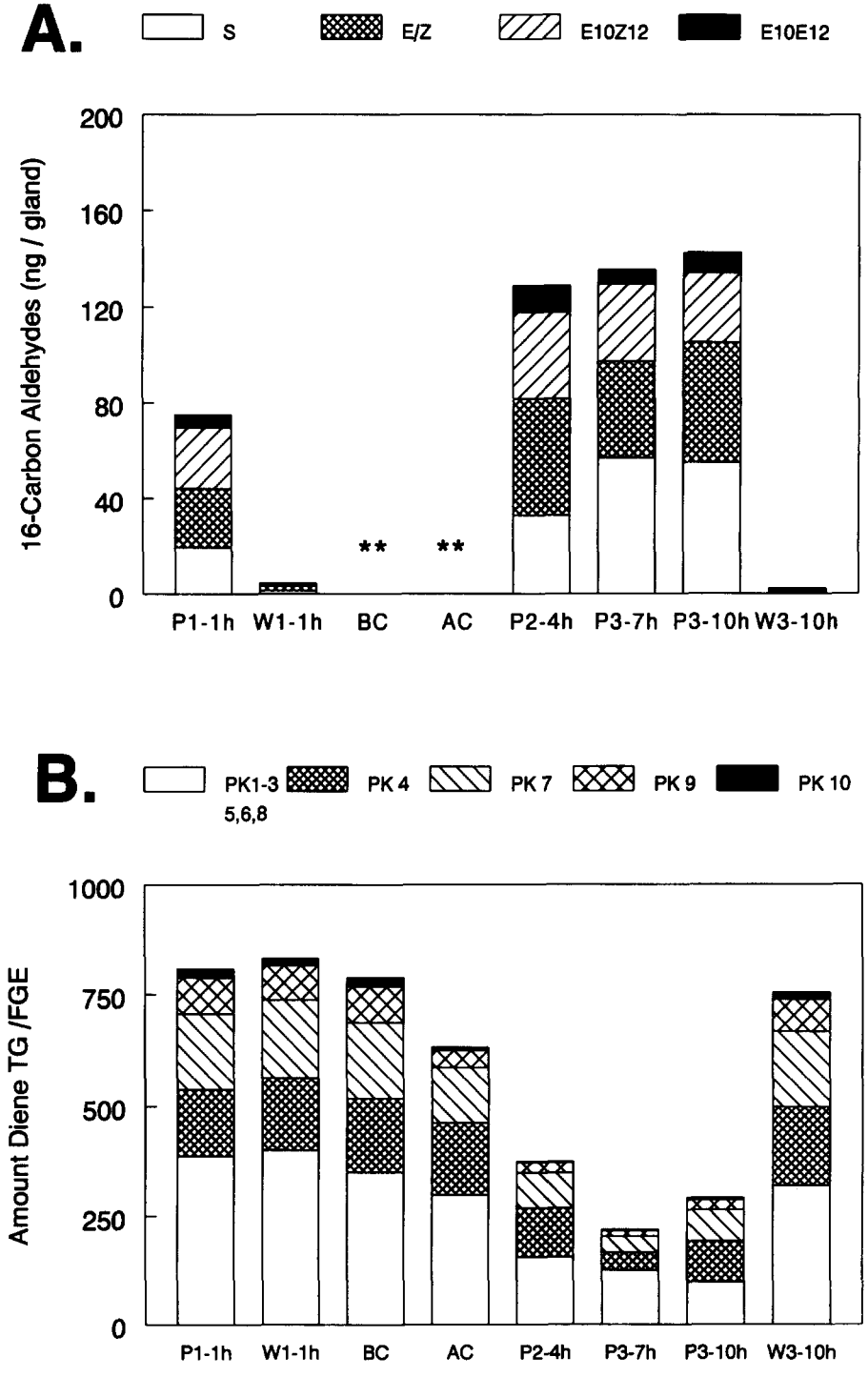

Figure 4. Effect of PBAN on pheromone aldehydes (A) and diene TG (B) in the glands of 2 day-old females. P1–1h, PBAN was injected 2 h before dark for a 1 h incubation; W1-1h, water (control) was injected 2 h before dark for a 1 h incubation; BC, glands were excised 10 min after the beginning of natural pheromone release; AC, glands were excised at the completion of ca. 4 h of natural pheromone release; P2–4h, P3–7h, P3–10h and W3-10h, PBAN or water was injected into the moths after completion of natural pheromone release: P2–4h = two PBAN injections with 4 h incubation, P3–7h = three PBAN injections with 7 h incubation, P3–10h = three PBAN injections with 10 h incubation, W3–10h = three water injections with 10 h incubation. Data for C-16-aldehydes are from GLC analyses and represent means of 18 replicate analyses of extracts of individual glands; ** signifies no aldehyde data was obtained for BC and AC. HPLC conditions and diene TG peak numbers are same as in Figure 3. Because appropriate TG standards were not available for calibration, absolute quantities of TG could not be determined by HPLC. Units on *Y* axis in B represent peak areas relative to 20 ng of methyl (*E*) cinnamate, assuming equivalent detector responses. Data for diene TG from HPLC analyses represent means of six replicates of extracts of three glands. FGE = female gland equivalents.

females incubated for 10 h were excised 4 h after the last PBAN injection, while in all other cases glands were excised 1 h after the last PBAN injection. Previous studies indicate that pheromone production begins to wane about 2.5 h after PBAN injection in this species.[37] Thus, after 2.5 h, the precursor acyl moieties in the donor TG may be replenished more rapidly than they are being converted to aldehydes.

Unlike 2 day-old moths, the proportions of diene TG in the glands of 5–7 day-old moths are variable.[36] After multiple injections of PBAN and 4, 7 and 10 h incubations, comparison of proportions of pheromone aldehydes with proportions of TG in the same glands of older moths revealed an apparent correlation between aldehyde and diene TG proportions.[36] Larger amounts of diene TG peaks 7, 9 and 10 (Fig. 3) were correlated with a larger amount of *E*10,*Z*12–16:AL in the pheromone blend in the same gland. Similarly, larger amounts of diene TG peak 4 corresponded to larger amounts of *E*/*Z*11-16:AL. No peak of diene TG has been found to correlate with S-16:AL. It is possible that the major TG donors of the acyl precursor for S-16:AL do not contain any diene acyl moieties and thus were not detected by the UV monitor set at 230 nm.

Although the total quantity of diene TG in the glands of moths was significantly lower at the end of the natural signaling period (Fisher LSD, $p = 0.05$: Fig. 4), this resulted primarily from large decreases in the levels of diene TG in peaks 7, 9 and 10.[36] These results suggest that only some of the diene TG, including those comprising peaks 7, 9 and 10, are donors of immediate acyl precursors of Δ10,12–16:AL during pheromone biosynthesis. When 2 day-old moths were forced to continuously produce pheromone for an additional 7 h by PBAN injection after completion of natural signaling, amounts of diene TG in all peaks decreased substantially (Fig. 4). This also occurs with older moths. It is conceivable that some of the TG containing conjugated diene acyl moieties are not direct donors of pheromone precursors, but instead are precursors of the actual TG donors. This might explain the considerable differences that have been found between the proportions of the putative acyl precursors and the components of the pheromone blend in the same gland of other species.[12,36] Thus, the proportion of aldehydes in the *M. sexta* pheromone blend may be determined by, and correlated with, the proportion of acyl precursors bound to the donor TG only.

PBAN mode of action

Normally, the aldehydes are produced and released only during the scotophase when the female is signaling. However, injection of PBAN at any time of the photoperiod into the abdomens of females of any age resulted in production of the pheromone aldehydes after ca. 1 h.[28] We found that injection of *M. sexta* with synthetic Hez-PBAN did not affect the quantities or proportions of deuterated fatty acids in glands treated with deuterated hexadecanoic, (*Z*)-11-hexadecenoic or (*E*)-11-hexadecenoic acids. Furthermore, during the photophase, endogenous and labeled fatty acids were converted to aldehydes only when PBAN was injected into the abdomens of females.[28,34] However, incubations of 24 h with labeled compounds were required to obtain sufficient incorporation of the label for analyses. Thus, we could not rule out the possibility that endogenous PBAN was affecting other steps in the biosynthetic pathway, particularly desaturation, in addition to conversion of fatty acids to aldehydes. It has been shown with other species that decapitation of females results in cessation of pheromone production, but that pheromone production can be induced by injection of PBAN for as long as 3 days after decapitation.[17] Our labeling studies indicated, but did not prove, that the role of PBAN in regulating pheromone biosynthesis in this species was to activate the enzymes that convert the fatty acids stored in the gland into the analogous aldehydes. Therefore, we designed experiments with decapitated *M. sexta* females to determine which steps in the pheromone biosynthetic pathway in this insect are controlled by PBAN.

When 2 day-old females were decapitated, none showed any signaling behavior, indicated by protrusion of the pheromone gland,[27] during the 5th to the 8th h of the dark phase following decapitation, when normal females with heads were signaling. Also, extracts of the glands excised during the same period from decapitated females did not contain measurable amounts of pheromone aldehydes as determined by GC analyses. Glands of normal females with heads, that were signaling during this period contained 13.7 (±2.3, SD) and 33.1 (±5.8, SD) ng of *Z*11–16:AL and *E*10,*Z*12–16:AL, respectively ($n = 6$).[38] These results are consistent with those of other studies of decapitated females,[17] in which pheromone biosynthesis ceased after removal of the females' heads. Since the source of PBAN is in the head, this would indicate that the natural peptide is no longer available to regulate pheromone biosynthesis in decapitated females.

The fate of D_3-16:FA and D_3-*Z*11–16:FA applied to the glands of females 24 h after they had been decapitated should not be affected by endogenous PBAN since it is not present. Both of these labeled acids were further desaturated directly, without the loss or gain of deuterium atoms, by enzymes in the pheromone gland. Also, both labeled acids were further desaturated, regardless of whether the treated insects had heads and whether or not they were treated with synthetic PBAN.[38] This is consistent with our results[28,34] that indicated that PBAN had no effect on the fatty acid content of the pheromone gland. Thus, the desaturases and isomerases that act on hexadecanoate and (*Z*)-11-hexadecenoate clearly are not affected by PBAN.

When either D_3-16:FA or D_3-*Z*11–16:FA was applied to the glands, labeled mono-, di- and triunsaturated 16-carbon aldehydes were produced during the day only in females subsequently injected with synthetic

PBAN, regardless of whether or not the females had been decapitated. However, the labeled fatty acid analogues of the aldehydes were produced in the glands of all females in relatively large quantities.[38] Therefore, PBAN is required to activate one or more enzymes involved in the conversion of fatty acyl precursors, stored in TG, to pheromone aldehydes in *M. sexta*.

HPLC analysis of diene TG showed that the total amounts and the composition of these compounds were the same in gland extracts of intact females and

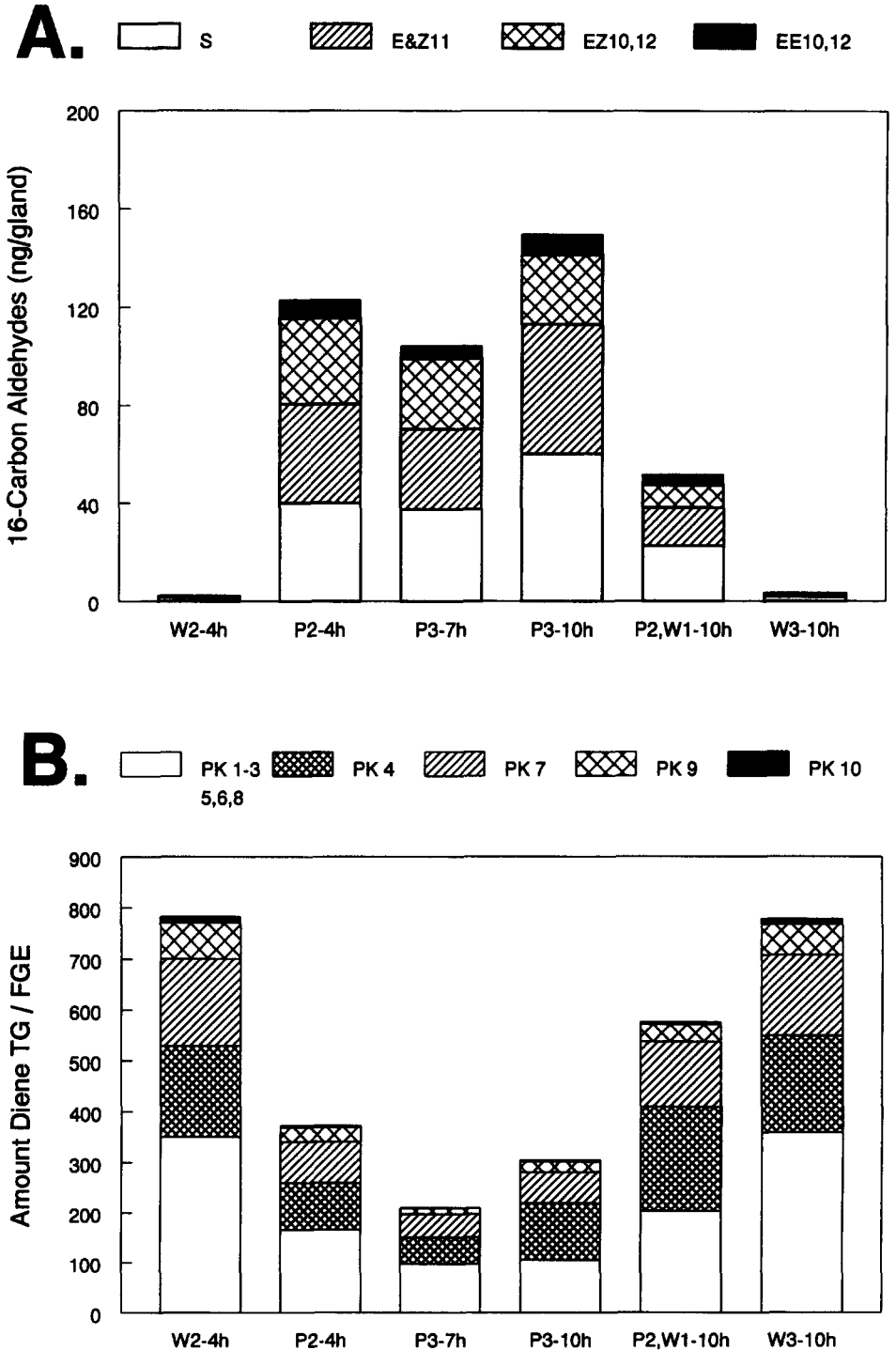

Figure 5. Comparison of mean total amounts of pheromone (A) with mean relative total amounts of diene TG (B) present in the glands of decapitated females (6 replicates/treatment). Data for pheromone aldehydes and diene TG were obtained from GLC and HPLC, respectively. Females (ca. 15 h-old) were decapitated 2 h after first scotophase and treated 24 h later. Treatments were: W2–4h, injected two times with H_2O, 4 h incubation; P2–4h, injected twice with PBAN, 4 h incubation; P3–7h, three PBAN injections and incubated 7 h; P3–10h and W3–10h, injected three times with PBAN or H_2O, respectively, 10 h incubation; P2W1–10h, injected two times with PBAN and a third time with H_2O, 10 h incubation. Diene TG peak numbers are same as in Figure 3. FGE = female gland equivalents.

those that had been decapitated 24 or 48 h. Thus, decapitation had no effect on the accumulation of diene TG in the gland. Although the decapitated insects did not produce pheromone naturally, there were no differences between decapitated and intact insects in either amounts or ratios of pheromone components produced when they were injected with PBAN. However, the amounts of the diene TG decreased significantly (Fig. 5) when decapitated females were stimulated to produce pheromone during a 7 h period by repeated injection of PBAN at 3 h intervals, but the amount of pheromone aldehydes obtained from glands 1 h after the third PBAN injection was as large as that produced 1 h after a single injection.[39] In contrast, extracts obtained 10 h after the initial PBAN treatment from decapitated females that received two PBAN injections followed by an injection of water 6 h after the first injection of PBAN contained significantly less pheromone than extracts from females receiving three PBAN injections and incubated the same amount of time. The amounts of diene TG were significantly larger in the extracts of glands from females receiving two PBAN and one water injections.

Conclusions

It is clear that the triacylglycerols play an integral role in the biosynthesis of the pheromonal aldehydes in *M. sexta*. Not only do they store the acyl precursors in readily accessible form for rapid conversion to aldehydes, but also the proportions of key TG appear to regulate the proportions of aldehydes in the final pheromone blend. Pheromone blend proportions are critical to speciation. It is also possible that the desaturation process occurs on fatty acyl moieties bound in TG. This would explain the occurrence of many different TG in the glands, containing different proportions of saturated, monoene, diene and triene acyl groups. Thus, some TG in the gland may actually be precursors of the key TG that are acted on by lipases to liberate acyl precursors of the pheromone aldehydes.

Also, the results of our studies on the induction of pheromone production by females of *M. sexta* indicate that PBAN affects the terminal steps in the biosynthesis of pheromones and has little or no effect on the de novo biosynthesis of fatty acyl precursors. If pheromone biosynthesis in *M. sexta* was regulated by PBAN at a step prior to or during fatty acid synthesis, as has been indicated in *A. velutinana*,[15,17] *H. zea*[40] and *M. brassicae*,[41] then all pheromone should have been produced by *de novo* biosynthesis of the fatty acyl precursors. Consequently, the levels of diene TG in the pheromone gland should have remained constant. However, the facts that (1) pheromone production is directly associated with declining levels of diene TG, (2) replenishment of the diene TG occurs after the effects of PBAN wane, (3) levels of diene TG in the glands of 3 and 4 day-old decapitated and control females are the same and (4) the ratio of pheromone components is closely correlated with ratio of triacylglycerols containing conjugated diene moieties[36] indicate that the triacylglycerides are a major source of pheromone precursors. Additionally, the data show that the biosynthesis of diene TG is not affected by PBAN but is regulated by the amounts of diene TG present in the pheromone gland. This suggests that PBAN acts to induce liberation of fatty acyl precursors from the triacylglycerols in the gland.

One of the questions that remains to be answered is how the bound fatty acids are converted to the aldehydes. Are they reduced directly to the aldehydes or are they first converted to alcohols and subsequently oxidized to aldehydes as in other species?[10,42] The absence of the corresponding alcohols in the gland of *M. sexta* females[28] suggests a direct conversion from fatty acids or acyl Co-A esters to the corresponding aldehyde by a reductase. This is supported by the absence of labeled alcohol during the conversion of the labeled acids to the labeled aldehydes, induced by injection of PBAN in vivo.[34] However, an oxidase that converts primary aliphatic alcohols into aldehydes was discovered in the cuticle of the sex pheromone gland and in the papillae anales (Fig. 1) on the tip of the abdomen of *M. sexta* females.[43] This suggests another possible pathway in which alcohols formed from the acids in the epidermal cells of the pheromone gland would be immediately oxidized to the pheromone aldehydes when they pass through the gland cuticle to the air. Thus, no detectable amounts of alcohol would ever exist in the pheromone gland. However, the role of the oxidase in the terminal step of biosynthesis of the pheromone aldehydes in *M. sexta* females is not yet understood and the specific enzymes involved in the terminal steps of pheromone biosynthesis in *M. sexta* have not been determined.

Several other questions remain to be answered regarding sex pheromone biosynthesis in *M. sexta*. We have yet to determine how the conjugated trienes are actually formed. They may result from further desaturation of (*E,Z*)-10,12-hexadecadienoate or they may be produced directly from (*Z*)-11-hexadecenoate. Also, the stereospecificity of the enzyme–substrate interactions involved in production of the conjugated dienes and trienes is unknown. Finally, it is not yet clear how fatty acid biosynthesis is regulated in the *M. sexta* pheromone gland.

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