BIOORGANIC CHEMISTRY 12, 279–298 (1984)

Biosynthesis of Lepidopteran Pheromones[1]

WENDELL ROELOFS AND LOUIS BJOSTAD

Department of Entomology, NYS Agricultural Experiment Station, Geneva, New York 14456

Received May 17, 1984

Research on lepidopteran sex pheromone component biosynthetic pathways has revealed general systems that may have significance in understanding the evolution of these moth mating communication signals. Studies with the redbanded leafroller moth, cabbage looper moth, and the domestic silkworm showed that they all possess a unique delta-11 unsaturated acid precursor. Radiolabeled precursor acids were used to show that various combinations of limited beta-oxidation chain-shortening or chain-elongation steps with the desaturase enzyme could produce most of the pheromone components identified for noctuid, pyralid, and tortricid moths. Evolution of the delta-11 desaturase enzyme from the ubiquitous delta-9 desaturase enzyme was suggested by finding primitive species that use the intermediate delta-10 desaturase enzyme. It is suggested that pheromone components of other primitive species are produced by using only the chain-shortening steps on available oleate, linoleate, and linolenate. Pheromone componets of some more advanced species appear to be produced by chain elongation of these available acids, with subsequent reductive decarboxylation to hydrocarbon.

INTRODUCTION

Sex pheromone research has concentrated heavily on moth species because many of them compete for man's food and fiber. In the past two decades hundreds of lepidopteran sex pheromones and attractants have been identified, and are now available for use in pest management programs. Decoding these chemical communication systems has generated other basic studies on odor perception, behavior, neurophysiology, and chemical synthesis. Surprisingly, few studies have been conducted on the biosynthesis of pheromone components and possible evolutionary relationships among those from various lepidopteran families. In this paper we will discuss our efforts in defining some of these biosynthetic pathways, and will use these data as a basis for speculation on sex pheromone evolution in the Lepidoptera.

SEX PHEROMONE BIOSYNTHESIS IN THE REDBANDED LEAFROLLER MOTH (TORTRICIDAE)

Sex pheromone identification of many leafroller species (Lepidoptera : Tortricidae) had revealed that many species utilize common components, such as (*E*)-11-

[1] Presented as a paper at a conference on "Molecular Messengers in Nature," Fogarty International Center, NIH, May 16–18, 1984.

0045-2068/84 $3.00

tetradecenyl acetate (*E*11-14 : Ac) and (*Z*)-11-tetradecenyl acetate (*Z*11-14 : Ac), and that specificity can be effected by specific component ratios (*1*). This implies a narrow-band chemical communication system involving tight control over the production of each unique isomeric ratio. We initiated our studies on the redbanded leafroller in quest of an understanding of this important enzymatic regulatory step.

The redbanded leafroller moth, *Argyrotaenia velutinana,* was chosen because the pheromone was found to have a precise 91 : 9 blend of *Z* and *E*11-14 : Ac's, along with a third component, dodecyl acetate (12 : Ac) (*2*). We also had carried out morphological, histological, and ultrastructural studies (*3*) on the development of the gland through the pupal and adult stages, and had correlated pheromone production to structural changes in the gland cells. The first appearance and prevalence of agranular endoplasmic reticulum and lipid droplets was coincident to the presence and increase of extractable pheromone in the glands (*4*). Analyses of individual female pheromone glands taken from wild and laboratory populations revealed that production of the *Z*/*E* blend was rigidly controlled around a 9/91 ratio with all blends falling within a narrow range of ratios between 4/96 to 12/88.

We suspected that the acetate pheromone compounds were produced from precursor fatty acyl intermediates, and so we carried out an acid or base methanolysis with the pheromone gland and analyzed for resulting methyl esters by GLC. The common fatty acyl groups palmitate (16 : Acyl), palmitoleate (*Z*9-16 : Acyl), stearate (18 : Acyl), oleate (*Z*9-18 : Acyl), linoleate (*Z*,*Z*-9,12-18 : Acyl), and linolenate (*Z*,*Z*,*Z*-9,12,15-18 : Acyl) were found in abundance since they are found in most insect tissue, but the pheromone fatty acyl precursors, (*Z*) and (*E*)-11-tetradecenoates (*Z*/*E*11-14 : Acyl), occurred only in the sex pheromone gland (*5*). Similar to the pheromone components, they were absent from the gland at adult emergence, and increased to a maximum over the next 4 days. The other common 16- and 18-carbon fatty acyl groups were all present at emergence in the pheromone gland, as well as in the rest of the insect.

We assumed initally that if *E* and *Z*11-14 : Acyl were present in the gland, they would occur in the same relative proportions as the pheromone components themselves. We were surprised to find instead that *E*11-14 : Acyl was even more abundant than *Z*11-14 : Acyl, in the ratio 58 : 42. We realized that this overall ratio might be different from the ratios in individual lipid classes in the gland, and that one or more of the lipid classes might have the expected 9 : 91 ratio. We separated the lipid classes in the gland by thin-layer chromatography (TLC), and found that triacylglycerols, diacylglycerols, choline phosphatides, and ethanolamine phosphatides contained most of the *E* and *Z*11-14 : Acyl in the gland, in the ratio 61 : 39. Smaller amounts were found in the choline phosphatides and ethanolamine phosphatides, but in both classes the *E*/*Z* ratio was 31 : 69, a ratio intermediate between that of the triacylglycerols (61 : 39) and that of the pheromone components (9 : 91). The diacylglycerols contained no detectable *E* or *Z*11-14 : Acyl.

The evidence at this stage indicated that the triacylglycerols, the choline phosphatides, and the ethanolamine phosphatides were all involved in sex pheromone biosynthesis, and that the regulation of the blend somehow involved all three of

them; but, none of them had the expected 9:91 ratio of *E* and *Z*11-14:Acyl. It seemed possible that the 9:91 ratio might occur at only one stereospecific position of a given glycerolipid, but phospholipase A_2 analysis of the choline phosphatides showed that both *E* and *Z*11-14:Acyl occurred exclusively at the *sn*-2 position, and lipase analysis of the triacylglycerols showed no selective distribution of the *Z* and *E* isomers among the *sn*-1, *sn*-2, and *sn*-3 positions.

Because the structures of the biosynthetic intermediates provided no obvious explanation of pheromone blend regulation or of the sequence of intermediates in the pathway, we began incubating glands with different radiolabeled precursors and analyzing incorporation of radiolabel into the biosynthetic intermediates and the pheromone components themselves (*5*). Glands incubated with sodium [1-^{14}C]acetate incorporated radiolabel into the pheromone components and into *E* and *Z*11-14:Acyl with similar specific activities. Tetradecanoate, hexadecanoate, and octadecanoate also incorporated appreciable radiolabel; but, it was striking that unsaturated fatty acyl groups with 16 or 18 carbon atoms incorporated almost no radiolabel, despite their abundance in the gland.

Having established that *E* and *Z*11-14:Acyl were biosynthesized from acetate in the gland (but that unsaturated 16- and 18-carbon fatty acids were not), we wished to determine if tetradecanoate was their immediate precursor. In most animal tissues, the biosynthesis of unsaturated fatty acyl groups involves the action of specific desaturases on the corresponding saturated compounds. In glands incubated with [1-^{14}C]tetradecanoic acid, *E* and *Z*11-14:Acyl both showed excellent incorporation of radiolabel (but 16- and 18-carbon fatty acyl groups incorporated almost no radiolabel). This implied the presence of two desaturases specific for tetradecanoate, a (*Z*)-11 desaturase and an (*E*)-11 desaturase, but we wished to test the possibility that only one desaturase might actually exist and that an isomerase might then produce the other geometric isomer. We synthesized [1-^{14}C](*Z*)-11-tetradecenoic acid and [1-^{14}C](*E*)-11-tetradecenoic acid. Glands incubated with either of these isomers failed to produce appreciable amounts of the other isomer. We concluded that both (*Z*) and (*E*)-11 desaturases occur in the gland, and that isomerases are not involved. Alternatively, a single delta-11 desaturase may exist that produces both geometric isomers in approximately equal proportions.

The origin of tetradecanoate from acetate was the part of the pathway that we understood least at this point. There was precedent in the literature for two very different mechanisms. The first possibility involved the production of hexadecanoate from acetate by fatty acid synthetase in the usual way (*6*), followed by chain shortening of hexadecanoate to generate tetradecanoate. Evidence for chain shortening of fatty acyl groups in insects had already been found (*7*, *8*). The second possibility entailed a modified fatty acid synthetase that produced tetradecanoate directly. Ryan *et al.* (*9*) showed that tetradecanoate in aphids arises by the action of a special thioesterase that cleaves nascent fatty acyl chains from fatty acid synthetase when they are 14 carbon atoms in length. We reasoned that, if chain shortening occurred in *A. velutinana*, glands incubated with [1-^{14}C]hexadecanoic acid would not produce radiolabeled tetradecanoate, but we expected that the specific activity of tetradecanoate in glands incubated with [U-^{14}C]hexa-

decanoic acid would be the same as in glands incubated with sodium [1-^{14}C]acetate. When these experiments were performed, the specific activity of tetradecanoate produced by glands incubated with [U-^{14}C]hexadecanoic acid was about twice that of glands incubated with [1-^{14}C]hexadecanoic acid. This implied that chain shortening of hexadecanoate to tetradecanoate did occur in *A. velutinana*, but also indicated that, in both experiments, some of the radiolabeled hexadecanoate had been degraded to radiolabeled acetate, which in turn was incorporated into tetradecanoate by de novo synthesis.

Further evidence for chain shortening of hexadecanoate to tetradecanoate was found in a closely related species, *Argyrotaenia citrana* (*10*). Incorporation of radiolabel into 14-carbon fatty acyl intermediates was 0.96% after incubation with [U-^{14}C]hexadecanoic acid and 0.84% after incubation with [9,10-^{3}H]hexadecanoic acid, but was only 0.01% after incubation with [1-^{14}C]hexadecanoic acid. A second demonstration of chain shortening in this insect involved incubation of gland homogenates with [3-^{14}C]hexadecanoic acid. Radiolabeled 14-carbon fatty acyl intermediates were recovered, and it was shown by decarboxylation that 80% of the radiolabel was in the first carbon atom, verifying that chain shortening was the principal (perhaps the only) mechanism for producing tetradecanoate. We could now propose the complete sequence of acyl intermediates in the pathway and the enzymes involved in their manufacture (Fig. 1).

We found additional support for the steps outlined in Fig. 1 when we determined the time course of incorporation of radiolabeled acetate into the pheromone components and the fatty acyl groups in each of the glycerolipid classes (*1*). We incubated pheromone glands with sodium [1-^{14}C]acetate for different lengths of time, and analyzed incorporation of radiolabel by the fatty acyl groups of each glycerolipid class. Earlier intermediates in the pathway were expected to have the most radiolabel after short incubation times. Incubations were performed for 8, 15, 40, and 240 min. A striking change in pattern of incorporation was observed

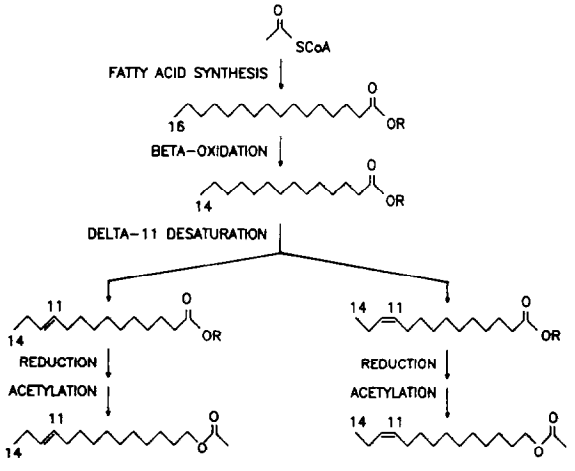

FIG. 1. Sequence of fatty acyl intermediates in biosynthesis of sex pheromone components of the redbanded leafroller moth.

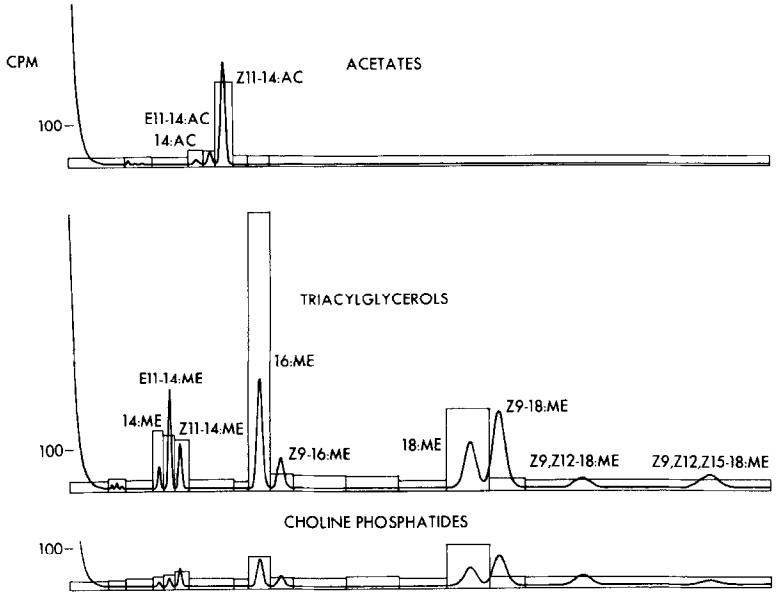

FIG. 2. Incorporation of radiolabel from sodium [1-^{14}C]acetate after 8 min incubation in redbanded leafroller moth pheromone gland. AC, acetates; ME, methyl esters.

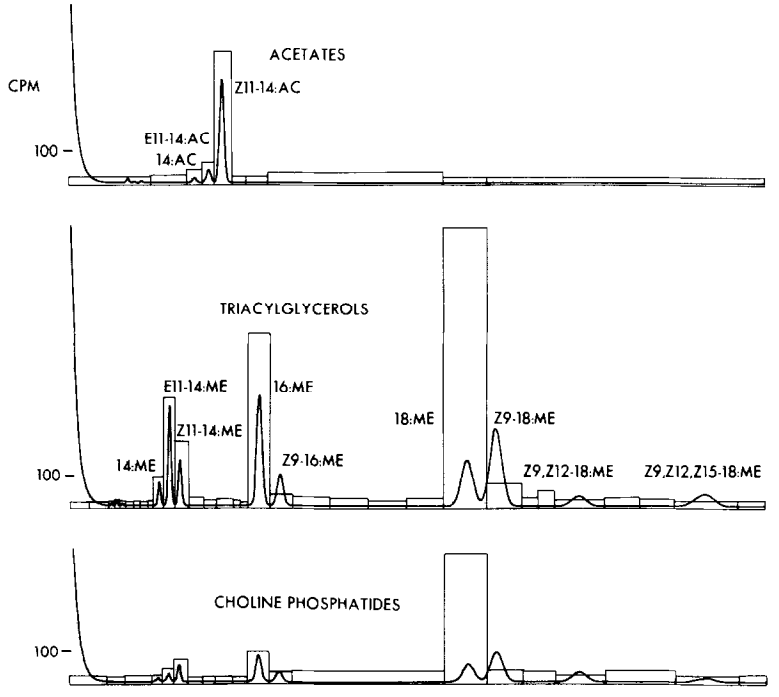

FIG. 3. Same as Fig. 2 except after 240 min incubation.

with respect to time of incubation. The relative proportion of radiolabel in hexadecanoate was much greater after 8 min incubation (Fig. 2) than after longer incubations (Fig. 3), in both the triacylglycerols and the choline phosphatides, consistent with our conclusion that hexadecanoate is a distal precursor for the pheromone components. Moreover, in the triacylglycerols, the proportion of radiolabeled tetradecanoate relative to *E* and *Z*11-14 : Acyl was higher after 8 min incubation than after longer incubations, supporting our conclusion that tetradecanoate is a precursor for the tetradecenoates.

We suspected that, after radiolabel began to appear in *E* and *Z*11-14 : Acyl in the glycerolipids, there would be a time lag before radiolabel would begin to appear in the pheromone components. Instead, the relative amounts of radiolabel in the pheromone components were high even after the shortest incubation time it was practical to test. This implied that, once hexadecanoate becomes available, the remaining steps in the biosynthesis of the sex pheromone components proceed rapidly. This is apparently because hexadecanoate is produced by fatty acid synthetase, which is soluble in the cytosol (*6*), whereas the subsequent steps (chain shortening, desaturation, reduction, and acetylation) are all likely to involve membrane-bound enzymes (*12, 13*) that may be closely associated with one another.

It was intriguing to find that the amounts of radiolabeled *E* and *Z*11-14 : Acyl in the triacylglycerols were about equal after 8 min incubation, but that the relative proportion of radiolabeled *Z* decreased progressively with longer times of incubation (Fig. 4). We concluded that, from the pool of *E* and *Z*11-14 : Acyl fatty acyl precursors in the gland, the *Z* isomer is preferentially selected for conversion to the corresponding acetate, to the extent that the pheromone component *Z*11-

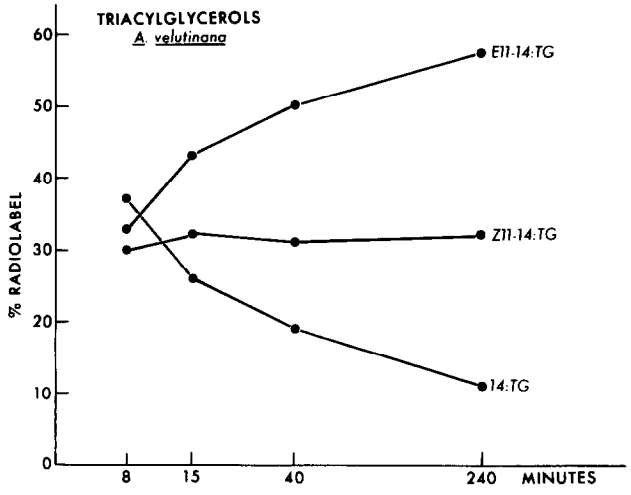

FIG. 4. Relative amounts of radiolabel in 14-carbon fatty acyl groups of triacylglycerols (TG) in redbanded leafroller moth pheromone gland, after different incubation times with sodium [1-^{14}C]acetate.

14 : Ac is more than ten times as abundant as the *E* component. The unselected *E* fatty acyl precursor accumulates, and this accounts for its relative increase with time of incubation.

The change in the proportions of radiolabeled *Z* and *E* fatty acyl precursors in the triacylglycerols is also important because it implies that the triacylglycerols are, in fact, the lipid class from which preferential selection of *Z* fatty acyl precursors is made. If selection occurred prior to their introduction into triacylglycerols, the proportions would have remained constant with time. With this piece of information, we can tentatively assess the role of the triacylglycerols in dealing with a fundamental problem in the regulation of the pheromone blend. Both the *Z* and *E* fatty acyl precursors are biosynthesized in large amounts, but the selection of *Z* precursors for conversion to pheromone components is ten times that of *E* precursors. As the *E* precursor accumulates, it might be expected that the *E*/*Z* ratio of the pheromone components would increase as well, but this does not occur (*4*). One way the gland could achieve this would be to select fatty acyl groups from one of the glycerolipid intermediates in triacylglycerol biosynthesis, and simply allow the unselected remainder to be converted to triacylglycerols. If this were the case, the triacylglycerol spheres in the gland could function as a chemical dump, assuring that selection would always be made from a fresh pool of the glycerolipid intermediate. Our evidence does not support this interpretation, however, because in a pathway of this sort the ratio of radiolabeled *E* and *Z* fatty acyl groups in the triacylglycerols would not change with time after introduction of radiolabeled acetate. A pathway in which selection of fatty acyl precursors is made from the triacylglycerols themselves is more consistent with the temporal change we observed in the ratio of radiolabeled *E* and *Z* fatty acyl groups in the triacylglycerols.

One novel mechanism that may be involved in maintaining the constant 9 : 91 blend of *E* and *Z* pheromone components involves the ultrastructure of the lipid spheres in sex pheromone gland cells, which are expected to contain the bulk of the triacylglycerols. Because the triacylglycerols consist largely of hexadecanoate and octadecanoate, moieties that would be expected to raise the melting point of the triacylglycerols greatly, it is possible that triacylglycerols elaborated on the surface of a lipid sphere undergo very little mixing with the interior of the sphere. If this is true, newer triacylglycerols may bury older triacylglycerols, effectively presenting a newly synthesized pool of *Z* and *E*11-tetradecenoyl groups to enzymes at the surface of the sphere. The great increase in size of the lipid spheres throughout the life of the adult female (*3*) is consistent with this interpretation.

SEX PHEROMONE BIOSYNTHESIS IN THE CABBAGE LOOPER MOTH, *Trichoplusia ni* (NOCTUIDAE)

After we showed that the redbanded leafroller, a moth in the family Tortricidae, has delta-11 desaturases that produce the immediate fatty acyl precursors of the delta-11 unsaturated sex pheromone components, we wished to find out if the same thing was true for species of other families. We decided to analyze the

cabbage looper moth, *Trichoplusia ni,* whose main pheromone component is (*Z*)-7-dodecenyl acetate (*Z*7-12 : Ac). We hypothesized that the immediate fatty acyl precursor was (*Z*)-7-dodecenoate (*Z*7-12 : Acyl), and that this was produced by a (*Z*)-7 desaturase acting on dodecanoate in the gland. We found instead that a much more interesting pathway was involved (*14*). We analyzed gland extracts, and found that *Z*7-12 : Acyl was in fact present, but we were surprised to find a large amount of another uncommon fatty acyl group, (*Z*)-11-hexadecenoate (*Z*11-16 : Acyl) (Fig. 5).

The abundance of this unusual group suggested a role in pheromone biosynthesis, and it occurred to us that chain shortening by beta-oxidation of *Z*11-16 : Acyl could produce (*Z*)-9-tetradecenoate (*Z*9-14 : Acyl), and further beta-oxidation could produce *Z*7-12 : Acyl. We synthesized [16-^{3}H](*Z*)-11-hexadecenoic acid and applied this to sex pheromone glands of *T. ni.* After 4 hr incubation, the glands were found to contain the expected radiolabeled intermediates *Z*9-14 : Acyl and *Z*7-12 : Acyl, and the pheromone component *Z*7-12 : Ac had incorporated a large amount of radiolabel as well. We recovered the radiolabeled *Z*7-12 : Ac, performed ozonolysis, prepared benzyloximes from the ozonolysis fragments, and analyzed the benzyloximes of both ozonolysis fragments by scintillation counting. Only the terminal fragment contained radiolabel, demonstrating unequivocally that chain shortening of radiolabeled *Z*11-16 : Acyl was solely responsible for the appearance of radiolabel in the pheromone component (Fig. 6). Degradation of radiolabeled *Z*11-16 : Acyl to acetyl coenyme A, followed by *de novo* synthesis of the pheromone, had not occurred. We also showed that glands incubated with sodium [1-^{14}C]acetate produced radiolabeled *Z*7-12 : Ac, and ozonolysis indicated

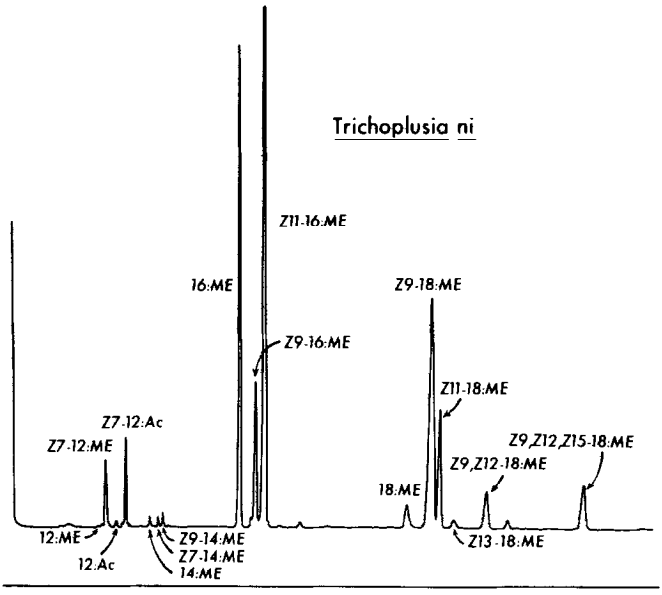

FIG. 5. Capillary GLC analysis of fatty acyl complement and pheromone components in the cabbage looper moth sex pheromone gland.

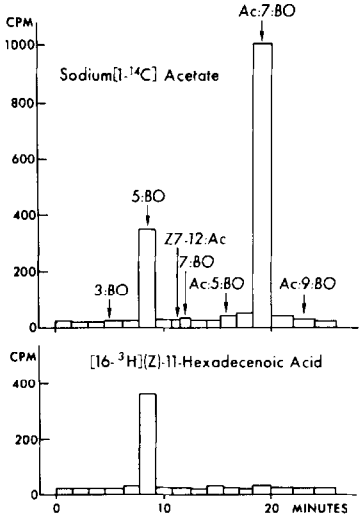

FIG. 6. Radiolabel in benzyloximes (BO) of ozonolysis fragments of (*Z*)-7-dodecenyl acetate in the cabbage looper pheromone gland after incubation with radiolabeled substrates. AC, acetates.

that both the smaller and the larger fragments were radiolabeled in this case, in the 1 : 2 ratio expected. This verified that, if radiolabeled acetate had been produced in the experiment with [16-^{3}H](*Z*)-11-hexadecenoic acid, we would have been able to detect it.

Our serendipitous discovery of the chain-shortening pathway offered an explanation for several features of the ultrastructure of the pheromone gland cell whose functional significance had previously been unclear. Percy (*15*) demonstrated that large lipid spheres are abundant in the pheromone gland cells of *T. ni*. The triacylglycerols, which contain the bulk of the *Z*11-16 : Acyl, are expected to occur in lipid spheres. Percy observed that each lipid sphere was completely surrounded by a "halo" of microperoxisomes. Peroxisomes in mammalian cells are able to carry out beta-oxidation of fatty acids, a process previously thought to take place exclusively in the mitochondria (*16*). Unlike mitochondrial beta-oxidation, peroxisomal beta-oxidation is incomplete. At most, five beta-oxidation cycles are involved, and products of the first two or three beta-oxidation cycles predominate (*17*). It seems likely that the principal function of the microperoxisomes in the sex pheromone gland of *T. ni* is to carry out two cycles of beta-oxidation (that is, chain shortening) of *Z*11-16 : Acyl to produce *Z*7-12 : Acyl, which is then reduced and acetylated to produce the main sex pheromone component *Z*7-12 : Ac.

This pathway also offered an explanation for a puzzling pattern that had become evident in the 150 species of Noctuidae whose pheromones had been characterized; that different double bond positions seemed to predominate in different chain lengths. (*Z*)-5-Decenyl, (*Z*)-7-dodecenyl, (*Z*)-9-tetradecenyl, and (*Z*)-11-hexadecenyl groups comprise 80% of the known sex pheromone components in the Noctuidae (*18*). It is clear that all these groups can be produced from *Z*11-16 : Acyl simply by altering the product specificity of the chain-shortening system.

(*Z*)-11-Octadecenoate (*Z*11-18 : Acyl) was also abundant in the sex pheromone gland of *T. ni*, and chain-shortened products of this can account for an additional 10% of the sex pheromone components known for the Noctuidae (*18*). In preliminary work with *Xestia dolosa*, in which (*Z*)-7-tetradecenyl acetate is the principal sex pheromone component, we have observed that large quantities of the expected precursors *Z*11-18 : Acyl, *Z*9-16 : Acyl, and *Z*7-14 : Acyl are present, but that *Z*11-16 : Acyl is absent.

We realized that the same biosynthetic steps that produced *Z*7-12 : Ac from *Z*11-16 : Acyl might be expected to produce small amounts of other acetates that could be additional pheromone components in *T. ni* (Fig. 7). Capillary GLC analy-

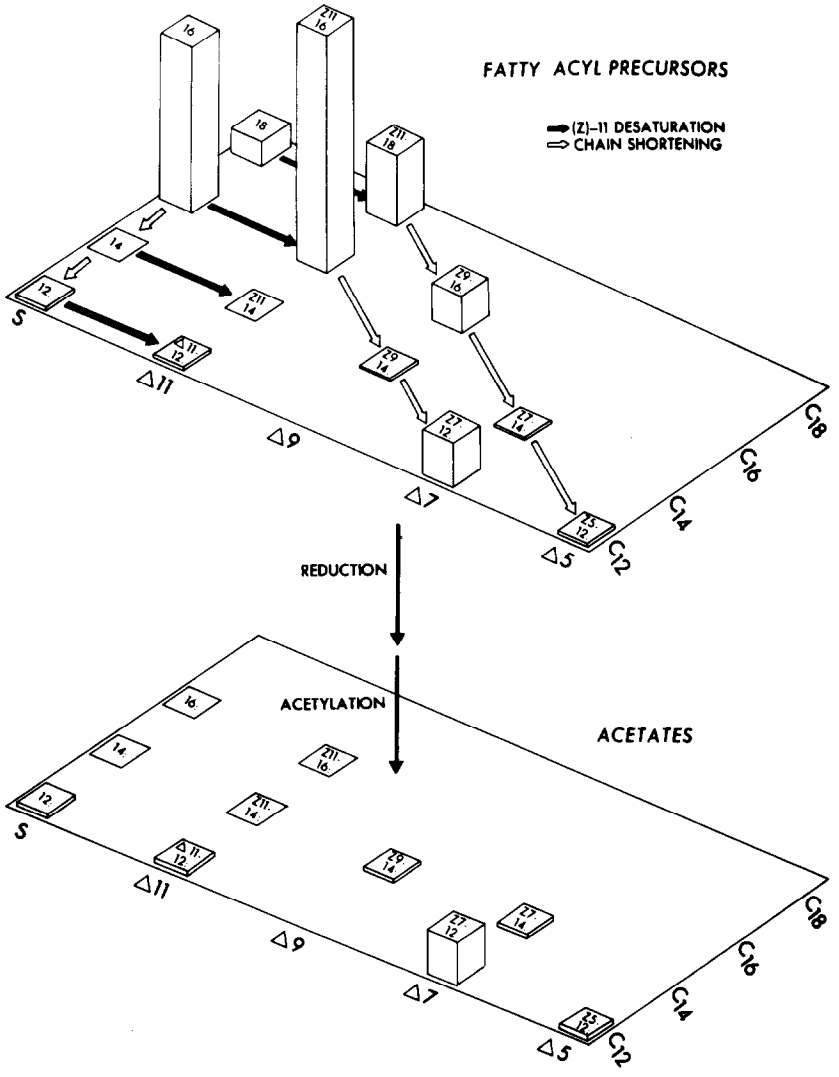

FIG. 7. Proposed biosynthetic routes for additional pheromone components in the cabbage looper moth.

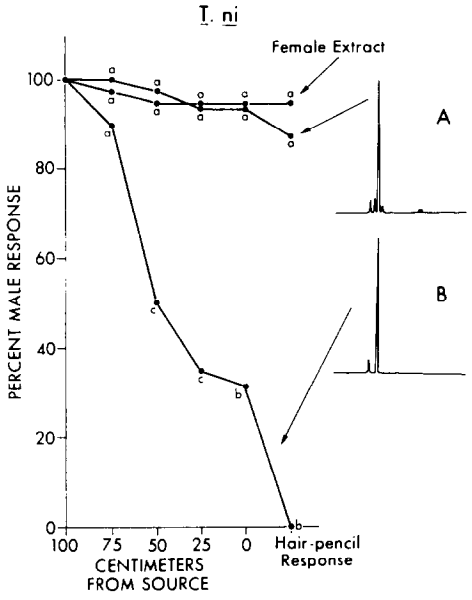

FIG. 8. Responses of cabbage looper male moths in a flight tunnel to (A) new six-component blend, and (B) old two-component blend.

ses indicated that this was true. In addition to the main pheromone component *Z*7-12 : Ac, glands of *T. ni* produced small amounts of dodecyl acetate, (*Z*)-5-dodecenyl acetate, 11-dodecenyl acetate, (*Z*)-7-tetradecenyl acetate, and (*Z*)-9-tetradecenyl acetate (*19*). Behavioral analyses of male responses in a flight tunnel (Fig. 8) showed that a synthetic blend of these six compounds (Blend A) elicited complete courtship flights from 95% of the males tested (not significantly different from the 98% responses to female extracts). Males exhibit a characteristic hair-penciling behavior after completing flights to virgin females (*20*). The six-component synthetic blend elicited hair-penciling from 91% of the males tested. In contrast, the previously identified pheromone complement, *Z*7-12 : Ac plus 12 : Ac (Blend B), elicited complete flights from only 33% of the males tested, and none of these males exhibited the characteristic hair-penciling response (*21*).

SEX PHEROMONE BIOSYNTHESIS IN THE SILKWORM MOTH, *Bombyx mori* (BOMBYCIDAE)

Our analyses of the redbanded leafroller moth, the cabbage looper moth, and many other species (*22*) indicated that, in every species analyzed, the expected fatty acyl precursors for the known pheromone components were abundant in the gland, usually in greater quantities than the pheromone components themselves. The pheromone components of the silkworm moth include (*E*,*Z*)-10,12-hexadecadienol, (*E*,*E*)-10,12-hexadecadienol, and (*E*,*Z*)-10,12-hexadecadienal (*23*), for which the expected fatty acyl precursors are (*E*,*Z*)-10,12-hexadecadienoate and

(*E,E*)-10,12-hexadecadienoate. We became interested in this moth because a report appeared in the literature indicating that the expected fatty acyl precursors were not present (*24*), although the uncommon fatty acyl group Z11-16: Acyl was present. We suspected that the acid methanolysis procedure used to make methyl ester derivatives from the fatty acyl groups in the pheromone gland might have destroyed compounds with conjugated double bond systems. We performed base methanolysis of gland extracts of *B. mori*, and found that the expected fatty acyl precursors (*E,Z*)-10,12-hexadecadienoate and (*E,E*)-10,12-hexadecadienoate were present (*25*). The distribution of these fatty acyl groups among the glycerolipids in the gland was determined by TLC separations of the glycerolipids, base methanolysis of each glycerolipid class, and analysis of the base methanolysis products by capillary GLC (Fig. 9).

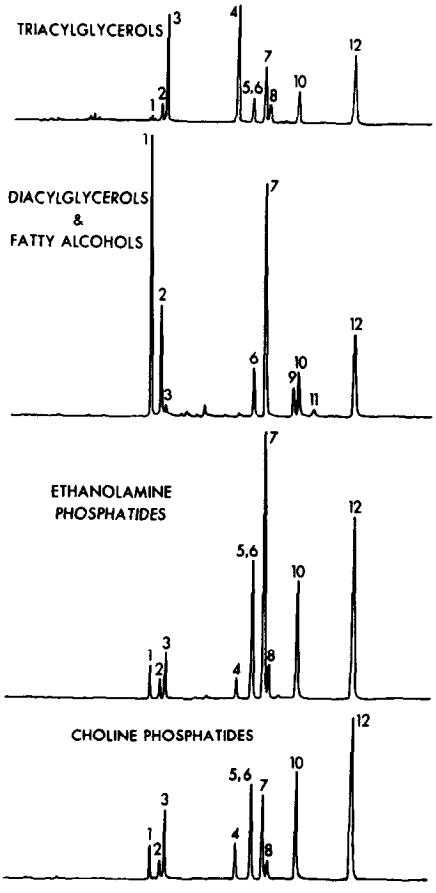

FIG. 9. Capillary GLC analyses of glycerolipids in sex pheromone glands of *B. mori* after base methanolysis and treatment with acetyl chloride. Compounds are numbered as follows: 1, methyl hexadecanoate; 2, methyl (*Z*)-9-hexadecenoate; 3, methyl (*Z*)-11-hexadecenoate; 4, methyl (*E,Z*)-10,12-hexadecadienoate; 5, methyl (*E,E*)-10,12-hexadecadienoate; 6, methyl octadecanoate; 7, methyl (*Z*)-9-octadecenoate; 8, methyl (*Z*)-11-octadecenoate; 9, (*E,Z*)-10,12-hexadecadienyl acetate; 10, methyl (*Z,Z*)-9,12-octadecadienoate; 11, (*E,E*)-10,12-hexadecadienyl acetate; 12, (*Z,Z,Z*)-9,12,15-octadecatrienoate.

We also performed acid methanolysis on gland extracts of *B. mori,* and confirmed that the conjugated pheromone components and their fatty acyl precursors were apparently destroyed, although nonconjugated fatty acyl groups formed methyl esters as readily as by base methanolysis. In addition to (*E,Z*)-10,12-hexadecadienoate and (*E,E*)-10,12-hexadecadienoate, the gland contained *Z*11-16 : Acyl. These three uncommon fatty acyl groups occurred principally in the triacylglycerols, were less abundant in the choline phosphatides and ethanolamine phosphatides, and were apparently absent from the diacylglycerols. The distribution of these fatty acyl groups among the lipid classes in the gland of *B. mori* bears a striking relationship to the fatty acyl distributions observed in sex pheromone glands of other species. In *A. velutinana,* the principal pheromone components are *Z* and *E*11-14 : Ac; the biosynthetic precursors *Z* and *E*11-14 : Acyl occur mainly in the triacylglycerols, are less abundant in the choline phosphatides and ethanolamine phosphatides, and are apparently absent from the diacylglycerols (*5, 11*). In *T. ni,* the fatty acyl precursor *Z*11-16 : Acyl is chain-shortened, reduced, and acetylated to produce the main pheromone component *Z*7-12 : Ac; this fatty acyl precursor occurs principally in the triacylglycerols, is less abundant in the choline phosphatides and ethanolamine phosphatides, and is apparently absent from the diacylglycerols (*14*). The role that each glycerolipid class plays in the biosynthesis of the sex pheromone components is not yet known for any of these species, but in all three species (occurring in three different Lepidopteran families) the fatty acyl biosynthetic precursors predominate in the triacylglycerols, are present in smaller amounts in phospholipids, and are apparently absent from the diacylglycerols. In *B. mori* this was true for the *Z*11-16 : Ac in the sex pheromone gland as well as for the expected biosynthetic precursors (*E,Z*)- and (*E,E*)-10,12-hexadecadienoate. This tends to confirm the suggestion by Yamaoka and Hayashiya (*24*) that *Z*11-16 : Acyl may be a biosynthetic precursor for the pheromone components, ostensibly involving conversion of *Z*11-16 : Acyl to the 10,12-hexadecadienoates. Such a conversion would be quite interesting, in that it would seem to entail an isomerization about the (*Z*)-11 double bond to generate the 10,12 double bond system.

THE DELTA-11 DESATURASE ENZYME IN LEPIDOPTERAN PHEROMONE BIOSYNTHESIS

The previous sections reveal that pheromone biosynthesis for the common pheromone components in the Tortricidae and Noctuidae can involve limited chain-shortening steps in the microperoxisomes and a unique delta-11 desaturase enzyme. This enzyme apparently can desaturate saturated 12- to 18-carbon chain acyl groups to produce *Z*11-18 : Acyl, *Z*11-16 : Acyl, *Z*11-14 : Acyl, and 11-12 : Acyl intermediates. Chain-shortening sequences with these precursors can yield many additional pheromone component intermediates (e.g., see the cabbage looper biosynthesis).

The presence of small amounts of chain-shortened intermediates provides the basis for evolutionary changes in pheromone components. For example, the

Tortricidae family contains two major subfamilies—the Tortricinae, in which many species utilize *Z* and *E*11 14-carbon acetates, alcohols, or aldehydes, and the Olethreutinae, in which many species utilize *Z* and *E*9 12-carbon compounds (*1*). The redbanded leafroller moth (a Tortricinae species), using *Z* and *E*11-14 : Ac's, also has traces of 12 : Ac, *Z*9-12 : Ac, *E*9-12 : Ac, and 11-12 : Ac in the pheromone gland and uses these in the overall pheromone blend (Fig. 10). It should be possible for mutations in evolutionary time to produce more of the 12-carbon chain compounds and less of the 14-carbon chain compounds until the *Z* and *E*9-12 : Ac's are the main pheromone components of another species. This would require very little change in the overall biosynthetic pathways, but would result in a large change in the chemical structure of the pheromone components. A good example of this type of change can be found with the pheromones of the sibling species *Zeirophera diniana* Larch (using *E*11-14 : Ac) and *Z. diniana* Cembran (using *E*9-12 : Ac) (*26, 27*).

It is possible that additional pheromone components could be generated by a second interaction of an unsaturated fatty acyl group with the delta-11 desaturase enzyme. In the Tortricidae some species produce *Z*9-14 : Acyl by chain-shortening the *Z*11-16 : Acyl intermediate (Fig. 10). A second desaturation of the *Z*9-14 : Acyl product with the delta-11 desaturase enzyme could produce the doubly unsaturated 9,11-14 : Acyl intermediate, which could be converted to acetate pheromone components as found in some Tortricinae species, or chain-shortened to the 7,9-12 : Acyl precursor of an Oletheutinae species (*1*).

Although chain shortening is an important step in producing many of the pheromone components, chain elongation also can be used. A common pheromone component in the Pyralidae is (*Z*)-13-octadecenal, whose precursor can be produced simply by adding two carbons to the *Z*11-16 : Acyl intermediate. Species of moths in the Sesiidae family apparently have capitalized on this chain-elongation step to generate their specialized pheromone components. Many species have been found to use (*E*,*Z*) or (*Z*,*Z*)-3,13-octadecadienyl acetate or alcohol or (*E*,*Z*)-2,13-octadecadienyl acetate (*28–30*). As shown in Fig. 11, these compounds also can be made from the *Z*11-16 : Acyl intermediate. In this case the chain-elongation sequence is interrupted before the final reduction. The probable steps involve addition of the two-carbon unit, reaction with a beta-ketoacyl reductase to produce the 3-hydroxy intermediate compound, and then reaction with special hydrase enzymes to produce either the *E*3, *Z*3, or *E*2 double bonds found in the

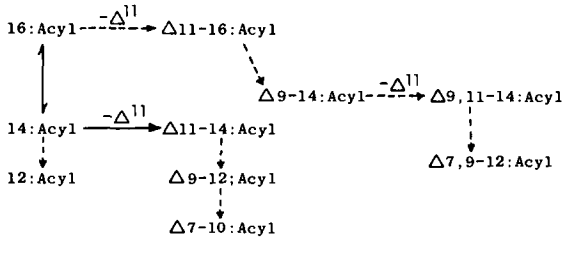

FIG. 10. Proposed biosynthetic steps for Tortricid pheromone components using a delta-11 desaturase enzyme and limited beta-oxidation chain-shortening steps.

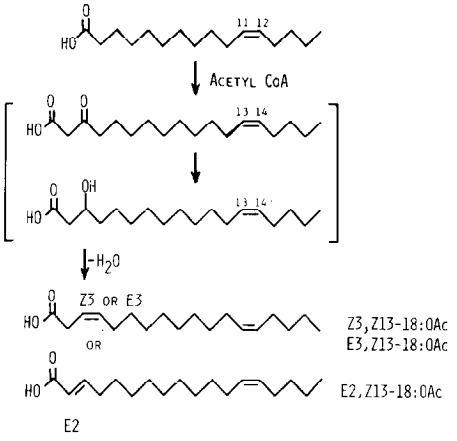

FIG. 11. Proposed bisynthetic routes for the 3,13- and 2,13-octadecadienyl acetate pheromone components identified in the family Sessiidae.

pheromone component—the other double bond always would be *Z*13 if the starting compound is pure *Z*11-16 : Acyl.

EVOLUTION OF DESATURASE ENZYMES

As mentioned above, common fatty acyl groups found in insect tissue include *Z*9-16 : Acyl (palmitoleate) and *Z*9-18 : Acyl (oleate). It is possible that the unique delta-11 desaturase enzyme evolved from the *Z*9 desaturase enzyme commonly found in insect cells. An intermediary stage in this evolutionary process could be a delta-10 desaturase enzyme. Evidence for this enzyme has been found in some of the more primitive Tortricid species. One species, *Bactra verutana* Zeller of the Olethreutini tribe, was sent to us from Mississippi by Dr. Kenneth Frick because the *Bactra* genus was derived by some taxonomists to be very primitive. Analyses of EAG, GLC-EAG, and capillary GLC revealed that all detectable EAG activity was with an unsaturated 14-carbon acetate, and that the active compound had a retention time on a 40-m capillary Carbonwax 20 M column identical to that of *Z*10-14 : Ac. EAG testing of all 14-carbon isomers showed that the *Z*10 isomer elicited the greatest response. Base methanolysis of the sex pheromone gland extract showed that methyl (*Z*)-10-tetradecenoate and methyl tetradecanoate were the only obvious unusual acyl groups in the extract, indicating that this primitive species does use a delta-10 desaturase enzyme in its pheromone biosynthetic pathway.

Additional evidence of the delta-10 desaturase enzyme was found in a primitive Tortricid species from New Zealand. The greenheaded leafroller, *Planotortrix excessana,* was found to use 14 : Ac and *Z*8-14 : Ac as its pheromone components (*31*). An analysis of the biosynthetic intermediate in the pheromone gland revealed that the major unusual fatty acyl group in the triacylglycerols, ethanolamine phosphatides, and choline phosphatides was *Z*10-16 : Acyl (Lofstedt and

Roelofs, unpublished). These data support a pathway in which the delta-10 desaturase enzyme operates on palmitic acid, and the resulting Z10-16 : Acyl intermediate is chain shortened and reduced to acetate to yield the pheromone Z8-14 : Ac.

The above studies support the existence of a delta-10 desaturase enzyme. There are many other lepidopterous species utilizing pheromone components that suggest the involvement of a delta-10 desaturase enzyme, particularly a large number of Tortricid species in the Olethreutinae subfamily that use Z8-12 : Ac, whose precursor would be produced from Z10-14 : Acyl.

PHEROMONE BIOSYNTHESIS USING OLEIC, LINOLEIC, OR LINOLENIC ACIDS

Lepidopteran species in many families have specific chemical mating signals because of their unique combinations of delta-10 or delta-11 desaturase enzymes and chain-shortening or -elongation steps. Some species, however, apparently produced pheromone compounds by chain-shortening the ubiquitous Z9-16 : Acyl and Z9-18 : Acyl groups. Simple chain shortening of the former would produce Z7-14 : Ac, Z5-12 : Ac, and Z3-10 : Ac, and the latter would give Z7-16 : Ac, Z5-14 : Ac, and Z3-12 : Ac. It should be noted that the former series also could be produced by involvement of the delta-11 desaturase enzyme to give Z11-18 : Acyl and subsequent chain shortening of that intermediate. The compounds Z7-14 : Ac and Z5-12 : Ac are used by a number of noctuid species (*18*), but are most likely all produced from Z11-18 : Acyl. All noctuid species analyzed have had the appropriate Z11 fatty acyl precursor compound, and the one noctuid species, *Xestia*

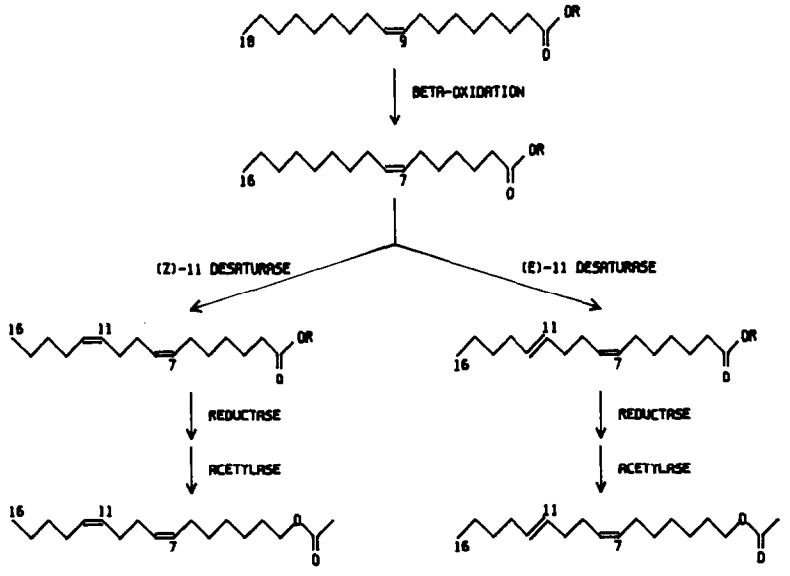

FIG. 12. Proposed steps in biosynthesis of sex pheromone components of the pink bollworm moth.

dolosa, that uses *Z*7-14 : Ac as a pheromone component was found to have large quantities of the expected *Z*11-18 : Acyl precursor (*14*).

Other, perhaps more primitive, species appear to produce their pheromone component without the involvement of a special desaturase. The European goat moth, *Cossus cossus* (Lepidoptera : Cossidae), uses *Z*5-12 : Ac and *Z*5-14 : Ac as pheromone components—probably produced directly from *Z*9-16 : Acyl and *Z*9-18 : Acyl, respectively. Some primitive leafroller species in New Zealand were found to use *Z*7-14 : Ac and *Z*5-14 : Ac as pheromone components (*31*). In both examples just cited the *Z*5-12 : Ac and the *Z*7-14 : Ac are most likely produced from common *Z*9-16 : Acyl, rather than *Z*11-18 : Acyl, because the companion compounds appear to come from oleate (*Z*9-18 : Acyl). Simple chain-shortening sequences may have evolved first in pheromone biosynthesis before the appearance of the delta-10 and delta-11 desaturase enzymes.

An interesting combination of oleate chain shortening and the involvement of the delta-11 desaturase enzyme is possible in pheromone production with the pink bollworm, *Pectinophora gossypiella* (Lepidoptera : Gelechiidae). The components *Z,E* and *Z,Z*-7,11-16 : Ac could be biosynthesized (Fig. 12) by chain shortening oleate to give *Z*7-16 : Acyl intermediate, and then action of the delta-11 desaturase enzyme would give the appropriate delta-7,11-16 : Acyl precursors to the pheromone components.

Some other gelechiid moths use pheromones that also suggest the available oleate, linoleate, or linolenate as starting compounds. The tomato pinworm, *Keiferia lycopersicella,* uses *E*4-13 : Ac as a pheromone. This unusual odd-num-

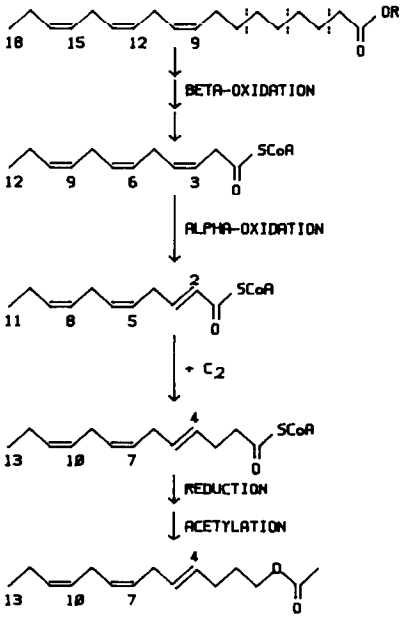

FIG. 13. Proposed steps in biosynthesis of sex pheromone components of the potato tuberworm moth.

bered carbon chain compound could be produced by chain shortening oleate (Z9-18 : Acyl) until the carboxyl group is only one carbon from the double bond. The enoyl system isomerizes the double bond to *E*, and addition of a two-carbon unit produces the appropriate precursor fatty acyl compound. A similar sequence of reactions could account for the pheromone components (*E*,*Z*-4,7- and *E*,*Z*,*Z*-4,7,10-13 : Ac) of the potato tuberworm from linoleate and linolenate (Fig. 13).

The examples discussed above represent pheromones that could be more primitive than those involving the special desaturase enzymes. However, a number of more advanced arctiid, noctuid, and geometrid species use pheromones that appear to be produced by chain elongation of linoleate with subsequent reductive decarboxylation to a hydrocarbon. The common compounds found in these species are (*Z*,*Z*,*Z*)-3,6,9-eicosatriene and -heneicosatriene (*32–34*), as well as the 9,10-epoxy derivative of these compounds (*35, 36*) and some related tetraene compounds (*37, 38*). The intermediate steps in these biosyntheses are not known, but they could involve a series of steps shown in Fig. 14. Reductive decarboxylation of long-chain fatty acid has been suggested as the principal mechanism for production of cuticular hydrocarbons in insects (*39*). Introduction of the epoxy group may be due to a mixed-function oxidase such as cytochrome P_{450} (*40*). It is interesting that we have not found the expected fatty acyl intermediate (*Z*,*Z*,*Z*)-13,16,19-dodosatrienoate in the gland, nor have such intermediates been found in some other species that produce long-chain hydrocarbons. These pheromone components appear in more advanced lepidoptera, and probably evolved after the routes discussed previously in this paper.

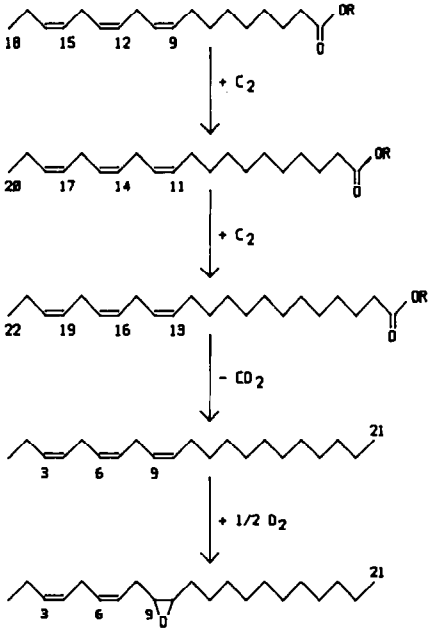

FIG. 14. Proposed steps in biosynthesis of sex pheromone components of the salt marsh caterpillar moth.

Many of the proposed biosynthetic pathways presented in this paper still need to be verified by rigorous research, and the more advanced species may use pathways completely different from that proposed. It appears that comparative analyses of biosynthetic pathways among lepidopteran species could provide much more information on possible evolutionary relationships than comparisons of just the pheromone components. It is obvious that there are many highly interesting and important studies to be carried out in the area of pheromone biosynthetic pathways.

ACKNOWLEDGMENT

Financial support was provided by National Science Foundation Grant PCM-8109850.

REFERENCES

1. ROELOFS, W. L., AND BROWN, R. L. (1982) *Annu. Rev. Ecol. Syst.* **13,** 395–422.
2. ROELOFS, W., HILL, A., AND CARDE, R. (1975) *J. Chem. Ecol.* **1,** 83–89.
3. FENG, K. C., AND ROELOFS, W. L. (1977) *Ann. Entomol. Soc. Amer.* **70,** 721–732.
4. MILLER, J. R., AND ROELOFS, W. L. (1977) *Ann. Entomol. Soc. Amer.* **70,** 136–139.
5. BJOSTAD, L. B., AND ROELOFS, W. L. (1981) *J. Biol. Chem.* **256,** 7936–7940.
6. VOLPE, J. J., AND VAGELOS, P. R. (1976) *Physiol. Rev.* **56,** 339–417.
7. LAMBREMONT, E. N., ERNST, N. R., FERGUSON, J. R., AND DIAL, P. F. (1976) *Comp. Biochem. Physiol. B* **54,** 167–169.
8. KEITH, A. O. (1967) *Comp. Biochem. Physiol.* **21,** 587–600.
9. RYAN, R. O., DE RENOBALES, M., DILLWITH, J. W., HEISLER, C. R., AND BLOMQUIST, G. J. (1982) *Arch. Biochem. Biophys.* **213,** 26–36.
10. WOLF, W. A., AND ROELOFS, W. L. (1983) *Insect Biochem.* **13,** 375–379.
11. BJOSTAD, L. B., AND ROELOFS, W. L. (1984) *J. Chem. Ecol.* **10,** 681–691.
12. BELL, R. M., AND COLEMAN, R. A. (1980) *Annu. Rev. Biochem.* **49,** 459–487.
13. HOLUB, B. J., AND KUKSIS, A. (1978) *Adv. Lipid Research* **16,** 1–125.
14. BJOSTAD, L. B., AND ROELOFS, W. L. (1983) *Science (Washington, D.C.)* **220,** 1387–1389.
15. PERCY, J. (1979) *Canad. J. Zool.* **57,** 220–236.
16. LAZAROW, P. B., AND DE DUVE, C. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 2043–2046.
17. OSMUNDSEN, H., NEAT, C. E., AND BORREBAEK, B. (1980) *Int. J. Biochem.* **12,** 625–630.
18. STECK, W., UNDERHILL, E. W., AND CHISHOLM, M. D. (1982) *J. Chem. Ecol.* **8,** 731–754.
19. BJOSTAD, L. B., LINN, C. E., DU, J. W., AND ROELOFS, W. L. (1984) *J. Chem. Ecol.* **10,** 1309–1323.
20. GOTHILF, S., AND SHOREY, H. H. (1975) *Environ. Entomol.* **5,** 115–119.
21. LINN, C. E., BJOSTAD, L. B., DU, J. W., AND ROELOFS, W. L. (1984) *J. Chem. Ecol.* **10,** 1635–1658.
22. WOLF, W. A., BJOSTAD, L. B., AND ROELOFS, W. L. (1981) *Environ. Entomol.* **10,** 943–946.
23. KASANG, G., AND SCHNEIDER, D. (1978) *Naturwissenschaften* **65,** 337.
24. YAMAOKA, R., AND HAYASHIYA, K. (1982) *Japan. J. Appl. Entomol. Zool.* **26,** 125–130.
25. BJOSTAD, L. B., AND ROELOFS, W. L. (1984) *Insect Biochem.* **14,** 275–278.
26. ROELOFS, W. L., CARDE, R., BENZ, G., AND VON SALIS, G. (1971) *Experientia* **27,** 1438.
27. BAITERSWEILER, W., PRIESNER, E., ARN, H., AND DELUCCHI, V. (1978) *Mitt. Schweiz Entomol. Ges.* **51,** 133–142.
28. TUMLINSON, J. H., YONCE, C. E., DOOLITTLE, R. E., HEATH, R. R., GENTRY, C. R., AND MITCHELL, E. R. (1974) *Science (Washington, D.C.)* **185,** 614–616.
29. NEAL, J. W. (1979) USDA Agr. Res. Results ARR-NE-6.

30. SCHWARZ, M., KLUN, J. A., LEONHARDT, B. A., AND JOHNSON, D. T. (1983) *Tetrahedron Lett.*, 1007–1010.
31. GALBREATH, R., BENN, M. H., YOUNG, H., AND HOLT, V. A. (1984) *Z. Naturforsch.*, in press.
32. CONNER, W. E., EISNER, T., VANDER MEER, R. K., GUERRERO, A., GHIRINGELLI, D., AND MEINWALD, J. (1980) *Behav. Ecol. Sociobiol.* **7,** 55–63.
33. HEATH, R. R., TUMLINSON, J. H., LEPPLA, N. C., MCLAUGHLIN, J. R., DUEBEN, B., DUNDULIS, E., AND GUY, R. H. (1983) *J. Chem. Ecol.* **9,** 645–656.
34. UNDERHILL, E. W., PALANISWAMY, P., ABRAMS, S. R., BAILEY, B. K., STECK, W. F., AND CHISHOLM, M. D. (1983) *J. Chem. Ecol.* **9,** 1413–1423.
35. HILL, A. S., AND ROELOFS, W. L. (1981) *J. Chem. Ecol.* **7,** 655–668.
36. HILL, A. S., KOVALEV, B. G., NIKOLAEVA, L. N., AND ROELOFS, W. L. (1982) *J. Chem. Ecol.* **8,** 383–396.
37. ROELOFS, W. L., HILL, A. S., LINN, C. E., MEINWALD, J., JAIN, S., HERBERT, H. J., AND SMITH, R. F. (1982) *Science (Washington, D.C.)* **217,** 657–658.
38. JAIN, S. C., DUSSOURD, D. E., CONNER, W. E., EISNER, T., GUERRERO, A., AND MEINWALD, J. (1983) *J. Org. Chem.* **48,** 2266–2270.
39. HOWARD, R. W., AND BLOMQUIST, G. J. (1982) *Annu. Rev. Entomol.* **27,** 149–172.
40. HODGSON, E. (1983) *Insect Biochem.* **13,** 237–246.