

Biosynthesis of 2-ketones in Drosophila buzzatii
by Paul Joseph Skiba

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry
Montana State University
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Abstract:
The analysis of pheromone biology in Drosophila is in its infancy, but promises to provide important information about many aspects of the signalling system which appear to have evolved during speciation. While there have been numerous studies on the identification and characterization of Drosophila pheromones, little was known about the biosynthesis of these important compounds.

Using radiolabeled acetate, the ejaculatory bulbs from mature male D. buzzatii were found to be the only tissues capable of biosynthesizing the 2-ketones in vitro. Although radiolabel was incorporated into lipids at all ages tested, label was not detected in pheromone components until the insects were four days post eclosion. The testes and the accessory glands, which are both closely associated with the ejaculatory bulb, did not incorporate label into the 2-ketones.

All of the subcellular fractions from the ejaculatory bulbs from mature males incorporated label from [1-14C]-acetate into the 2- ketones to some extent. However, the microsomes incorporated the most radiolabel into the 2-ketones, with about 7% of the recovered label present in 2-tridecanone and (Z)-10-heptadecen-2-one.

The use of specific heavy atom labeled fatty acyl-CoA esters allowed the biosynthetic pathways for the production of 2-ketones to be determined. The incorporation of [2H23]-lauroyl-CoA into 2-tridecanone and [l,2-13C]-palmitoleoyl-CoA into (Z)-10-heptadecen-2-one, indicated that D. buzzatii produce their 2-ketones via a type of chain elongation.

Furthermore, the incorporation of the fatty acyl-CoA esters in the presence and absence of the acetyl-CoA carboxylase inhibitor avidin indicated that the 2-ketones were biosynthesized by a microsomal chain elongation system and not by a microsomal fatty acid synthetase. The in vitro production of 2-tridecanone and (Z)-10-heptadecen-2-one by the microsomal fraction from ejaculatory bulbs required malonyl-CoA and lauroyl-CoA and palmitoleoyl-CoA, respectively.

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ABSTRACT

The analysis of pheromone biology in Drosophila is in its infancy, but promises to provide important information about many aspects of the signalling system which appear to have evolved during speciation. While there have been numerous studies on the identification and characterization of Drosophila pheromones, little was known about the biosynthesis of these important compounds.

Using radiolabeled acetate, the ejaculatory bulbs from mature male D. buzzatii were found to be the only tissues capable of biosynthesizing the 2-ketones in vitro. Although radiolabel was incorporated into lipids at all ages tested, label was not detected in pheromone components until the insects were four days post eclosion. The testes and the accessory glands, which are both closely associated with the ejaculatory bulb, did not incorporate label into the 2-ketones.

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The use of specific heavy atom labeled fatty acyl-CoA esters allowed the biosynthetic pathways for the production of 2-ketones to be determined. The incorporation of [$^{2}H_{23}$]-lauroyl-CoA into 2-tridecanone and [1,2-^{13}C]-palmitoleoyl-CoA into (Z)-10-heptadecen-2-one, indicated that D. buzzatii produce their 2-ketones via a type of chain elongation.

Furthermore, the incorporation of the fatty acyl-CoA esters in the presence and absence of the acetyl-CoA carboxylase inhibitor avidin indicated that the 2-ketones were biosynthesized by a microsomal chain elongation system and not by a microsomal fatty acid synthetase. The in vitro production of 2-tridecanone and (Z)-10-heptadecen-2-one by the microsomal fraction from ejaculatory bulbs required malonyl-CoA and lauroyl-CoA and palmitoleoyl-CoA, respectively.

INTRODUCTION

General

Chemical communication is an information channel that arthropods have nearly perfected. Chemicals participate in recruitment, defense, caste determination, sexual attraction, aggregation, and other behaviors. Insects also use chemicals for interacting with other arthropods, their plant and animal hosts, and their general environment. These chemicals, which convey information between organisms are called semiochemicals.

If a semiochemical conveys intraspecific information (i.e. aggregation or sexual attraction), it is called a pheromone. Therefore, pheromones are substances which are secreted to the outside by an individual and received by a second individual of the same species in which they elicit a specific reaction.

Pheromones are excellent transmitters of messages because they can pass around obstacles and are potentially capable of being detected at very low dilutions and so convey messages over long distances. They are also useful over extremely short distances, even to the level of contact chemoreception. In addition, their ability to be effective in very small quantities makes them energetically efficient, as does their ability to endure and to continue to convey their signal for extended periods, as in scent trails.

Bossert and Wilson (1963) reasoned that because of the nature of chemical communication, only a limited range of molecules would be suitable. They should have between 5 and 20 carbons and a molecular weight between 80 and 300. This was because, at low molecular weights, only a small number of molecules are feasible signals, while above a certain weight, the diversity of alternatives is more

than is required and the cost of synthesizing these molecules becomes progressively high. On the functional side, large molecules have lower volatility, which makes them unsuitable for certain roles.

The first pheromone identified was the sex pheromone of Bombyx mori, the silkworm moth (Butenandt et al., 1959). The elucidation of bombykol [(E,Z)-10,12-hexadecadien-1-ol] required twenty years and 500,000 female abdomens. Thirty years after the discovery of bombykol, hundreds of insect pheromones have been characterized and purified. Biological studies of their behavioral function and specificity have frequently revealed that a group of compounds, a pheromone blend, rather than a unique molecule are involved. These mixed compounds are usually structurally related.

Although all insect orders use pheromones for communication, the highly social Hymenoptera and Isoptera have developed the most complex and sophisticated pheromone systems. Among subsocial insects, pheromones have been shown to play major roles in the initiation of gregarious behavior during group oviposition among certain mosquitoes (Hudson and Mclintock, 1967) and the desert locust (Norris, 1963); mate attraction among species that maintain a solitary lifestyle(Inscoe, 1977); dispersal behavior among generally gregarious species during predator attack (Nault and Phelan, 1984); the synchronization of gamete maturity among species exhibiting aggregative behaviors (Blum, 1981, 1983); and the formation of aggregations at food sites, particularly among scolytid beetles (Birch, 1984) and Drosophila species (Bartelt and Jackson, 1984). An aggregation pheromone elicits behavior in conspecifics leading to an increase in their density in the vicinity of the pheromone source. Aggregation can benefit the individual insect in a number of ways including defense against predators, overcoming the resistance of a host, location of food, and mate selection.

Aggregation pheromones are widespread in insects, being found in cockroaches, social Hymenoptera (bees and wasps), and many beetles in addition to Drosophila.

Pheromones play a role in many important behavioral and physiological processes of Drosophila (Bartelt et al., 1985; Jallon, 1984; Brieger and Butterworth, 1970; Manning, 1967). These pheromones and the responses they mediate are adaptions that increase reproductive success or survival. All of the Drosophila species studied to date use intraspecific chemical communication to locate food, habitats, and mates. These pheromones are believed to play a role in the reproductive isolation of individual Drosophila species (Brieger and Butterworth, 1970).

Pheromone Biosynthesis

Initial studies on the biosynthesis of pheromones were begun in the 1970's when Kasang et al. (1974) induced female gypsy moths to synthesize tritiated dispalure and Jones and Berger (1978) succeeded in inducing female cabbage looper moths to produce ^{14}C-labeled (Z)-7-dodecenyl acetate by injecting [1-^{14}C]-acetate. Although many insect pheromones have been isolated and their structures determined, until recently relatively little was known about their biosynthesis (Bjostad and Roelors, 1983, 1984; Chu and Blomquist, 1980).

Lipids have assumed considerable functional significance during the evolutionary history of the class Insecta. They are essential structural components of the cell membrane and cuticle, they provide a rich source of metabolic energy for periods of sustained energy demand, they facilitate water conservation both by the formation of an impermeable cuticular barrier and by yielding metabolic water upon oxidation, and they include important hormones and pheromones.

Most insect pheromones are lipids and are believed to be derived from fatty acids. The chemicals used as pheromones exhibit considerable diversity, reflecting a variety of unique biochemical processes that occur in pheromone-producing tissues. The biosynthetic pathways for the pheromones from a number of species have been determined, and work is progressing toward description of the enzymes involved. From the results of studies to date, it appears that these specific and unique chemicals are produced by the addition of one or two ancillary enzymes to alter the products of "normal" metabolism, rather than the elaboration of an entire set of unique enzymes in the pheromone producing glands.

Over the past few years, a substantial amount of information of fatty acid derived pheromone biosynthesis in a number of Dipteran and Lepidopteran species has become available. The method used by most insects seems to be that of de novo synthesis, although food components may be used by some species (Blomquist and Jackson, 1979).

By characterizing which types of reactions are involved and in what order, a number of different biosynthetic pathways have been shown to exist Pennanec'h et al., 1992 for a review). There are a variety of common reactions, such as fatty acid synthesis, elongation, chain shortening, and desaturation. A few steps are specific, depending on the functional group involved.

Insects produce a vast number of compounds which are basically straight-chain, and can be formally derived from fatty acids. Most of these compounds probably originate in the classic acetate-malonate pathway.

Acetyl-CoA and malonyl-CoA are the basic building blocks used for the de novo biosynthesis of fatty acids in living organisms (Volpe and Vagelos, 1973, 1976). Acetyl-CoA normally arises in the cell as a product of the glycolytic cycle or by the ß-oxidation of fatty acids in the mitochondria. In addition, acetyl-CoA

can also be formed by direct esterification of acetate in the cytoplasm by acetyl-CoA synthetases. The synthesis of malonyl-CoA is catalyzed by acetyl-CoA carboxylases which condense carbon dioxide with acetyl-CoA in an ATP-dependent reaction. Fatty acids are then synthesized by condensation of two-carbon units from malonyl-CoA with a growing fatty acyl chain covalently linked to fatty acid synthase in eukaryotic systems; the release of carbon dioxide provides the driving force for the condensation reaction. The ß-ketoacyl derivative is reduced, dehydrated, and reduced again to form the fatty acyl derivative two carbons longer. The cycle is then repeated with condensation of another malonyl-CoA molecule with the elongated fatty acyl chain. Seven different activities are involved in the reaction catalyzed by fatty acid synthetase.

De novo biosynthesis of medium and long chain pheromones and of their fatty acyl precursors, especially palmitate and stearate from radiolabeled acetate have been documented in many insect species (Bade, 1964; Bjostad and Roelofs, 1984; de Renobales and Blomquist, 1984). These studies have shown the ability of insects to condense small carbon units such as acetyl- and malonyl-CoA to form medium size fatty acids, as it occurs in other eucaryotic organisms.

Insect fatty acid synthetases (FAS) have been isolated from the pea aphid, Acyrthosiphon pisum (Ryan, et al., 1982), and from two different fruit flies: Ceratitis capitata (Municio et al., 1979), and Drosophila melanogaster (deRenobales and Blomquist, 1984). In all three cases, the FAS activity was found in the 105,000 g particle-free supernatant. Acetyl-CoA and malonyl-CoA were substrates for the enzyme which in all cases required NADPH. Fatty acid products were all released in the free form. All of these insect FAS showed marked similarities with the few vertebrate enzymes which have been purified: complex structure, similar molecular weight, and a preference for NADPH.

The major proportion of the fatty acid complement of insect is represented by eight fatty acids. These are the saturated fatty acids: myristic acid, palmitic acid, and stearic acid; the monounsaturated fatty acids: myristoleic acid, palmitoleic acid, and oleic acid, and the polyunsaturated fatty acids, linoleic acid and linolenic acid. Examination of the fatty acids into which the label from [1-^{14}C]-acetate becomes incorporated indicates that the primary products of de novo fatty acid synthesis are palmitic, palmitoleic, stearic, and oleic acids Keith, 1967; Zebe and McShan, 1959).

Acetyl-CoA carboxylase contains covalently bound biotin as a prosthetic group, and catalyzes the carboxylation of acetyl-CoA to the three carbon compound malonyl-CoA. Malonyl-CoA then condenses with acetyl-CoA in a series of reactions catalyzed by the fatty acid synthetase complex to yield a four carbon butyryl intermediate. There follows a successive sequence of similar condensations with the growing acyl intermediate until an acyl chain of appropriate length has been achieved. It is evident that this pathway results in the production of fatty acids containing an even number of carbon atoms.

Following the synthesis of a long chain saturated fatty acid, the monounsaturated homologue may be formed by direct dehydrogenation (Bade, 1964; Lambremont et al., 1965;, 1966; Takaya and Miura, 1968; Stephen and Gilbert, 1969) and have demonstrated that, in locust this capacity resides within the microsomal fraction. Similar to other desaturases, the locust desaturase shows an absolute dependence on oxygen and requires reduced pyridine nucleotide (NADH being more active than NADPH), a finding that has been confirmed in Ceratitis capitata (Gonzalez-Buitrago et al., 1979).

Two insect orders have been the subject of intensive studies revealing two pheromone types which have different properties: volatile pheromones common

among Lepidoptera and contact pheromones common in Diptera. Most have aliphatic chains with a limited number of double bonds in a variety of positions and usually of the cis (Z) configuration. Volatile pheromones show chain lengths which are usually less than eighteen carbons and bear a functional group, very often an alcohol, acetate, aldehyde, or ketone. For contact pheromones, the chains are usually longer than twenty-one carbons and often very long, with an odd number of carbons and no functional group; most are linear hydrocarbons. These pheromones have been proposed to be biosynthesized by either a chain elongation pathway or a chain shortening pathway.

A large number of examples of derivatives of long chain fatty acids are found in other insect groups, particularly the Hymenoptera (Blum et al., 1983; Williams, et al., 1980; Graham, et al., 1979). The Dufour's gland and some other abdominal glands of ants often contain long chain fatty acid derivatives. Hydrocarbons and saturated acetate esters predominate, but unsaturated acetates are occasionally detected. The other group of compounds found commonly is ketones, particularly methyl ketones but including some ethyl ketones. The methyl ketones usually have odd-carbon chains and range from 2-tridecanone to 2-nonadecanone.

Biosynthetic studies of the Dufour's gland have not demonstrated conclusively the incorporation of acetate units into either hydrocarbons or the main chain of the oxygenated compounds, although rapid incorporation into the acetate portion of the esters is evident (Graham et al., 1979).

The exocrine secretions of many species of bee contain very similar compounds as found in ants. Alcohols are particularly common, including a number of methyl carbinols and the related methyl ketones.

Several of the compounds produced in the defensive secretions of termites,

particularly from the family Rhinotermitidae, are also similar. Among these are several compounds with unusual structures, including a nitroalkene, a series of conjugated enones, and enolic ß-ketoaldehydes (Spanton and Prestwich, 1981).

Apart from these examples, derivatives of long-chain fatty acids have been found in a wide variety of insects from different orders. In some cases the same compound can have very different functions in different insects; for example, hexadecyl acetate is a pheromone in Lepidoptera, but is found, in larger amounts, in the defensive secretions of beetles, ants and bees.

Chain Shortening Pathway

Many Lepidopteran sex pheromones share the following features: a long nonbranched carbon chain with an oxygen function (alcohol, aldehyde, ester) on one terminal carbon, and one or more double bonds, usually far removed from the oxygenated carbon. Species specificity is achieved by varying these characteristics (chain length, oxidation state, double bond site) and the mixing of two or more components in specific ratios. The structures of these sex pheromones suggested that their synthesis may be linked to or similar to that of fatty acids Roelofs and Wolf, 1988).

Although the structural characteristics of typical lepidopteran sex pheromones suggest an origin in either the fatty acid synthesis or fatty acid degradation patyhways, studies to confirm this and elucidate the details of the insertion of unsaturation, and modification of the functional group, have only been performed recently (reviewed in Roelofs and Brown, 1982).

Total lipid analysis of the sex pheromone gland of the redbanded leafroller moth, Argyrotaenia velutinana, shows that a wide range of saturated and unsaturated fatty acids is present, but the use of radio-labeled precursors has

demonstrated that the primary route for biosynthesis of the sex pheromones is via a chain-shortening pathway (Roelofs and Wolf, 1988). Essentially, hexadecanoic acid can be thought of as the initial precursor, which is first shortened, possibly by hexadecanoyl-CoA oxidase, to tetradecanoic acid. The double bond is then introduced by two desaturase enzymes, one producing the trans (E)-isomer and one the (Z)-isomer. The resulting acids are then reduced and acetylated to give two of the pheromone components, Z11-14:Ac and E11-14:Ac (Figure 1). The acid moieties are normally bound to glycerol, usually as triacylglycerols. The E/Z ratio of the tetradecenoyl moieties was found to be 61:39 in triacylglycerols, and approximately 31:69 in the phosphatides, contrasting strongly with the observed 9:91 ratio in the acetates emitted in the pheromone. No evidence was found for an isomerase which could correct the ratio of precursors, and the fate of the excess (E)-component is at the present time unknown, as is the way in which the final ratio of acetates is controlled.

A central problem of lepidopteran pheromone biochemistry, the control of component proportions within a given blend, is still a mystery. The specificity and relative quantities of the various enzymes of a biosynthetic sequence could play a major role in determining the proportions of homologous compounds in a mixture.

The stereospecificity of individual enzymes for certain substrates and products might explain the fixed proportions of geometric isomers found in species like Argyrotaenia velutinana. Most of the enzymes involved in biosynthesis have not been purified and protein chemistry studies are thus very limited.

Similar processes can be postulated to produce very many of the common constituents of Noctuid and Totricid sex pheromones although evidence for most of these steps is sparse. Many of the known double bond positions can be accounted for by the involvement of either the 11-desaturase detected in A.

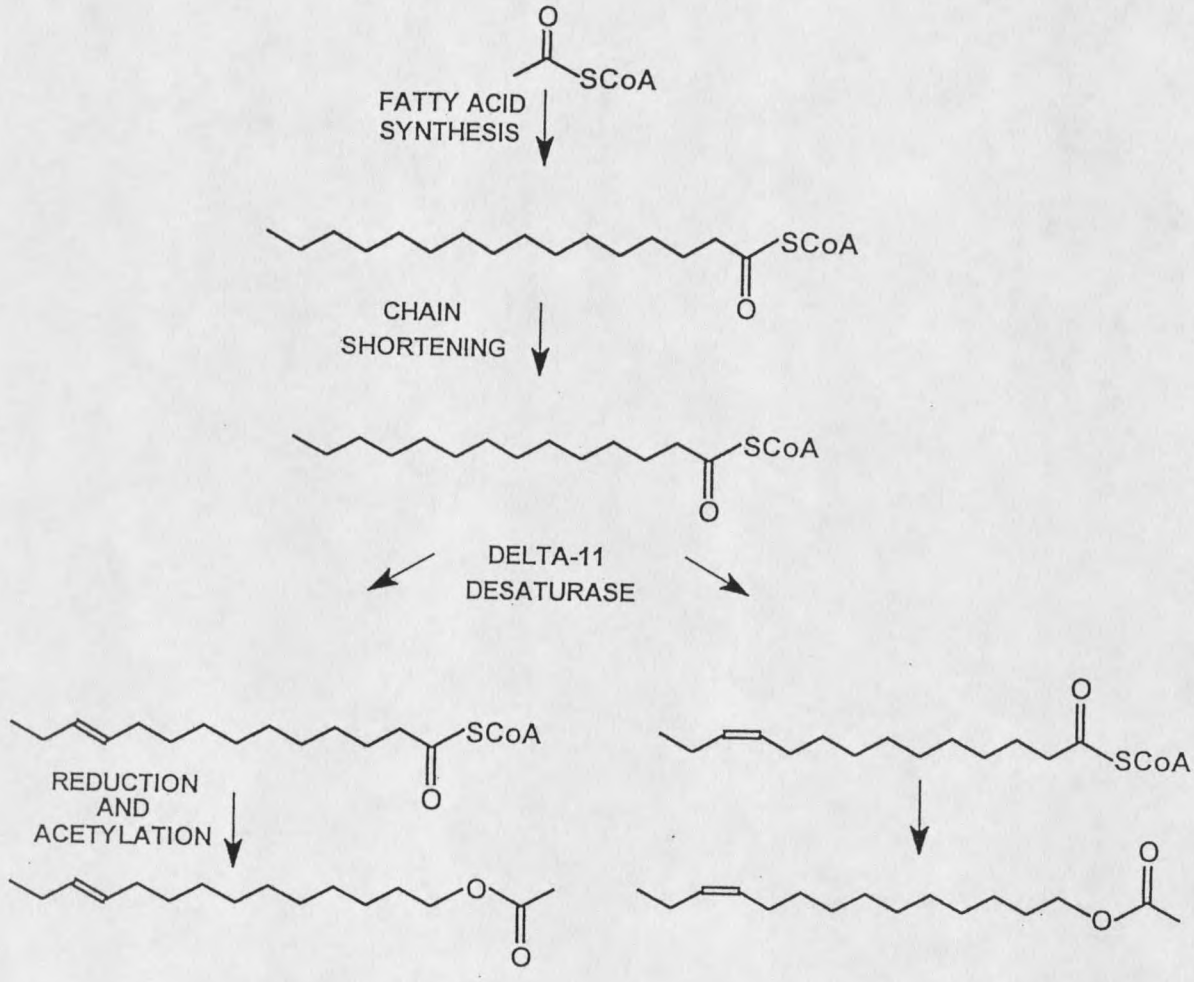


Figure 1. Biosynthesis of Z11-tetradecenyl acetate and E11-tetradecenyl acetate, the sex pheromone components of Argyrotaenia velutinana Bjostad and Roelofs, 1981).

velutinana (Bjostad and Roelofs, 1981), apparently unique to insects, or a 9-desaturase, examples of which are widespread, and which give rise, for example, to common fatty acids, oleic and palmitoleic. It is likely that, as more species are investigated, more diverse routes of biosynthesis will become apparent.

Chain shortening consists of the removal of one or more two-carbon units. Keith (1967) showed that D. melanogaster larvae reared on radioactive palmitic and stearic acids were able to shorten stearic acid to myristic acid but unable to shorten palmitic acid. The first in vivo evidence for chain shortening was found in Argyrotaenia citrana and Argyrotaenia velutinana where the pheromone gland was shown to be capable of shortening hexadecanoic acid to tetradecanoic acid (Bjostad and Roelofs, 1981). Further studies with cell free preparations from A. citrana showed that [U-^{14}C]-palmitate was converted to [^{14}C]-tetradecanoate while [1-^{14}C]-palmitate was not converted to labeled tetradecanoate (Wolf and Roelofs, 1983). In addition, in vivo studies on Trichoplusia ni (Bjostad and Rolofs, 1983) (Z)-11-hexadecenoate was chain-shortened to (Z)-9-tetradecenoate, which in turn was shortened to (Z)-7-dodecenoate, the immediate fatty acyl precursor to the pheromone, (Z)-7-dodecenyl acetate (Figure 2).

There is much evidence to suggest that the metabolic pathway for fatty acid oxidation in insects is the same as in other eucaryotes. The initial activation of fatty acid is catalyzed by the microsomal enzyme fatty acyl-CoA synthetase which has been demonstrated in several insect species (Hoskins, et al., 1956; Zebe and McShan, 1959; Domroese and Gilbert, 1964; Beenakkers and Henderson, 1967; Stevenson, 1972). In addition, ß-hydroxyacyl-CoA dehydrogenase and ß-ketoacyl-CoA thiolase have been demonstrated in mitochondrial preparations from several

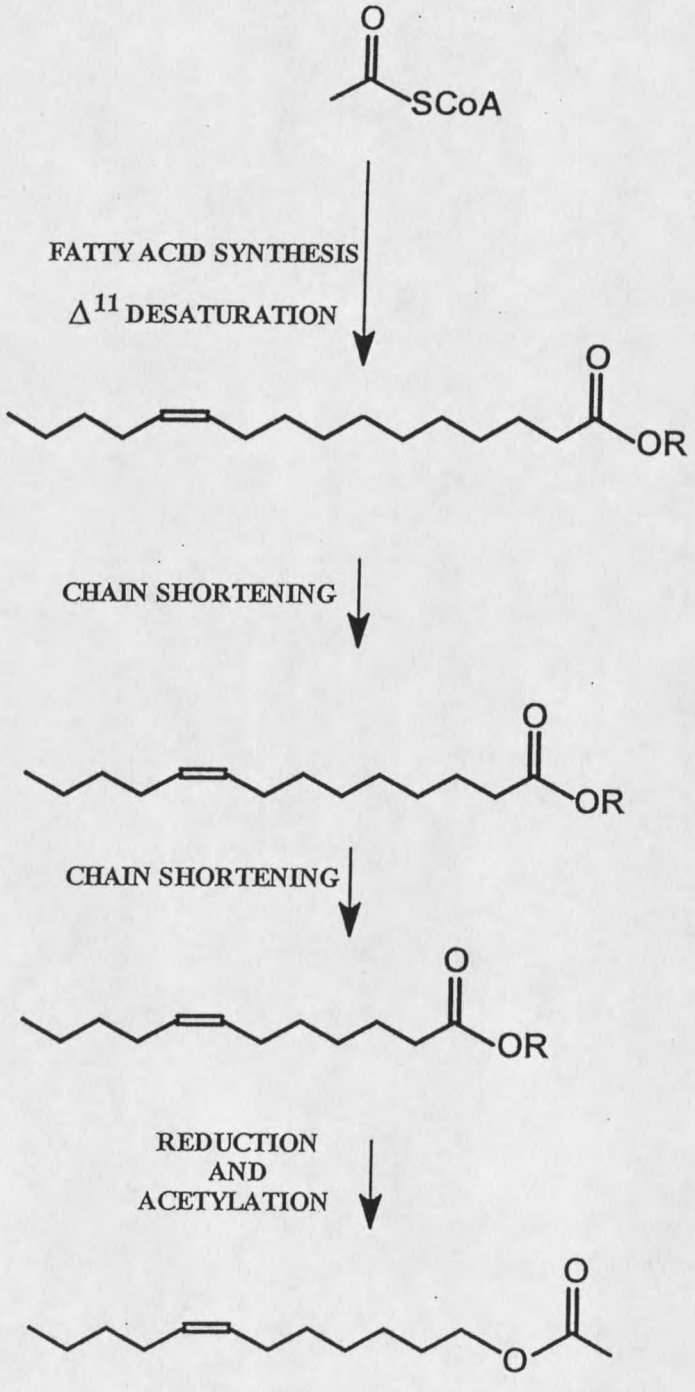


Figure 2. Biosynthesis of Z7-dodecenyl acetate, the sex pheromone of Trichoplusia ni (Bjostad and Roelofs, 1983).

species (Zebe and McShan, 1959; Beenakkers and Henderson, 1967; Crabtree and Newsholme, 1972), indicating that the complete complement of ß-oxidation enzymes are present in insects. Although the enzymatic mechanism of chain shortening in insects has not been studied, it appears to be similar to the ß-oxidation steps which occur in peroxysomes.

Chain Elongation Pathway

Fatty acyl elongation consists of successive additions of two carbon units to the medium size fatty acyl moieties which result from fatty acid synthesis. In some insects, the involvement of chain elongation has been shown to be involved in the biosynthesis of very long chain fatty acids which are precursors to hydrocarbons and acetates.

Only a few in vitro studies of the elongation reaction have been carried out. Gonzales-Buitrago et al. (1979) studied the microsomal elongation system of Ceratitis capitata, which utilized malonyl-CoA as a substrate.

Based on the structural similarity between cuticular hydrocarbons and the 2-ketones, it was suggested that the 27 and 29 carbon 2-ketones of the German cockroach may be formed from oxidation at the 2 position of pre-formed hydrocarbons, or alternatively from failure to reduce the carbonyl group during an elongation reaction by which the hydrocarbons would otherwise be synthesized (Jurenka et al., 1989).

Microsomes from Musca domestica were able to quickly transform stearoyl and oleoyl-CoA to fatty acids containing between 20 and 28 carbons (Vaz, et al., 1988a). The cofactors necessary for the elongation reaction are malonyl-CoA, and NADPH or NADH. Since fatty acids longer than 18 carbons are not usually observed in vivo, it appears that elongation and decarboxylation are closely

coupled.

To produce (Z)-9-tricosene and other cuticular hydrocarbons, long chain fatty acids have to be decarboxylated. The final decarboxylation of tetracosenoic acid to tricosene has not been directly tested, but a similar process has been demonstrated in termite and cockroach microsomes (Chu and Blomquist, 1980; Major and Blomquist, 1978). Very little is known about the decarboxylation reaction. It is suggested that a reductive decarboxylation through an alpha-hydroxy-intermediate takes place, by analogy with plants (Kolattukudy, 1976; Tchoy-Pek et al., 1986). All studies have found a condensation-reduction pathway similar to that found in bacteria, and have also described a strong link between elongation and decarboxylation (Jurenka et al., 1989; Chase et al., 1990; Juarez, et al., 1992).

3,11-Dimethyl nonacosanone, the sex pheromone of the German cockroach has been shown to be produced by a chain elongation reaction (Chase et al., 1990, Juarez, et al., 1992). This methyl branched ketone is formed by the insertion of methylmalonyl-CoA units in the microsomal chain elongation reaction (Figure 3).

As in other insects, much more is known about the biology and chemistry of dipteran pheromones than about their biosynthesis. Extensive biosynthetic studies on pheromones have been done only with the housefly, Musca domestica, and the pathways in this insect are now well understood (Figure 4). The sex pheromones of the housefly are closely related to the hydrocarbon components of the epicuticular wax layer. The biosynthetic pathways for cuticular hydrocarbons have been studied in a number of non-dipteran species and apply directly to dipteran sex pheromone biosynthesis (Blomquist and Dillwith, 1985).

The model systems for pheromone biosynthesis show several common steps involving similar enzymes: fatty acid synthesis, chain shortening, elongation,

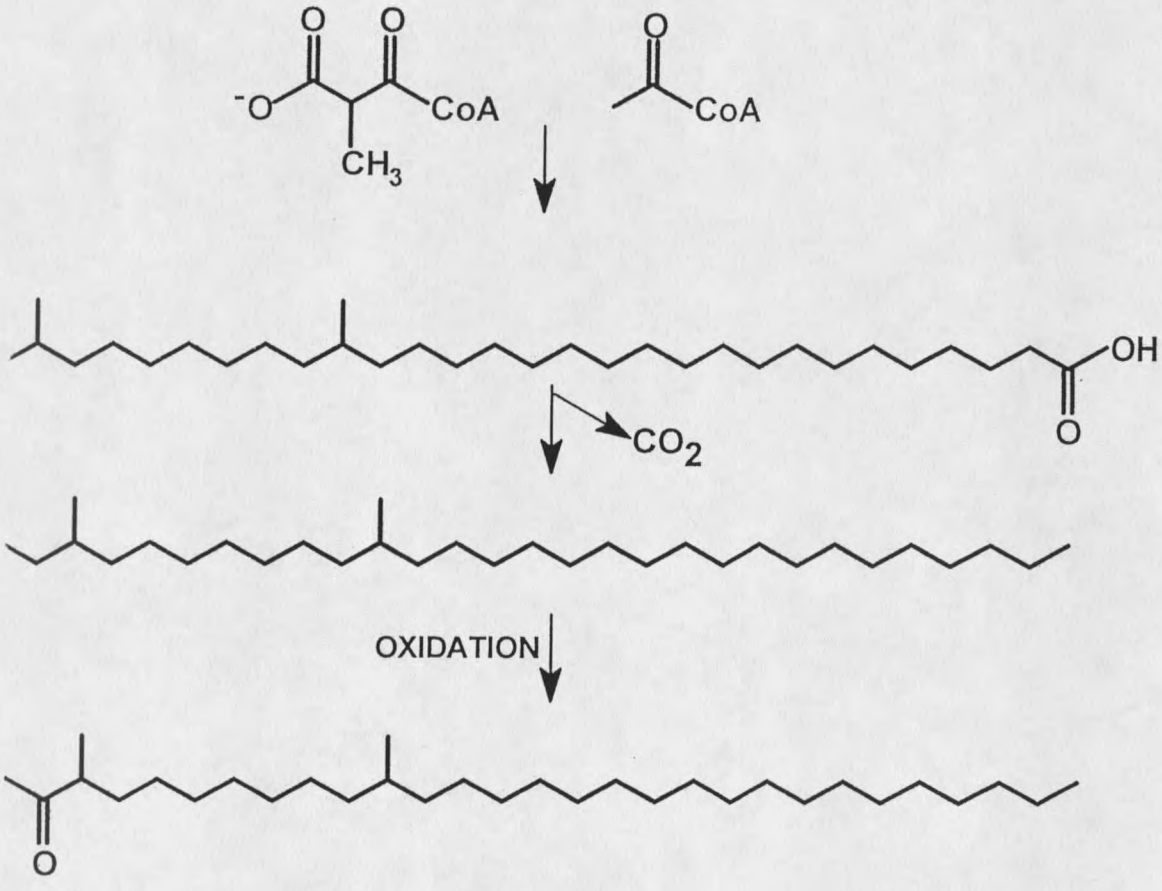


Figure 3. Biosynthesis of 3,11-dimethyl-2-nonacosanone, the sex pheromone of the German cockroach (Juarez, et al ., 1992).

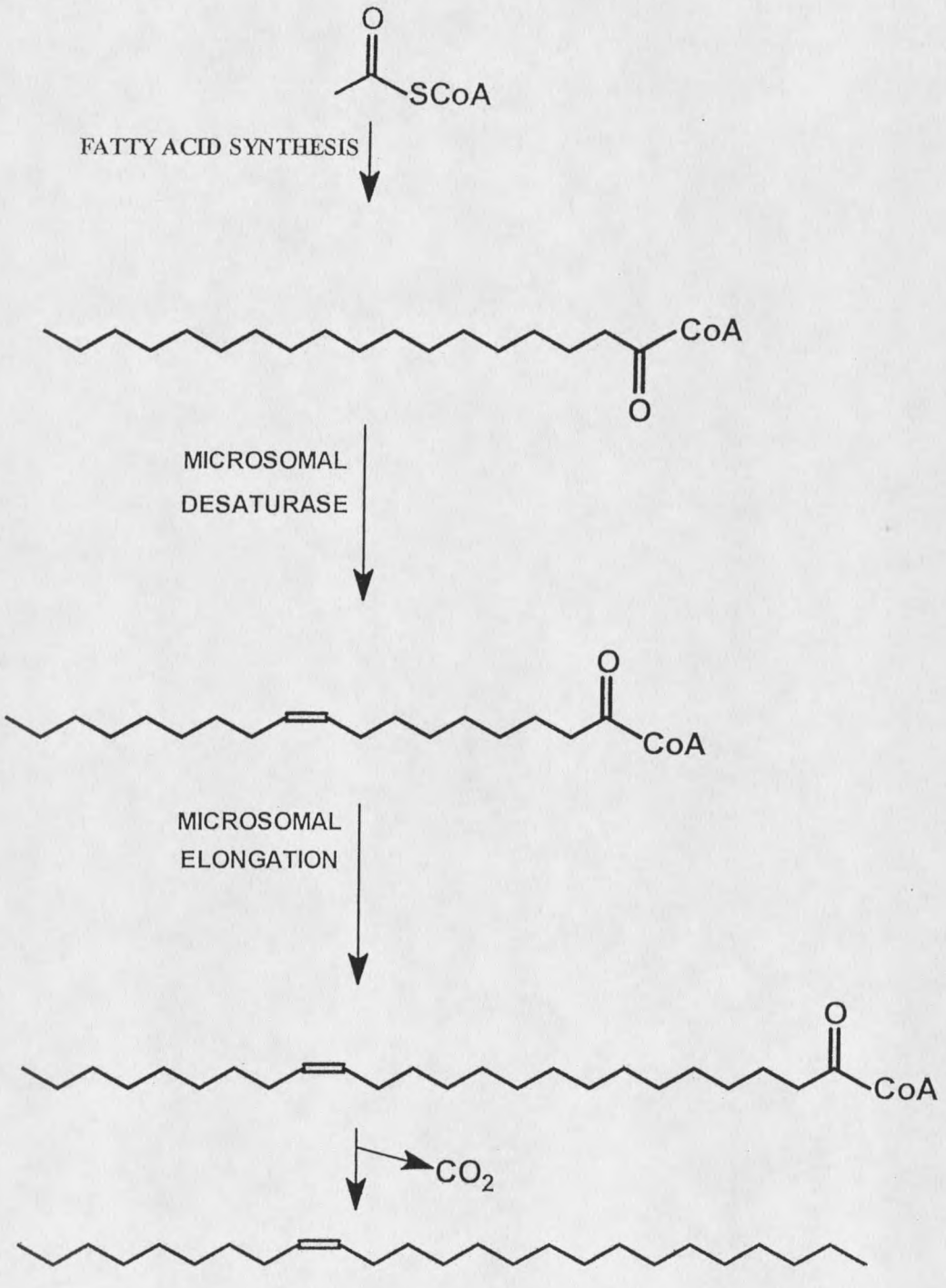


Figure 4. Biosynthesis of Z9-tricosene, the sex pheromone of the housefly (Vaz et al., 1988).

desaturation, and a few specific steps: decarboxylation, esterification, hydrolysis, oxidation, and reduction.

Drosophila Pheromones

Although a number of investigators have studied the involvement of pheromones in close range courtship behavior (Antony and Jallon, 1987; Antony et al., 1985; Jallon, 1984), less is known about how the flies initially come together in the field so that close range courtship stimuli can operate. The contribution of pheromones to this process had probably been overlooked because in most Drosophila species, feeding, mating, and oviposition occur at the same site and attraction to food odors could account for aggregation. However, in certain Hawaiian species, mating occurs in male mating territories away from the feeding site (Ringo, 1976). In these species, the males appear to emit pheromones which may be long range attractants. Further work (Bartelt, et al., 1985a, 1985b, 1986, 1988; Schaner, et al., 1987, 1989a, 1989b, 1989c, 1989d, 1989e) has shown that many of the Drosophila species which do not use separate feeding and mating sites, nevertheless, emit pheromones which contribute to the aggregation of individuals.

Just eight years ago, Bartelt ans Jackson (1984) demonstrated that aggregation behavior in Drosophila was regulated in part by chemicals produced by the flies. Since that first paper, the aggregation pheromones of over thirty species of Drosophila have been characterized and identified. The results are an exciting addition to the understanding of the chemical ecology, evolution, and molecular systematics of the species studied. The composition of the aggregation pheromones vary from single compounds that are common to a number of species to mixtures of a variety of components that are species-specific.

The use of aggregation pheromones to locate feeding sites and mating partners is probably a general phenomenon in the Drosophila . The presence of male-produced aggregation pheromones has been demonstrated in seven species of the D. virilis species group, in eight species of the D. melanogaster species group, and in six members of the D. repleta species group. In all cases, the pheromone attracts nearly equal numbers of both sexes and synergizes food odors.

Pheromones play a role in many important behavioral and physiological processes of Drosophila. These pheromones and the responses they mediate are adaptions that increase reproductive success or survival. All of the Drosophila species studied to date use intraspecific chemical communication to locate food, habitats, and mates. These pheromones are believed to play a role in the reproductive isolation of Drosophila species (Ewing, 1983) .

The pheromones produced by Drosophila species represent several different classes of compounds. Pheromone components can be hydrocarbons, both monounsaturated and diunsaturated, usually longer than twenty carbons. The functional groups identified in Drosophila thus far include tiglic esters (Bartelt et al., 1985), ketones (Moats et al., 1987), and acetate esters (Schaner et a:., 1989a, 1989e). The diversity of pheromone structure has been suggested to be responsible for the separation of species. At the chemical level, the biosynthesis of these pheromones has become so finely tuned that components are biosynthesized not only to a high degree of chemical purity, but geometrical and optical isomerism is also precisely controlled.

Mature males of the thirty Drosophila species studied to date produce relatively nonpolar aggregation pheromones which attract both males and females of any age (Table 1). The male-produced aggregation pheromones are synergistic with volatile compounds related to food odors and/or oviposition sites. These

chemical cues are not present in newly eclosed males or in virgin females of any age. However, over a two to three day period during sexual maturation there is a dramatic increase in the amount of pheromone stored in the male's ejaculatory bulb. In many Drosophila species, the pheromone components are stored in the male's ejaculatory bulb and transferred to the female during mating. The female later releases the pheromone onto the food and/or oviposition site (Schaner et al., 1989a, 1989b, 1989c).

Most of the species of the melanogaster group use the single component (Z)-11-octadecenyl acetate (ZVA) as their aggregation pheromone; however, in the evolution of the bipectanata complex there appears to be a mutation to production of (Z)-11-eicosenyl acetate which is two carbons longer. In addition, the pheromone perception by species studied in the bipectanata complex is not as specific as those in the melanogaster complex, since they will respond to both (Z)-11-eicosenyl acetate and (Z)-11-octadecenyl acetate (Schaner et al., 1989a; Schaner et al., 1989b).

Several Drosophila species produce two chemically different classes of chemicals in their aggregation pheromone blend. The aggregation activity of components of the two classes is usually synergistic. All species of the virilis group make esters of tiglic acid (mostly ethyl tiglate) as a low molecular weight component of their aggregation pheromone blend (Bartelt et al., 1985; Bartelt et al., 1986b). Most of the virilis subgroup also make a high molecular weight component, (Z)-9-heneicosene, as a coattractant (Bartelt et al., 1986b); however, it appears that when D. virilis split off, they developed the ability to make and respond to (Z)-10-heneicosene (Bartelt and Jackson, 1984). D. lummei and all of the montana subgroup lost the ability to produce the hydrocarbon component, but

Table 1. Phylogeny of the the melanogaster, virilis, and repleta groups and their aggregation pheromone components.

Species	Pheromone
melanogaster group	
suzuki subgroup	
rajasekarii	Z-11-18:Ac
melanogaster subgroup	
yakuba	Z11-18:Ac
simulans	Z11-18:Ac
mauritiana	Z11-18:Ac
melanogaster	Z11-18:Ac
ananassae subgroup	
malerkotliana	Z11-20:Ac
bipectinata	Z11-18:Ac
ananassae	Z11-18:Ac and Z11-20:Ac
virilis group	
virilis subgroup	
virilis	Z10-21 and ET
lummei	ET
novamexicana	Z9-21 and ET
americana	Z9-21 and ET
texana	Z9-21 and ET
montana subgroup	
borealis	ET
littoralis	ET
repleta group	
hydei subgroup	
hydei	2-tridecanone and ET
repleta subgroup	
repleta	2-tridecanone and ET
mulleri subgroup	
mulleri	(S)-2-tridecyl acetate
serido	(Z)-10-heptadecen-2-one
borborema	(Z)-10-heptadecen-2-one
buzzatii	(Z)-10-heptadecen-2-one

Z-11-18:ac= (Z)-11-octadecenyl acetate; Z-11-20:Ac= (Z)-11-eicosenyl acetate;

Z10-21= Z10-heneicosen; Z9-21= Z9-heneicosene; ET= ethyl tiglate

lummei and some species of the montana subgroup retained the ability to respond to both (Z)-9- and (Z)-10-heneicosenes which they do not make (Bartelt et al., 1988). These data suggest that production and perception of (Z)-9-heneicosene and ethyl tiglate are primitive traits for the virilis group. In speciation to virilis, there were mutations to production and perception of (Z)-10-heneicosene. However, in speciation to lummei and the montana subgroup, there was a loss of (Z)-9-heneicosene production, but not a loss of (Z)-9-heneicosene perception.

The hydrocarbon and ester components of the aggregation pheromone in all members of the virilis group are transferred directly to the food source by mature males (Bartelt, et al., 1985).The pheromone and food odors then act in concert to attract both males and females of any age. Virilis is the only group studied to date in which the pheromones are transferred to the food source directly by the male.

Although the production of tiglate esters in the pheromone blend extends into the repleta group (Moats et al., 1987), hydrocarbons have not been observed as pheromone components in this group. Instead, one or more 2-ketones and acetate esters of 2-alcohols are the major components of the aggregation pheromone in most species of the repleta group studied thus far (Bartelt et al., 1989; Schaner and Jackson.,1992).

In members of the mulleri subgroup of the repleta group, the ejaculatory bulbs contain saturated and unsaturated 13, 15, and 17 carbon 2-ketones and in some species (S)-2-tridecanyl acetate. The most abundant and most active component present in D. mulleri was (S)-2-tridecanyl acetate (Bartelt et al., 1989). This compound was unique as the first pheromone component with a chiral carbon observed in Drosophila. D. wheeleri and D. aldrichi also contain large quantities of (S)-2-tridecanyl acetate. Much smaller amounts of 2-pentadecanone,

(Z)-10-pentadecen-2-one, 2-heptadecanone, and (Z)-10-heptadecen-2-one were also observed in the ejaculatory bulbs of wheeleri and mulleri. Only the seventeen carbon ketones of mulleri were active in the pheromone blend.

In the martensis complex, D. martensis had nearly equal amounts of 2-tridecanone and (Z)-10-heptadecen-2-one in their ejaculatory bulbs. The (Z)-10-heptadecen-2-one had aggregation pheromone activity, while 2-tridecanone was not active (Schaner et al.,1992). D. buzzatii likewise had large quantities of 2-tridecanone and (Z)-10-heptadecen-2-one in their ejaculatory bulbs; however in this case, 2-tridecanone inhibited the aggregation activity promoted by (Z)-10-heptadecen-2-one (Schaner et al., 1992). This was the first observation of an ejaculatory bulb component from Drosophila inhibiting aggregation activity. The other two closely related species in the buzzatii cluster, D. serido and D. borborema, did not have 2-tridecanone in their ejaculatory bulbs, but only (Z)-10-heptadecen-2-one, which was their aggregation pheromone. 2-Tridecanone was present in most species of the mulleri cluster, but was not active as an inhibitor or as a component of the pheromone except in buzzatii.

The mulleri complex of the repleta group consists of 23 described species from six clusters. The buzzatii cluster consists of three South American species, D. buzzatii, D. serido, and D. borborema. Of the members of the mulleri complex, Drosophila buzzatii males store the largest quantities of 2-tridecanone (230 ng/male) and (Z)-10-heptadecene-2-one (690 ng/male) in their ejaculatory bulbs (Schaner and Jackson, 1992). From the study of insect pheromones to date, it appears that these specific and unique lipid molecules are biosynthesized via either the chain shortening or chain elongation pathways. While the biosynthesis of Drosophila aggregation pheromones has not been investigated, the 2-ketones made by males of the mulleri group could be produced by either one of these pathways.

In addition to Drosophila, medium chain length 2-ketones (13-19 carbons) have been identified as components of complicated mixtures of hydrocarbons, aldehydes, acids, esters, and terpenes in the Dufour's glands of a variety of Lasius (Blum et al., 1981; Bergstrom and Lofqvist, 1970), Acanthomyops (Regnier and Wilson, 1968), Formica (Bergstrom and Lofqvist, 1968), Gigantiops (Blum et al., 1983), and Myrmecocystus (Lloyd et al., 1989) species. 2-Ketones have also been found in two genera of termites (Quennedey et al., 1973; Prestwich et al., 1975), two genera of stingless bees (Luby et al., 1973; Franke et al., 1983), two species of beewolfs (Schmidt et al., 1985; McDaniel et al., 1987), and a caterpillar (Eisner et al., 1972). In most cases where the functions of the 2-ketones are known, they are components of the alarm pheromone or of the defensive secretion. However, the methyl branched 2-ketone 10-methyl-2-tridecanone is the sex pheromone of the southern corn rootworm, Diabrotica undecimpunctata howardi (Guss et al., 1983).

Similar to Drosophila, males of 2 species of beewolfs use saturated methyl ketones in their scent marking pheromone(McDaniel et al., 1987). A third species uses (Z)-11-eicosenol plus a small amount of the unsaturated 2-ketone 10-nonadecen-2-one (Schmidt et al., 1990). Unsaturated 2-ketones have also been isolated from the Argentine ant (Cavill et al., 1980), and from the defensive secretion of Uloma tenebrionedis (Gnanasunderam et al., 1985).

2-Ketones have also been identified in plants. Resistance of wild tomato (Lycopericom hirsutum f. glaboratum) to attack by important insect pests including the tobacco hornworm (M. sexta), the tomato fruitworm (Heliothis zea), the tobacco budworm (H. virescen), and the Colorado potato beetle (Leptiotarsa decemlineata) is due, in part, to the presence of the toxic methylketone, 2-tridecanone, in the glandular tricomes(Kaufman and Kennedy, 1989). As indicated above, the biosynthesis of 2-ketones is important to the exocrine

chemistry of a number of phytophagous insects and is also involved in plant protection against insects.

The chain shortening pathway has been demonstrated for the biosynthesis of unsaturated aldehydes, primary alcohols, and acetate esters which comprise a vast majority of the lepidopteran sex pheromones (Bjostad et al., 1987 for a review). The exact mechanism of the chain shortening is not known, but there is a loss of two carbons, which suggests a type of beta-oxidation. A presumed intermediate in this chain shortening would then be a beta-keto acid (presumably as the CoA-thioester), which if the free acid were formed could be decarboxylated to yield a 2-ketone (Figure 5).

The failure to reduce the beta-carbonyl group during an elongation reaction of fatty acids allows for the proposal of an "elongation pathway". Medium-chain fatty acids from the pheromone producing tissue and/or hemolymph could be elongated by addition of two carbons (acetyl-CoA or malonyl-CoA) without carbonyl reduction to give a beta-keto-acyl-CoA. If the free beta-keto acid were formed, it would decarboxylate to yield a 2-ketone (Figure 6). The chain elongation pathway has been demonstrated in the biosynthesis of pheromones in the housefly and the cockroach (Vaz et al., 1988; Juarez et al., 1992).

Common to both the proposed elongation pathway and the chain shortening pathway is the decarboxylation of a beta-keto-acid to yield the 2-ketones. The remainder of two proposed pathways are very different in putative precursors allowing for detailed examination as proposed pathways for 2-ketone biosynthesis.

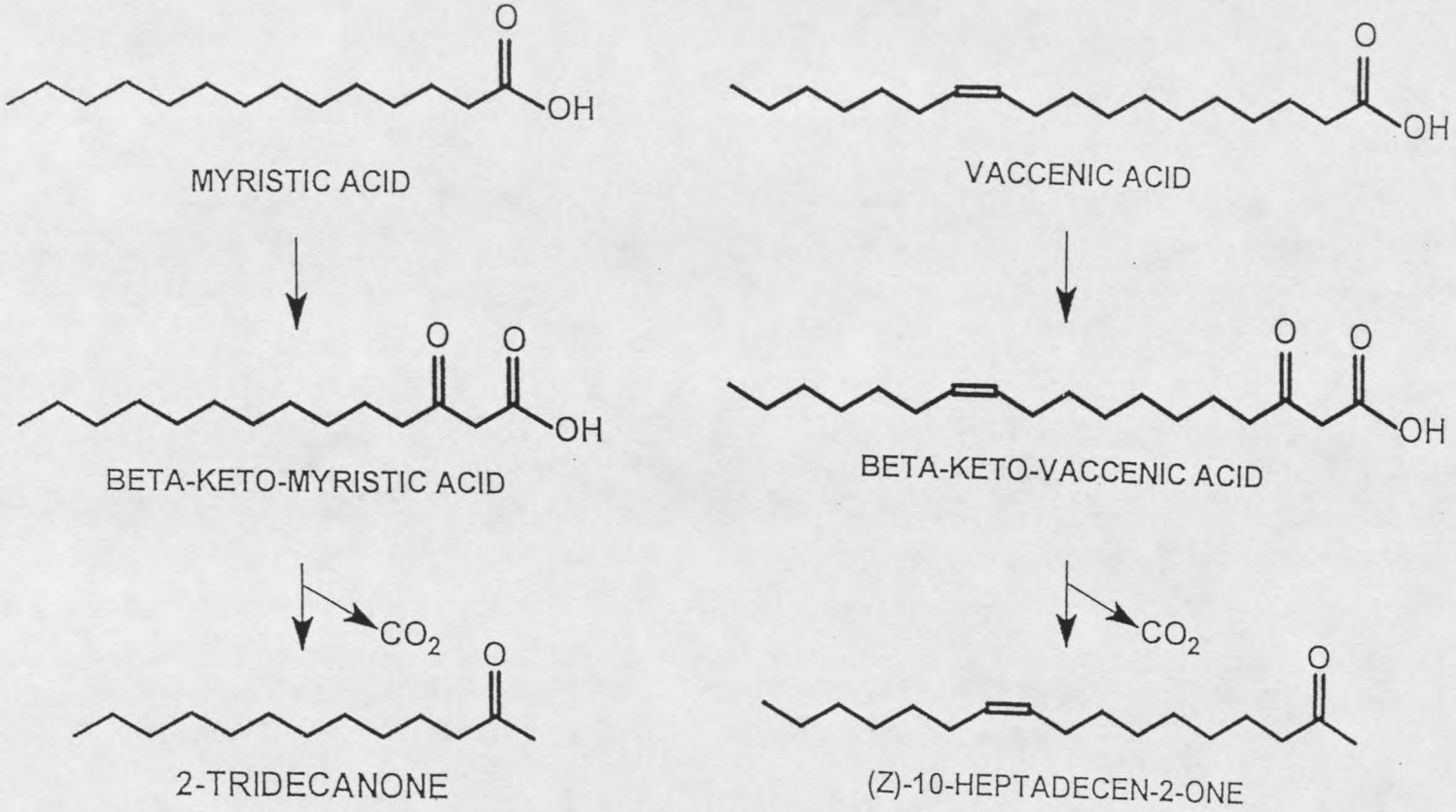


Figure 5. Biosynthesis of 2-ketones via the proposed chain shortening pathway.

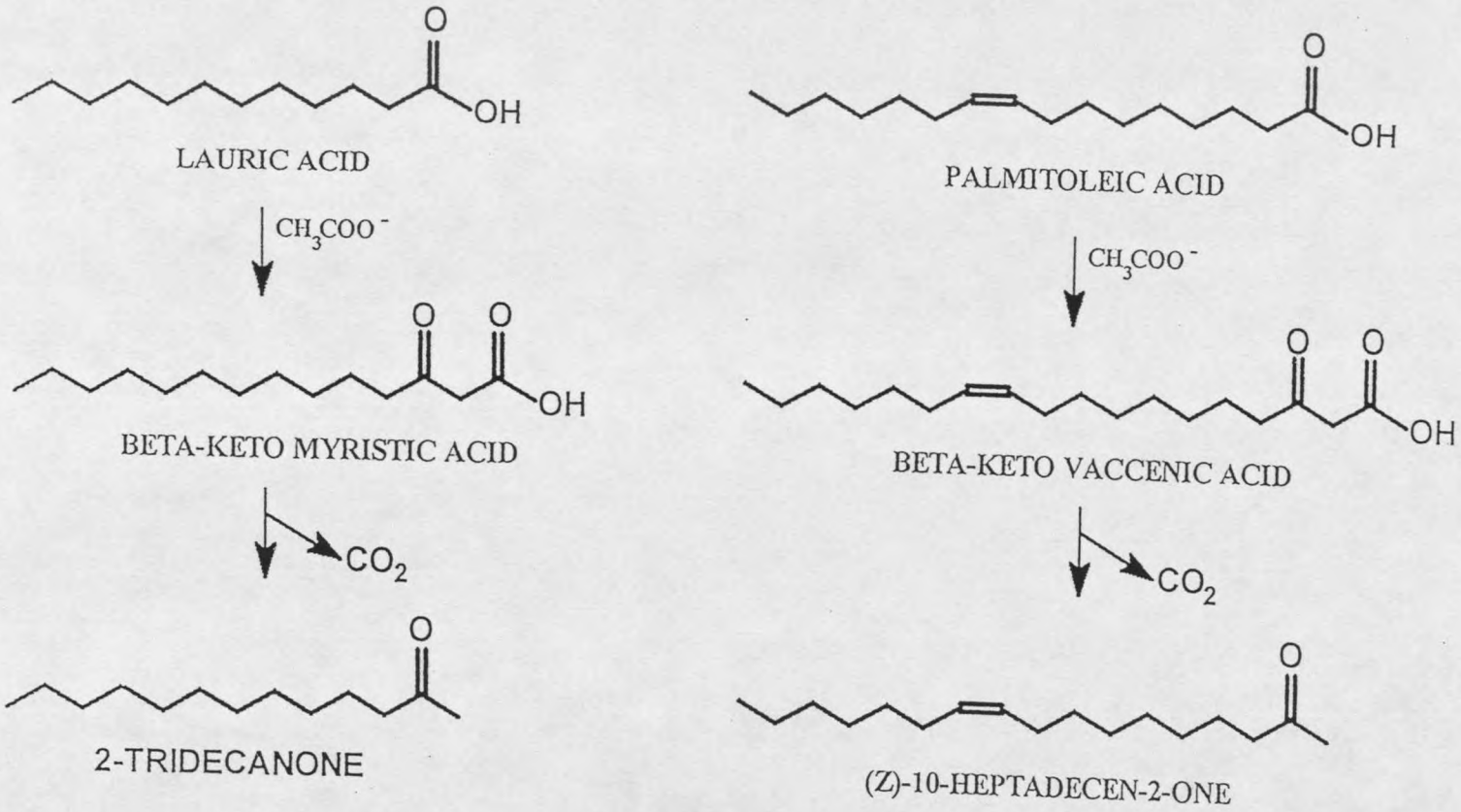


Figure 6. Biosynthesis of 2-ketones via the proposed chain elongation pathway.

STATEMENT OF THE PROBLEM

During the last thirty years, a great deal of effort has been devoted to chemically characterizing numerous insect pheromones. This basic research has been driven by the potential for using these compounds in insect control, and pheromones have proven to be useful in integrated pest management systems. Another strong control potential would be to develop methods of interrupting the normal biosynthesis of these essential chemicals in a way that would provide a unique method of insect control. To this end however, relatively little is known about insect pheromone biosynthesis (Prestwich and Blomquist, 1987; Tumlinson, 1988a; Tumlinson, 1988b).

Just eight years ago, Bartelt, et al.(1984) first demonstrated that aggregation behavior in Drosophila was regulated at least in part by chemicals produced by the flies. Since that first paper, the aggregation pheromones of over thirty species of Drosophila have been identified and characterized. These results are an exciting addition to the understanding of chemical ecology, evolution, and molecular systematics of the species studied.

While much is known about the presence of aggregation pheromones in Drosophila, little is known about Drosophila aggregation pheromone biosynthesis. The analysis of pheromone biology in Drosophila is in its infancy, but recent investigations are providing critical information about the molecular nature of the signalling systems and how they have evolved with speciation. While the studies on the biosynthesis of pheromones are just beginning, the field promises to be one of the most exciting areas of research for the future from both the purely scientific and practical standpoints. Knowledge of the biochemistry and enzyme systems involved in pheromone biosynthesis has been cited as a critical need for many

years. Understanding Drosophila chemical ecology at the molecular level is a major step toward fulfilling this need.

Based on the structural similarities between a number of insect pheromones and the fatty acyl moieties in the pheromone producing glands, a link between fatty acid synthesis and pheromone production was proposed. The 2-ketones produced by D. buzzatii have an odd number of carbons, suggesting that they may be formed via a decarboxylation reaction similar to that used for the biosynthesis of hydrocarbons in insects (Vaz et al., 1988; Jurenka, et al., 1989). Beta-keto acids are proposed intermediates in the pathways involving both fatty acid elongation and chain shortening. We propose that 2-ketones are formed by a branching of the pathways that produce beta-keto-acids to the appropriate 2-ketone by loss of the carboxyl group as CO_2.

Failure to reduce the beta-carbonyl group during an elongation reaction of fatty acids allows for the proposal of an elongation pathway. Likewise, although the exact mechanism of chain shortening in insects is not known, there is a loss of two carbons which suggests a type of beta-oxidation. A presumed intermediate in this chain shortening would be a beta-keto acid which could decarboxylate to produce the 2-ketone.

While the proposed elongation and chain shortening pathways share the beta-keto acid intermediate, the substrates for the production of this intermediate are different. The use of specific ^{13}C- and ^{2}H-labeled fatty acyl-CoA esters allows for detailed examination of the proposed pathways of 2-ketone biosynthesis in Drosophila buzzatii.

EXPERIMENTAL

Insects

Drosophila buzzatii (strain 15081-1291.0, originally collected from Cochabamba, Bolivia) from the Drosophila Species Resource Center (Bowling Green, Ohio) were reared in 1 liter glass containers on yeasted Instant Drosophila Medium 4-24 (Carolina Biological Supply) at ambient laboratory temperatures (20-23°C using a 16-8 hour light-dark cycle. At less than eight hours post-eclosion, flies were separated by sex under CO_2 anesthesia, and ca. 20 flies were put into a rearing vial (10X3 cm ID) until the specified age.

Extraction, Purification, and Identification of 2-Ketones

Eighteen hundred mature D. buzzatii males (5 days old) were extracted for 24 hours at room temperature in three ml of hexane. The extracted flies were washed four times with three ml of hexane and the hexane extracts combined and reduced to three ml under nitrogen. The concentrated hexane extract was then chromatographed on a 2.5 X 18 cm column filled with silicic acid. A five-fraction stepwise elution solvent system consisted of (a) 100 ml of hexane; (b) 100 ml of 5% ether in hexane; (c) 100 ml of 10% ether in hexane; (d) 100 ml of 50% ether in hexane; (e) 100 ml of 10% methanol in methylene chloride. Quantitation of the compounds present in each fraction was obtained by gas chromatography (GC) on a Varian 3700 gas chromatograph fitted with a 30 meter DB-1 Megabore column and a flame ionization detector (FID) using nonadecane as an internal standard. The temperature program was initial temperature 100°C, increased at 5°C per minute to 250°C, with a 10 minute hold at 250°C. The injector port was at 270°C and the detector at 280°C. Helium was used as the carrier gas at a rate

of six ml per minute. The 10% ether in hexane fraction contained two peaks which co-chromatographed with 2-tridecanone and (Z)-10-heptadecen-2-one on both a nonpolar 30 meter DB-1 column and a polar 30 meter DB-5 column. Fifty μl (containing ca. 300 ng of 2-tridecanone and ca. 1 ug of (Z)-10-heptadecen-2-one) of the 10% ether in hexane fraction was incubated with 100 μl of methyl disulfide and 50 μl of 5% iodine in ether at 50°C to prepare dimethyl disulfide adducts of the unsaturated compounds (Nichols et al., 1986). After 48 hours, 500 μl of aqueous sodium thiosulfate and 500 μl of hexane were added and the organic layer was removed. The aqueous layer was extracted twice with 500 μl of hexane-methylene chloride (4:1), the organic layers combined, and reduced to 10 μl under nitrogen. One μl of the reduced extract was subjected to gas chromatography-mass spectrometry (GC/MS) on a Varian 3700 GC fitted with a 30 meter DB-5 megabore column and interfaced with a VG/MM-16 mass spectrometer. The retention times and mass spectra of authentic 2-tridecanone and (Z)-10-heptadecen-2-one (on hand from previous work with D. mulleri, Bartelt, et al., 1989) were compared to those of the fly derived compounds.

Age of 2-Ketone Production

To determine the age of maximum 2-ketone production, flies (<2 hours old) were separated by sex under CO_2 anesthesia, and the males placed in rearing vials until the specified age. To obtain the ejaculatory bulbs, males were placed in the freezer for 10 minutes to facilitate handling, then fastened ventral side up on a corkboard with a dissecting pin through the thorax. The abdomens were removed by cutting with a scalpel and transferred to a drop (ca. 50 μl) of

Drosophila Ringers (Ephrussi and Beadle, 1936). Two dissecting pins were used to gently tease the ejaculatory bulb away from the surrounding tissues. The ejaculatory bulbs (five sets of five bulbs each) were transferred to a vial containing 1 μg of nonadecane as an internal standard in 100 μl of hexane and extracted for one hour at room temperature. After one hour, the extract was concentrated to 10 μl under nitrogen and one μl of the concentrated extract was analyzed by gas chromatography (GC). GC analyses were carried out using a Varian 3700 gas chromatograph fitted with a 30 meter Megabore DB-1 capillary column (J and W Scientific, Folsom, CA) and FID. The temperature program was initial temperature 100°C, increased at 5°C/minute to a final temperature of 250°C. The injector port was operated at 260°C and the detector at 270°C. Helium was used as the carrier gas at a rate of six ml per minute.

Total Lipid Analysis

To determine the change in fatty acyl moieties in the ejaculatory bulb, males were aged and the ejaculatory bulbs removed as described above. The bulbs (five sets of five bulbs each) were transferred to 200 μl of hexane-isopropanol (3:2, Hara and Radin, 1978) containing 2500 ng of nonadecane as an internal standard. After 1 hour at room temperature, the extract was evaporated to apparent dryness under nitrogen and the extracted lipids were transesterified by the addition of 50μl of 0.5M KOH in methanol (Litchfield, 1972). Using this mild alkaline methanolysis procedure, the glycerolipids react to form methyl esters while the free fatty acids and 2-ketones do not react. The methylation procedure was necessary to convert the fatty acyl groups in the glycerolipids to volatile

methyl esters to prevent them from being held up on the GC column. After 30 minutes at room temperature, 100 μl of 1M HCl and 200 μl hexane-methylene chloride (4:1) were added. The organic layer was removed and the aqueous phase was extracted twice with 200 μl of hexane-methylene chloride. The combined organic layers were removed and reduced to 25 μl prior to GC analysis. One μl of the concentrated extract was analyzed by GC on a Varian 3700 GC fitted with a 50 meter HP-1 capillary column and FID programmed from 100°C to 150°C at 5°C/min, after a 1 minute hold at 150°C, the temperature was increased at 2°C/min to a final temperature of 300°C. Retention times were compared to standards. Mass spectra were obtained on a VG/MM-16 instrument or on an HP 5971 series MSD, both operating at an electron energy of 70 eV. The double bond positions and configurations were determined by GC/MS of the dimethyl disulfide adducts as described above (Identification of 2-ketones section).

To obtain lipid classes, the ejaculatory bulbs were removed from cold anesthetized males and placed in a conical vial containing 150 μl of chloroform-methanol (2:1) and 1 μg of heptadecanoic acid methyl ester as a quantitative internal standard. After one hour at room temperature, the extract was taken to apparent dryness under nitrogen and brought to 100 μl with chloroform. The various lipid fractions were isolated by silicic acid chromatography on columns prepared from disposable Pasteur pipets plugged with glass wool. The columns were pre-washed with five ml of each of the eluting solvents. The lipid extracts were transferred to the head of the column in 100 μl of chloroform. Once loaded, five ml of chloroform was used to elute the neutral lipids. The glycolipids were then eluted with five ml of acetone, and the phospholipids eluted with five ml of

methanol (Chrisite, 1982). All of the lipid fractions were then transesterified as described above.

To obtain body segments, males were placed in the freezer for ten minutes to facilitate handling, then placed ventral side up on a corkboard. The head, thorax, and abdomen were separated by cutting with a scalpel. Each body segment was transferred to a separate vial containing 200 μl of hexane-isopropanol and extracted and derivitized as above.

Abdominal cuticles and internal tissues were obtained by placing cold anesthetized males ventral side up on a corkboard with a dissecting pin through the thorax. The abdomen was removed with a scalpel and placed in 20 μl of Drosophila Ringers. Under 45X magnification, two dissecting pins were used to remove the internal tissues from the cuticle. Dissecting pins were used to separate the internal tissues into fat body, G.I. tract, testes, accessory glands, and ejaculatory bulb. Each tissue type was transferred to a separate vial containing 200 μl of hexane-isopropanol and extracted and derivitzed as described above.

Site of Pheromone Biosynthesis

To determine the site of pheromone biosynthesis, the heads, thoraces, and abdomens of 4-5 day old males were transferred to vials containing 50 μl of incubation buffer and incubated for 16 hours at 22-24°C. The body segments and incubation media were extracted with 200 μl of hexane-isopropanol containing 1 μg of nonadecane and then transesterified and analyzed as above. The amounts of 2-ketones present were compared to the amounts present in body segments that were extracted and derivitized immediately after removal.

Tissue Experiments

Once the abdomen was identified as the site of 2-ketone biosynthesis, the abdomen was further dissected into different tissue types. Abdomens obtained as above were transferred to 50 μl of Drosophila Ringers and two dissecting pins were used to separate the tissues into cuticle, fat body, gastrointestinal tract, ejaculatory bulb, testes, and accessory glands. Each tissue type (five sets of five each) was transferred to a separate vial containing 50 μl of incubation buffer (0.2mM Coenzyme A, 7 mM $MgCl_2$, 1 mM ATP, 1 mM dithiothreitol, 0.4 mM NADPH, 2 mM ascorbic acid, 0.25 mM sucrose, in 0.1 M potassium phosphate buffer at pH 6.8) and incubated for 16 hours at room temperature. The tissues and incubation media were extracted, derivitized and analyzed as above and the amount of 2-ketones present compared to the amount present in tissues that were extracted and derivitized immediately after removal.

Subcellular Fractions

Once the ejaculatory bulb was identified as the site of 2-ketone biosynthesis, the subcellular fractions from the ejaculatory bulbs were tested for 2-ketone production. The ejaculatory bulbs from 50 male flies are removed as described above, added to 500μl of 4°C incubation buffer, and homogenized on ice in a Duall glass homogenizer. The homogenate is centrifuged at 1,000g for 5 minutes (cell debris), 10,000g for 20 minutes (mitochondria), and 240,000g for 30 minutes (microsomes) all at 4°C (Vaz et al, 1988). The pellets are each resuspended in 500μl of incubation buffer and 50 μl of each suspension (five male equivalents) were used for each assay.

[1-^{14}C]-Acetate Experiments

Sodium [1-^{14}C]-acetate (50 mCi/mmole) was obtained from Research Products International, Mount Prospect, IL. A stock solution of 100 μCi in 100 μl of water was prepared for addition to in vitro incubations.

Body segments, tissues, and subcellular fractions were obtained as described above and incubated in 50 μl of incubation buffer containing 0.2 μl (ca. 440,000 counts per minute (cpm)) of sodium [1-^{14}]-acetate. Unless indicated otherwise, all incubations were for 16 hours at pH 6.8 and 22-24°C in a shaking water bath. Incubation media were extracted and derivitized as above prior to radio-GC analysis. Radio-GC analysis used a Packard 437A GC fitted with a 30 meter DB-5 Megabore column and a flame ionization detector. The temperature program was initial temperature 100°C increased at 5°C/min to 150°C, after a one minute hold at 150°C, the temperature was increased at 3°C to a final temperature of 250°C. Helium was used as the carrier gas at a rate of 5.4 ml/min. The column effluent was split (1:100) between the FID and a Packard 894 gas proportional counter, providing both mass and radioisotope quantitation of each sample.

A control experiment was done to verify that the sodium [1-^{14}C]-acetate stock solution was not contaminated with other radioactive compounds that might appear in the analysis. A sample of 0.5 μCi of the sodium [1-^{14}C]-acetate stock solution was extracted with 3:2 hexane-isopropanol and evaporated to apparent dryness under nitrogen. Alkaline methanolysis was performed as described above, and the sample analyzed by radio-GC. Less than 70 cpm were observed for the entire run by radio-GC (detection limit: 60 cpm).

Optimization of Incubation Conditions

To determine the pH optimum for in vitro 2-ketone biosynthesis, ejaculatory bulbs and subcellular fractions from 4-5 day old males were incubated for 16 hours in 50 μl of incubation buffer of varying pH containing 0.2 μl of [1-^{14}C]-acetate, extracted and derivitized as above prior to radio-GC analysis.

In addition to pH experiments, the ejaculatory bulbs and subcellular fractions were also tested using commercial tissue incubation media instead of the potassium phosphate buffer system. Ejaculatory bulbs and subcellular fractions from 4-6 day old males were incubated for 16 hours in 50 μl of Grace's Insect Media (Gibco, Grand Island, New York), Schneider's Drosophila Media (Gibco), or potassium phosphate buffer containing 0.2 μl of [1-^{14}C]-acetate. The incubation media were extracted, derivitized, and analyzed as above.

To determine the optimal incubation time for in vitro 2-ketone biosynthesis, ejaculatory bulbs and subcellular fraction from 4-5 day old males were incubated for varying lengths of time in pH 6.8 incubation buffer containing 0.2μl of [1-^{14}C]-acetate, extracted and derivitized as above prior to radio-GC analysis.

To determine the age of maximum incorporation of [1-^{14}C]-acetate incorporation, flies were separated by sex within two hours after eclosion and placed in rearing vials until the specified age. Body segments, tissues, and subcellular fractions were obtained, incubated at pH 6.8 for 16 hours, extracted, and derivitized as above prior to radio-GC analysis.

To determine whether the 2-ketones were being synthesized via a FAS or chain elongation system, the incorporation of [1-^{14}C]-acetate into the 2-ketones in the presence and absence of avidin was determined. Avidin is a potent inhibitor

of acetyl-CoA carboxylase which prevents the formation of malonyl-CoA from acetate (Goodridge, 1985).

Stable Isotope Experiments

[^{2}H]-Labeled Compounds

[$^2H_{23}$]-labeled dodecanoic acid and [$^2H_{27}$]-labeled tetradecanoic acid were obtained from Matreya, Inc., Pleasant Gap, PA. Coenzyme A was purchased from Sigma Chemicals. The [^{2}H]-labeled dodecanoic and tetradecanoic coenzyme A thioesters were synthesized by the method of Kawaguchi et al. (1981) which is summarized below. A solution of the fatty acid (5 umoles) in 100 μl of freshly distilled tetrahydrofuran was added to a solution of 6 umoles carboxyldiimidazole (Aldrich) in 100 μl of tetrahydrofuran. After one hour at room temperature, the solvent was evaporated under nitrogen and the residue was dissolved in 200 μl of tetrahydrofuran-water (2:1). This solution was allowed to react with 5 umoles of Coenzyme A which had been dissolved in 500 μl of tetrahydrofuran-water (2:1). The pH of the reaction mixture was maintained at 7.0-7.5 by the addition of 1 N NaOH. The reaction was carried out at room temperature under a nitrogen atmosphere for 4 hours. The tetrahydrofuran was then evaporated under nitrogen and the residual aqueous solution was acidified to pH 3-4 by adding small amounts of Dowex 50 (H^+). The Dowex 50 (H^+) was removed by filtration, and the filtrate was extracted three times with 500 μl of ether to remove any unreacted fatty acid. The thioester content was also measured by GC quantitation of the amount of fatty acid methyl ester formed upon reaction with methanolic potassium hydroxide.

[^{13}C]-Labeled Compounds

The [^{13}C]-labeled fatty acids were synthesized as described by Roelofs and Wolf, 1988. [1,2-^{13}C]-(Z)-9-Palmitoleic acid was synthesized by stepwise addition of [^{13}C]-KCN , as follows. (Z)-7-tetradecenyl bromide was prepared from 100 mg (471 μmoles) of (Z)-7-tetradecenyl alcohol by the addition of triphenylphosphine dibromide (400 mg, 471 μmoles) in 200 ml of methylene chloride. After 30 minutes at room temperature, the reaction mixture was taken to apparent dryness under nitrogen and 100 ml of hexane was added. The hexane extract was filtered through a Kimwipe wad in a Pasteur pipet and reduced to 2 ml under nitrogen. The reduced hexane extract was added to a Florisil column in a Pasteur pipette and the bromide was eluted with 10 ml of hexane. [1-^{13}C]-(Z)-8-pentadecenonitrile was prepared by addition of 471 μmole (31 mg) of [^{13}C]-KCN to the (Z)-7-tetradecenyl bromide in 25 ml of freshly distilled dimethyl sulfoxide in a 500 ml round bottom flask. After one hour at 25°C, 100 ml of water was added and the mixture was extracted three times with hexane (25 ml each). The hexane extract was purified by Florisil chromatography in a Pasteur pipet. The column was eluted with 10 ml of hexane to remove any unreacted bromide, then with 10 ml of methylene chloride to elute the nitrile. The nitrile was hydrolyzed to the corresponding carboxylic acid by reaction with 1 gram of sodium hydroxide in 100 ml of ethanol and 10 ml of distilled water. After 16 hours at 100°C, 100 ml of distilled water was added and the mixture was extracted three times with 100 ml each of hexane to remove any unreacted nitrile. Five ml of sulfuric acid in 100 ml of distilled water was added and the carboxylic acid was extracted with three 100 ml portions of hexane. The combined hexane extracts were evaporated under

reduced pressure to yield the carboxylic acid as a pale yellow oil. To the neat acid was added 50 ml of Red-Al (sodium bis(2-methoxyethoxy)aluminum hydride in toluene, Aldrich Chemical). Excess Red-Al was destroyed by adding 2M sodium hydroxide until the mild fizzing ceased, and the 1-[^{13}C]-(Z)-8-pentadecenol was extracted three times with 100 ml of hexane. The hexane solution was washed twice with 50 ml of distilled water and evaporated under reduced pressure. This procedure resulted in a fatty alcohol one carbon longer than the starting alcohol and containing one [^{13}C]-atom. By repeating this procedure without the reduction of the carboxylic acid, [1,2,-^{13}C]-palmitoleic acid was produced. Likewise, 3 rounds of elongation with K^{13}CN followed by one elongation with KCN resulted in the synthesis of [2,3,4,-^{13}C]-vaccenic acid. The purity of the [^{13}C]- labeled fatty acids was determined by GC and GC/MS of the methyl esters formed by acid methanolysis of the free fatty acids (Christie, 1982). The Coenzyme A thioesters of the[^{13}C]- labeled fatty acids were synthesized as described above and their purity determined by GC of the methyl esters produced by alkaline methanolysis.

To study the biosynthesis of 2-tridecanone, the microsomal fraction from the ejaculatory bulbs of 4-6 day old D. buzzatii was incubated with 1 nmole of [$^{2}H_{23}$]-labeled dodecanoyl-CoA (containing ca. 250 ng of the labeled acid) or with 1 nmole of [$^{2}H_{27}$]-labeled tetradecanoyl-CoA (containing ca. 250 ng of the labeled acid) at pH 6.8. After 16 hours at 20-23°C, the microsomal incubation media were extracted with 200 μl of hexane-isopropanol (3:2) containing 100 ng of heptadecanoic acid methyl ester as an internal standard. The extract was taken to apparent dryness under nitrogen and reacted with 50 μl of 0.5M KOH in methanol. After 30 minutes at room temperature, 100 μl of 1M HCl was added

and the mixture was extracted with three 100 μl portions of hexane-methylene chloride (4:1). The combined extracts were reduced under nitrogen to ten μl and one μl of the reduced extract was used for GC/MS analysis.

Likewise, to study the biosynthesis of (Z)-10-heptadecen-2-one, the microsomal fraction from 4-6 day old male ejaculatory bulbs was incubated with 250 ng of [1,2-^{13}C]-(Z)-9-hexadecenoyl-CoA or 250 ng of [2,3,4-^{13}C]-(Z)-11-octadecenoyl-CoA as described above.

GC\MS Analysis

GC/MS analysis of the incorporation of heavy atom labeled fatty acyl-Coenzyme A esters utilized a Hewlett Packard 5890 series II gas chromatograph fitted with a 12 meter HP-1 capillary column (Hewlett Packard) and a Hewlett Packard 5971 series mass selective detector. The extracts were analyzed in both the total ion mode and the selective ion monitoring (SIM) mode to evaluate the incorporation of heavy atom labeled precursors into the 2-ketones. Selective ion monitoring is a technique that allows an additional increase in sensitivity, by programming the mass spectrometer to analyze for only a few masses, unlike the complete spectrum mode of operation in which the mass spectrometer sequentially evaluates the abundance of each of a few hundred masses. When a complete spectrum is being obtained, the time spent analyzing for a particular mass is usually less than one percent of the total analysis time, and the sensitivity of the instrument for any one mass is reduced accordingly.

In the SIM mode, the mass spectrometer was programmed to scan initially for the M^+ peaks of only two compounds, the labeled and unlabeled isomers of 2-

tridecanone (the first compounds of interest to emerge from the column). In microsomes incubated with [$^2H_{23}$]-labeled lauroyl-CoA, chain elongation would produce [$^2H_{23}$]-labeled 2-tridecanone. The labeled isomer (molecular weight 221) has a mass 23 units higher than that of the unlabeled 2-tridecanone (molecular weight 198). The mass spectrometer was therefore initially programmed to scan for the masses 198 and 221, corresponding to the M^+ values for the two compounds (alternately scanning for mass 198 for 10 msec, then for mass 221 for 10 msec).

The first GC peak was well resolved from the second peak that emerged from the GC column, which was methyl dodecanoate (Figure 23). Just before this second GC peak began to emerge into the source of the mass spectrometer, the program changed to scan for the masses 214 and 237 corresponding, respectively, to the M^+values for the labeled and unlabeled methyl dodecanoate. The program changed in this way just before the emergence of each GC peak to scan for the labeled and unlabeled isomers of 15 compounds in all, which emerged from the GC column in the following order: 2-tridecanone, methyl dodecanoate, free dodecanoic acid, methyl (Z)-9-tetradecenoate, methyl tetradecanoate, free tetradecanoic acid, (Z)-10-heptadecen-2-one, methyl (Z)-9-hexadecenoate, methyl hexadecanoate, free (Z)-9-hexadecenoic acid, free hexadecanoic acid, methyl (Z)-11-octadecenoate, methyl (Z)-9-octadecenoate, methyl octadecanoate, and free octadecenoic acid (the free (Z)-9 and (Z)-11 isomers were not resolved by the GC column).

For microsomes incubated with[$^2H_{23}$]-labeled dodecanoyl-CoA, incorporation into any of the saturated compounds of interest would result in an

isotopomer with 23 additional mass units. Likewise, incorporation into any of the unsaturated compounds of interest would result in an isotopomer with 21 additional mass units. Sequential plotting of the intensity of the M^+ of the most abundant isotopomer (MAI) of each of the fifteen compounds of interest allows a GC trace of the unlabeled native compounds in the microsomes to be generated. Alternatively, scanning for MAI+21 and MAI+23 generates a GC trace allowing detection of mass-labeled compounds resulting from the incorporation of $[^2H_{23}]$-labeled dodecanoyl-CoA. By alternately scanning for MAI and MAI+21 (or MAI+23) every 10msec, it is possible, within a single GC run, to detect the unlabeled native compounds and also to detect any mass-labeled isotopomers of those compounds that might be formed.

To make a meaningful assessment of incorporation of mass-labeled compounds, it is important to compare the MAI and MAI+23 and MAI+21 GC traces for unincubated control microsomes with corresponding traces for microsomes incubated with $[^2H_{23}]$-labeled dodecanoic acid.

Ideally, each microsomal extract would be expected to contain the same overall amounts of the compounds of interest, because 50 ejaculatory bulbs were used to make each extract. In practice, handling losses and individual male variation can cause the overall amounts to vary. The use of methyl heptadecanoate as an internal standard was used to control this variation. The amounts of the MAI, MAI+21, and MAI+23 compounds in the microsomal extract were represented as a proportion of the amount of the MAI of methyl heptadecanoate. Therefore, the MAI, MAI+21, and MAI+23 traces for unincubated control microsomes and for microsomes incubated with a mass-

labeled precursor could be directly compared with one another. Likewise, for the incubations containing [$^2H_{27}$]-myristoyl-CoA, the mass spectrometer was set to monitor for MAI, MAI+25, and MAI+27.

[$^2H_{23}$]-2-tridecanone was synthesized from [$^2H_{23}$]-lauric acid as described by Bartelt et al. (1989). Forty five mg (0.2 mmol) of the deuterated fatty acid in 2 ml of dry ether was cooled to 0°C. Methyllithium in ether (0.8 mmol, 0.6 ml) was added in one portion. The mixture was warmed to room temperature, refluxed for 20 minutes, then cooled to 0°C, after which 1 ml of water was added very slowly. The ether layer was washed with dilute HCl, water, and $NaHCO_3$ solution, then dried over Na_2SO_4. The resulting deutero-2-tridecanone was purified on an open column of silicic acid. The purity was 99+% by capillary GC and by GC/MS. Similarly, for microsomes incubated with [1,2-^{13}C]-palmitoleoyl-CoA or [2,3,4-^{13}C]-vaccenoyl-CoA, the mass spectrometer analyzed for MAI and MAI+2 or MAI+3, respectively.

Acetyl-CoA Carboxylase Inhibition

To differentiate between the chain elongation reaction and fatty acid synthesis, the microsomal fraction from ejaculatory bulbs was incubated with [1-^{14}C]-acetate in the presence and absence of avidin. Avidin is an inhibitor of acetyl-CoA carboxylase. In the presence of avidin, acetyl-CoA cannot be converted into malonyl-CoA, which is required for microsomal chain elongation.

Likewise, the microsomal fraction from ejaculatory bulbs was also incubated with acetyl-CoA or malonyl-CoA, and [1,2-^{13}C]-palmitoleoyl-CoA or [$^2H_{23}$]-lauroyl-CoA in the presence and absence of avidin.

RESULTS AND DISCUSSION

Extraction and Identification of 2-Ketones

The 10% ether in hexane fraction from mature males contained two components which co-chromatographed with 2-tridecanone and (Z)-10-heptatdecen-2-one standards on both polar (DB-5) and nonpolar (DB-1) capillary GC columns. The two compounds were identified based on their mass spectra. By comparing the retention times, mass spectra, and mass spectra of the DMDS adducts of the D. buzzatii 10% ether in hexane extract with known standards, the compounds were identified as 2-tridecanone and (Z)-10-heptadecen-2-one. For 2-tridecanone, the key spectral peaks were at m/z 43 (67%), 58 (100%), and 198 (4%) (Figure 7). For (Z)-10-heptadecen-2-one, the key spectral peaks were at m/z=43 (100%), 58 (27%), and 252 (4%) (Figure 8). (Z)-10-Heptadecen-2-one was further identified on the basis of the GC/MS of the dimethyldisulfide (DMDS) adduct. The DMDS adducts identify the double bond position by causing the adduct to fragment preferentially at the site of the thiomethyl additions (Nichols et al., 1986). In addition, the (Z) and (E) isomers of the DMDS adducts are also resolved on the GC. The key spectral peaks for the DMDS adduct of (Z)-10-heptadecen-2-one were at m/z=346 (18%), 201 (100%), 145 (41%) (Figure 9).

The change in the amount of 2-ketones stored in the ejaculatory bulb with age (Figure 10), shows that 2-ketones were not present in newly eclosed males or in virgin females of any age. However, as the males aged, the amount of 2-ketones stored in ejaculatory bulb increased. (Z)-10-Heptadecen-2-one was first detected in the ejaculatory bulb when males reached three days of age. Likewise,

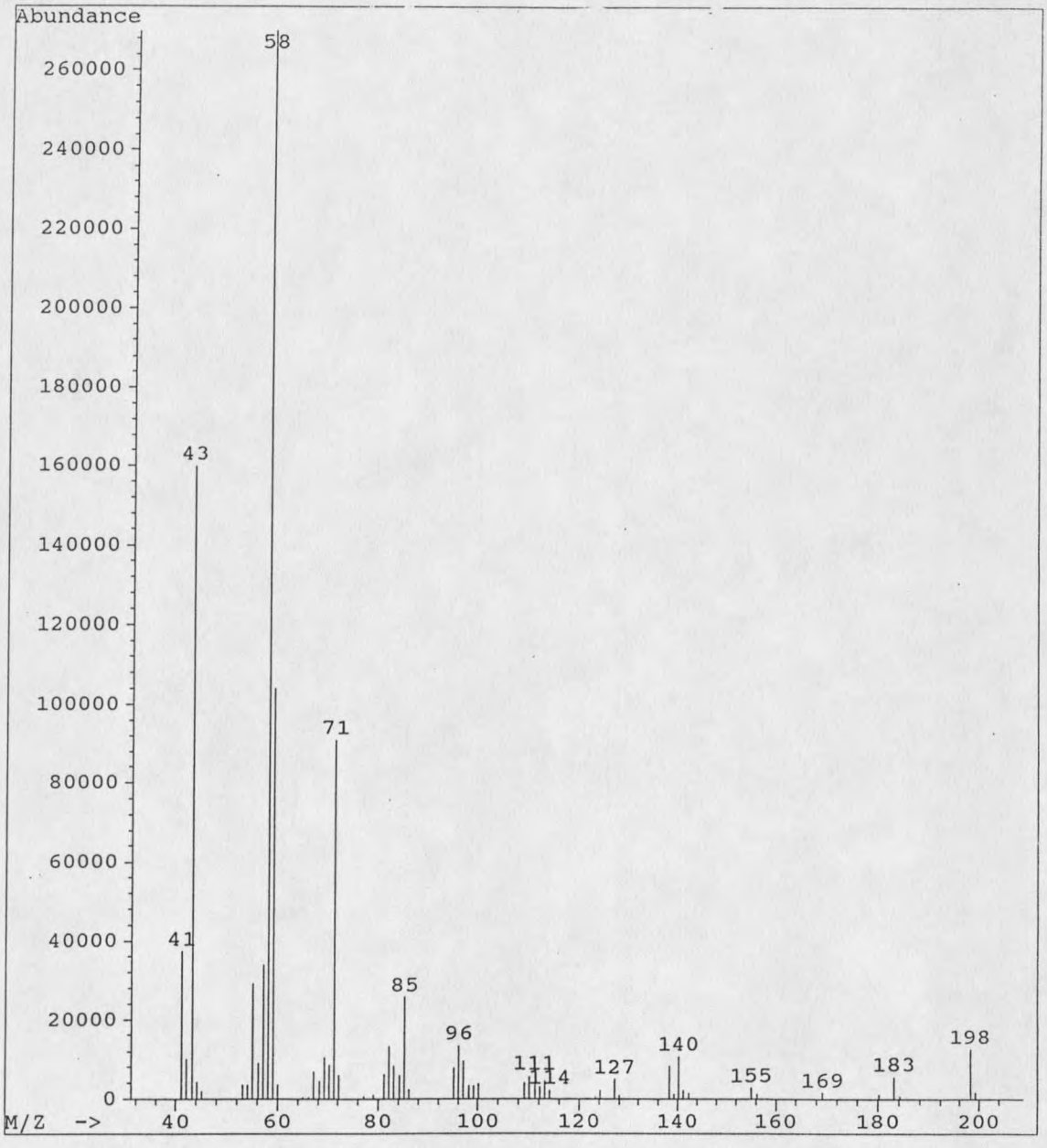


Figure 7. Mass spectrum of 2-tridecanone obtained from D. buzzatii ejaculatory bulbs.

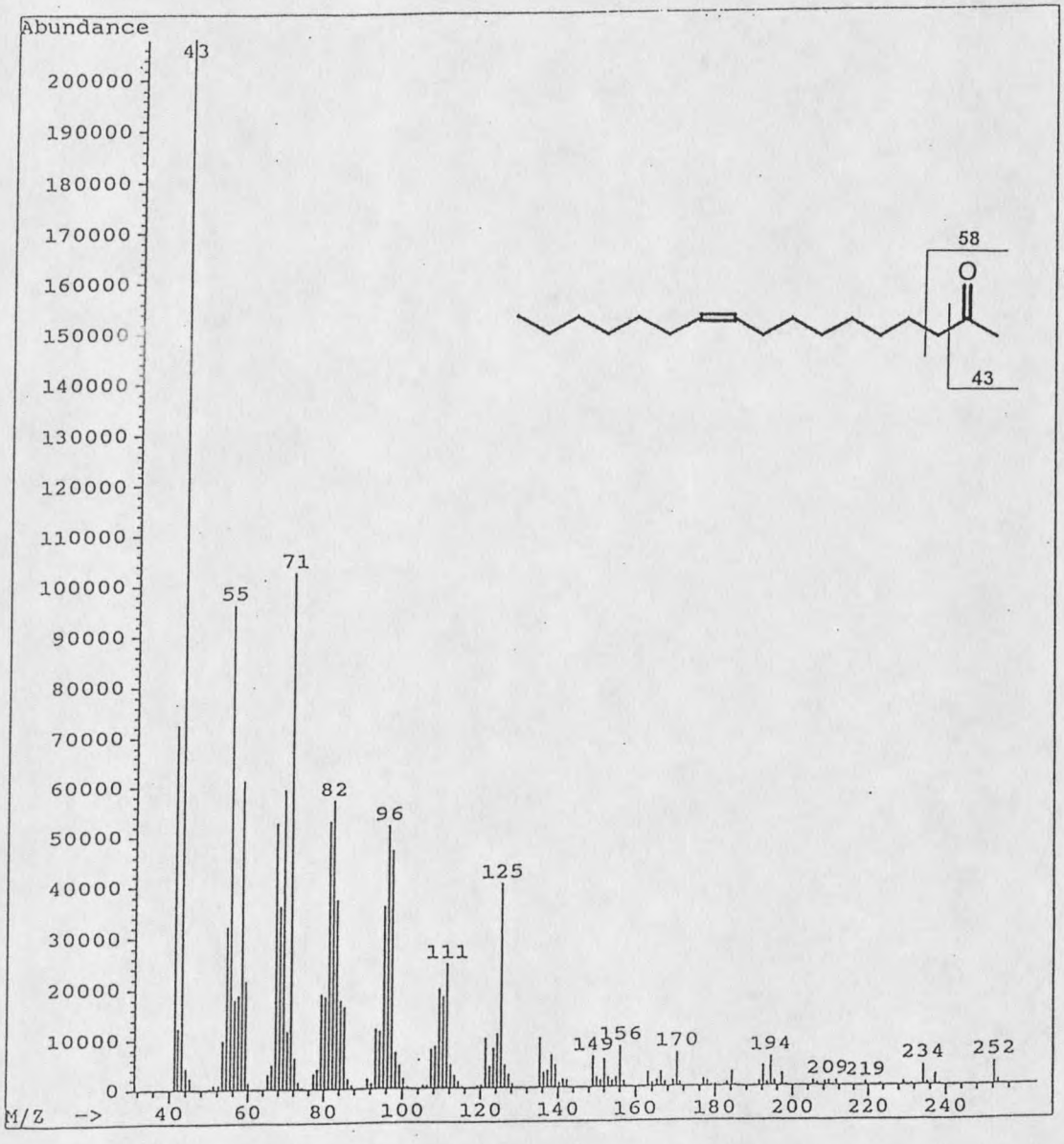


Figure 8. Mass spectrum of (Z)-10-heptadecen-2-one obtained from D. buzzatii ejaculatory bulbs.

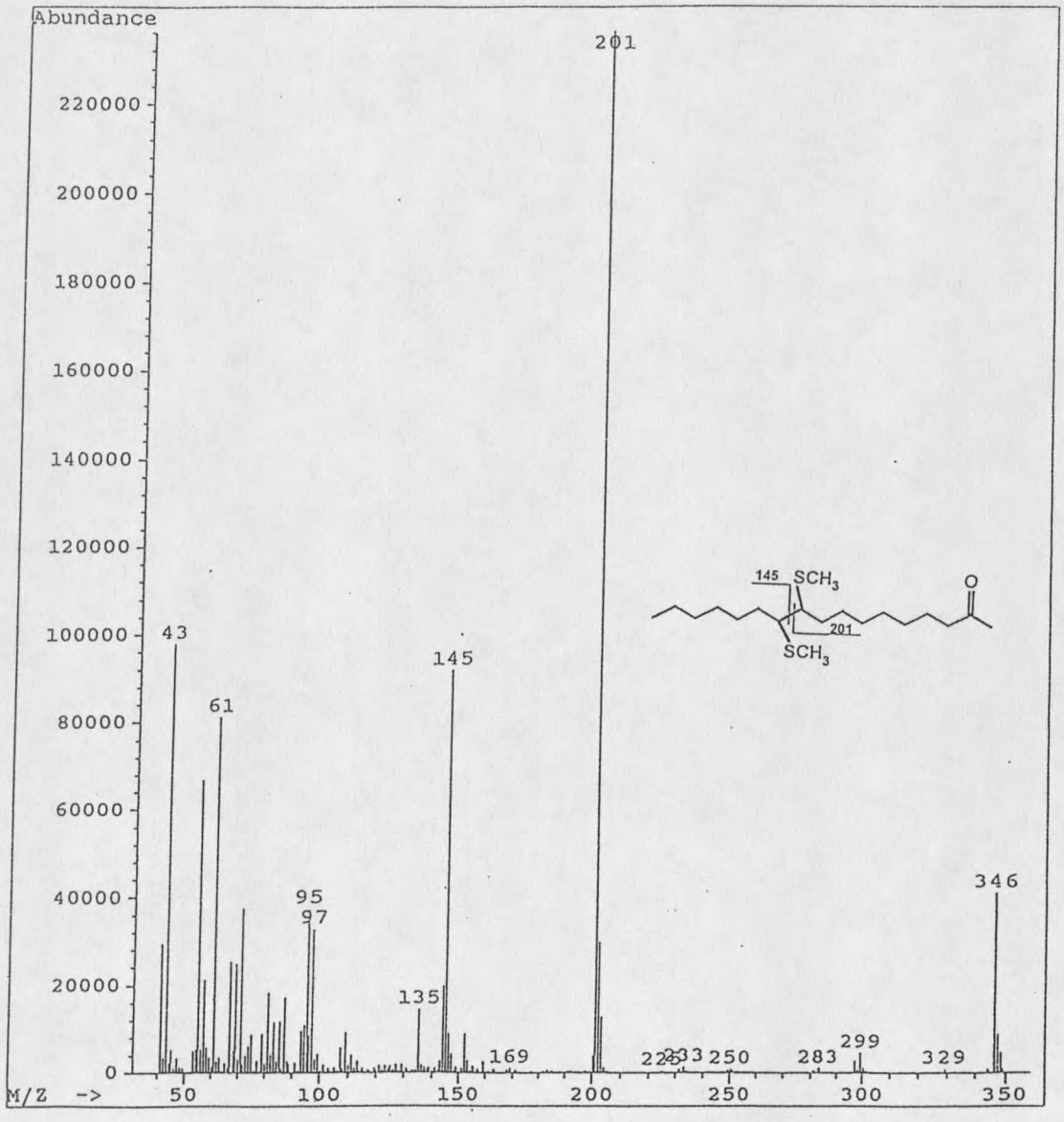


Figure 9. Mass spectrum of the dimethyldisulfide adduct of (Z)-10-heptadecen-2-one from D. buzzatii ejaculatory bulbs.

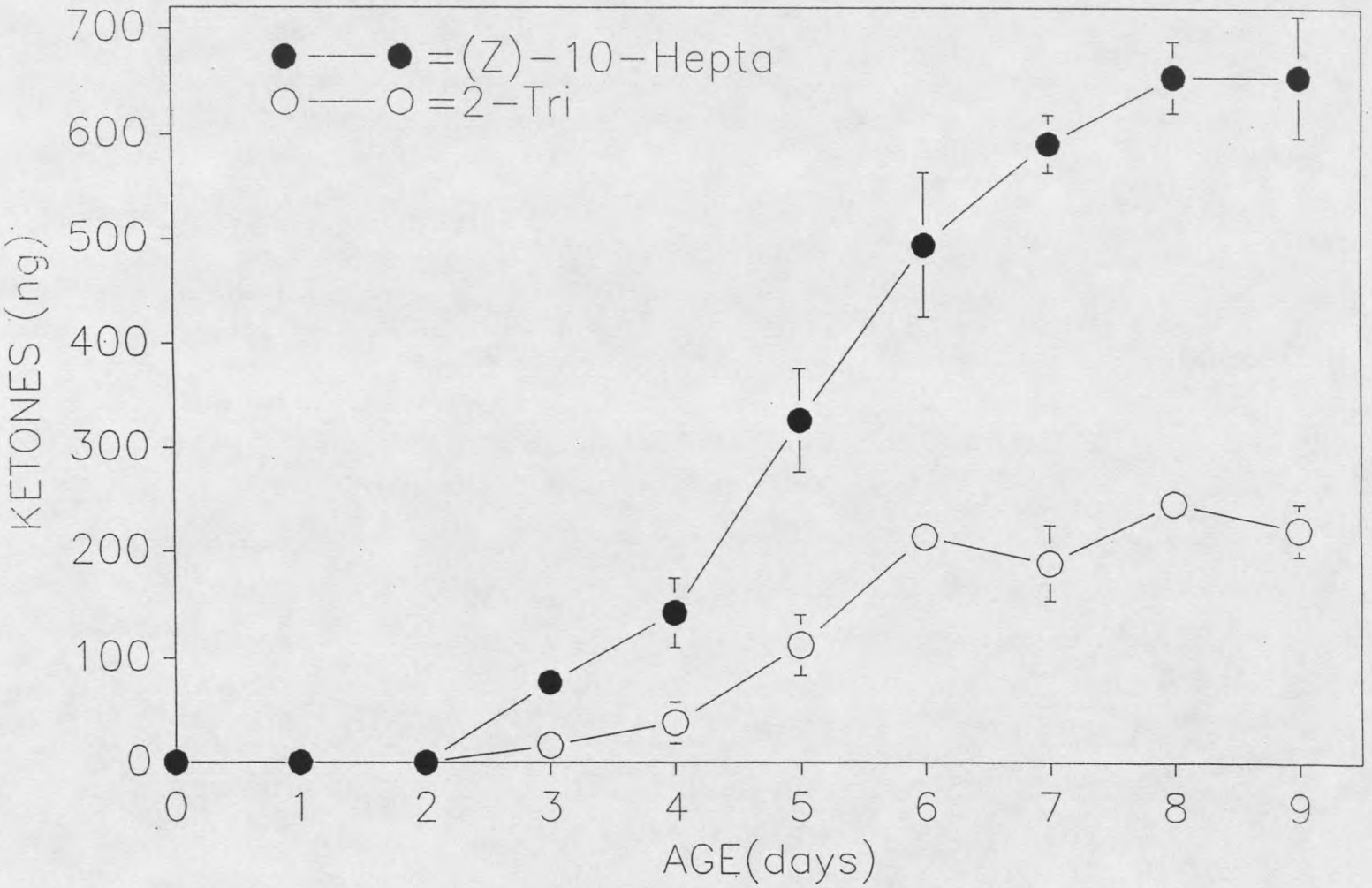


Figure 10. Change in the amounts of 2-ketones stored in the ejaculatory bulb with age. Values are means ± standard deviations per male for 5 sets of 5 ejaculatory bulbs each.

2-tridecanone was first detected when the males reached three days of age. The amounts of the 2-ketones increased most rapidly between four and six days of age before reaching a plateau at 8 days. D. buzzatii males reach sexual maturity at 4-5 days of age (Markow, 1982), coinciding with the beginning of pheromone production.

Site of 2-Ketone Biosynthesis

Body Segment

Although the ejaculatory bulb has been shown to be the site of aggregation pheromone storage in a number of Drosophila (Bartelt et al., 1989; Schaner et al., 1989a, 1989b, 1989c), the site of pheromone biosynthesis remained unknown. Since the site of 2-ketone storage, the ejaculatory bulb, is located in the abdomen, we had expected that the abdomen would produce the 2-ketones. However, it was possible that the pheromones were biosynthesized in another region and then transported to the ejaculatory bulb for storage.The site of pheromone biosynthesis in D. buzzatii was investigated by determining the increase in the amounts of 2-ketones produced during an overnight (16 hour) incubation. The first experiments were designed to determine if the site of biosynthesis could be localized to a particular body segment.The amounts of 2-ketones present in the incubated body segments were compared to the amounts present in body segments that were extracted immediately after removal. Of the three body segments, only the abdomens from mature males were capable of producing the pheromone in vitro (Table 2). The 2-ketones were never detected in the heads or thoraces and were not detected in any of the incubations of these body segments.

Table 2. Amounts of (Z)-10-heptadecen-2-one ((Z)-10-hepta) and 2-tridecanone (2-tri) produced by the body segments of mature male D. buzzatii during a 16 hour incubation at pH 6.8. Values are the mean amount ± standard deviatiom of 2-ketone produced per male for 5 sets of 5 males each.

Body Segment	ng of (Z)-10-hepta	ng of 2-tri
HEAD	0	0
THORAX	0	0
ABDOMEN	132± 27	58± 16

Organs

Once the abdomen was identified as the site of pheromone biosynthesis, we attempted to determine if a particular abdominal tissue or organ could be identified as the site of 2-ketone synthesis. Of the tissues/organs tested, only the ejaculatory bulbs from mature males were capable of producing 2-ketones in vitro (Table 3). Neither the testes nor the accessory glands, which are both closely associated with the ejaculatory bulb, were capable of 2-ketone production. The 2-ketones were not detected in the cuticle, fat body, the G.I. tract, or in the extracts from the incubations of these organs. Therefore, in D. buzzatii, the site of pheromone storage and biosynthesis is the ejaculatory bulb.

Table 3. Amounts of 2-ketones produced by the abdominal tissues from mature male D. buzzatii during a 16 hour incubation at pH 6.8 potassium phosphate buffer. Values are the means ± standard deviations per male for 5 sets of 5 males each.

ABDOMINAL TISSUE	ng of (Z)-10-hepta	ng of 2-tri
Cuticle	0	0
G.I. Tract	0	0
Fat Body	0	0
Testes	0	0
Accessory Glands	0	0
Ejaculatory Bulb	178± 38	93± 21

Optimization of 2-Ketone Production

pH Optimum

Once the ejaculatory bulb was identified as the site of 2-ketone biosynthesis, attempts were made to increase the amounts of the ketones produced during the incubations. The amounts of the 2-ketones produced during a 16 hour incubation varied with pH. The 2-ketones were produced over a narrow range from pH 6.0 to 7.5, being optimal at pH 6.8 in 0.1M potassium phosphate buffer (Figure 11).

Cofactor Requirements

Various cofactors were used in an attempt to increase the amounts of 2-ketones produced during the incubations. Of the cofactors tested, only ATP exhibited an effect on in vitro 2-ketone production (Table 4). ATP increased the amounts of 2-ketones produced, with the greatest increase in production occurring

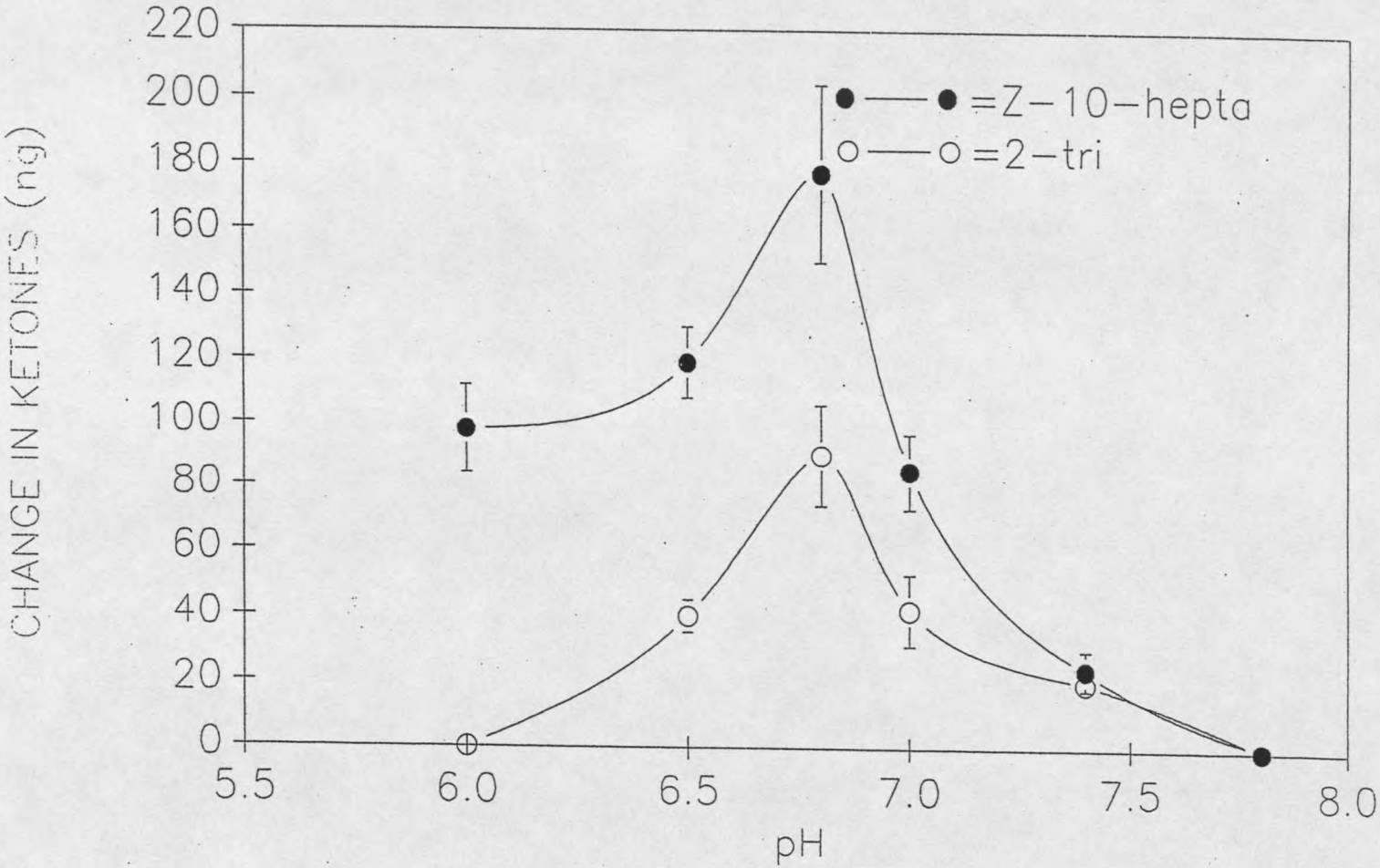


Figure 11. Change in the amounts of 2-ketones produced by ejaculatory bulbs during a 16 hour incubation with pH. Values are means ± standard deviations per ejaculatory bulb for 5 sets of 5 males each.

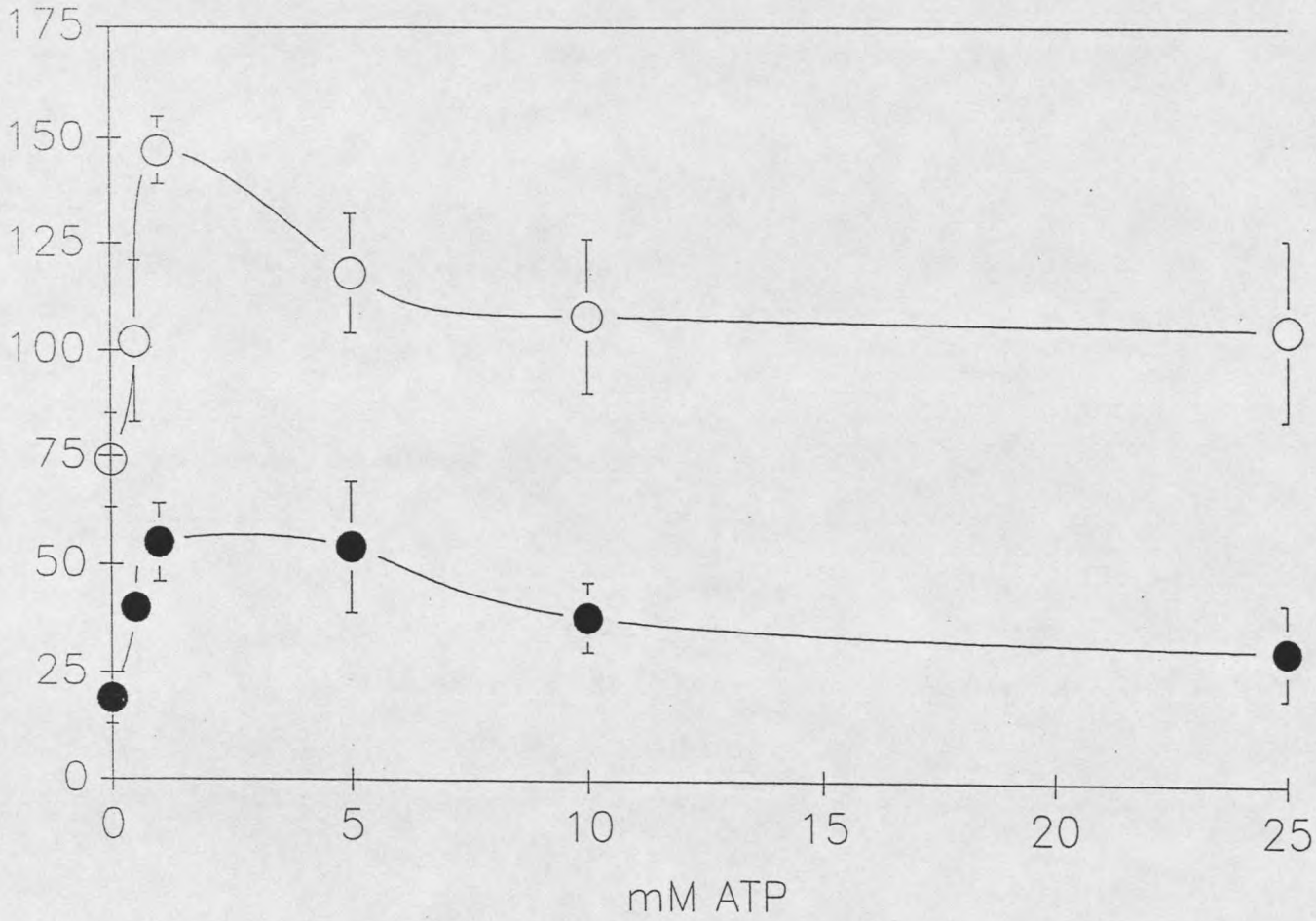


Figure 12. Change in the amounts of 2-ketones produced by ejaculatory bulbs during a 16 hour incubation with ATP concentration. Values are means ± standard deviations per ejaculatory bulb for 5 sets of 5 males each.

at 1mM ATP (Figure 12). The amounts of 2-ketones produced during an incubation in the presence of 1mM ATP were comparable to the amounts produced by live males of the same age during the same time period.

Table 4. Amounts of 2-ketones (ng) produced by the ejaculatory bulbs from mature male D. buzzatii during a 16 hour incubation at pH 6.8 in the presence of various cofactors (1mM). Values are the means ± standard deviations per ejaculatory bulb for 5 sets of 5 bulbs each.

COFACTOR	2-TRI	Z-10-HEPTA
NONE	29±12	74±19
ATP	72±10	147±24
NAD^+	22±6	89±33
NADH	37±14	91±17
NADPH	41±9	86±27
FAD	19±5	67±18

In addition to cofactor experiments, the ejaculatory bulbs were also tested for 2-ketone production when incubated in commercial tissue incubation media. Although the ejaculatory bulbs incorporated radiolabel from [1-^{14}C]-acetate into the 2-ketones in all of the media tested, 2-ketone production was greatest in potassium phosphate buffer containing 1mM ATP (Table 5).

Age of Biosynthesis

Since the amounts of 2-ketones stored in the ejaculatory bulb varied dramatically with age, we expected that the time of maximum pheromone biosynthesis is when the rate of increase in amount of stored pheromone is maximum. The amounts of 2-ketones produced by ejaculatory bulbs from males of different ages during a 16 hour incubation are shown in Figure 13. As

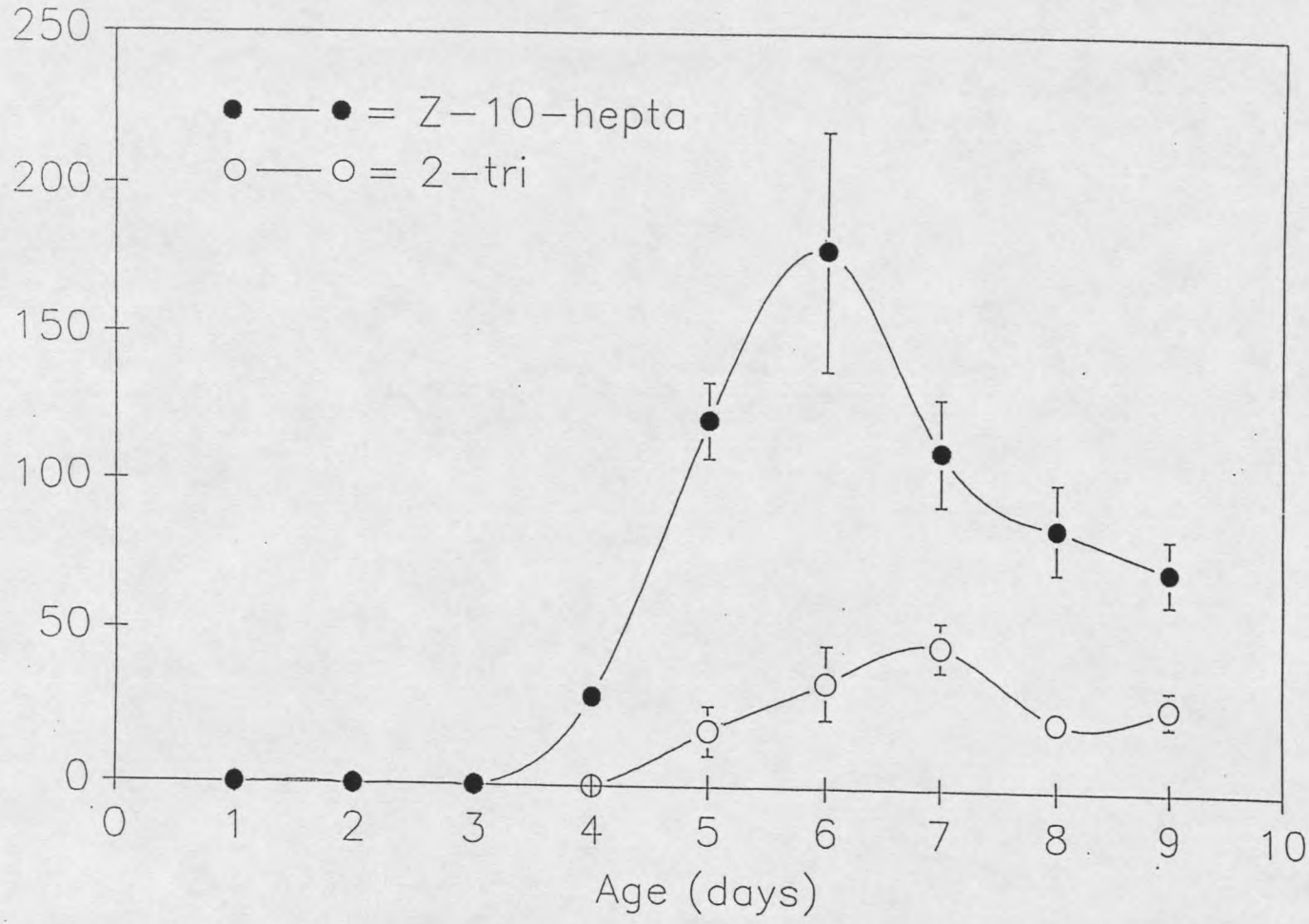


Figure 13. Change in the amounts of 2-ketones produced by ejaculatory bulbs during a 16 hour incubation with age. Values are means ± standard deviations per ejaculatory bulb for 5 sets of 5 males each.

expected, newly eclosed males which did not have any 2-ketones stored in the ejaculatory bulb, did not produce 2-ketones. However, during a two to three day

Table 5. Incorporation of [1-^{14}C]-acetate into 2-ketones in various tissue culture media with and without 1mM ATP. Values are means ± standard deviations for 5 sets of 5 ejaculatory bulbs each.

BUFFER	(Z)-10-HEPTA	2-TRIDECANONE
Potassium Phosphate	819±116	366±88
Potassium Phosphate+ ATP	1472±108	805±117
SCHNEIDER'S	682±143	258±58
SCHNEIDER'S + ATP	1037±219	580±131
GRACE'S	711±107	306±62
GRACE'S + ATP	1024±203	671±107

period during sexual maturation, the 2-ketones were produced in vitro. Between four and six days of age, when the amounts of stored pheromone increase most dramatically, the amounts of 2-ketones produced in vitro also increased dramatically. Also, as the increase in the amount of pheromone components stored in the ejaculatory bulb decreased between 7 and 10 days of age, the amounts of 2-ketones produced during these ages likewise decreased.

[1-^{14}C]-Acetate Studies

Body Segments

The in vitro incorporation of [1-^{14}C]-labeled acetate into 2-ketones and other lipids in the head, thorax, and abdomen of mature male D. buzzatii was determined. Of the three body segments, only the abdomens from mature males were able to incorporate the radiolabeled acetate into the 2-ketones (Table 6).

All of the body segments incorporated the label into extractable lipids. Of the three insect body segments, only the abdomens from mature males were able to incorporate label from [1-^{14}C]-acetate into (Z)-10-heptadecen-2-one, with 1.1% of the recovered label incorporated into the aggregation pheromone. In addition, 0.2% of the recovered label was present in 2-tridecanone. Although both the heads and thoraces incorporated label into lipids, label was not detected in the 2-ketones in these body segments.

Table 6. Incorporation of [1-^{14}C]-acetate into 2-ketones in the three body segments of male D. buzzatii. Incubations were for 16 hours at 23°C in incubation buffer containing ca. 440,000 cpm of acetate. Values are the means per male of 5 sets of 5 segments each.

Body Segment	Total Recovered Label (cpm± SD in thousands)	Label in Z-10-hepta (cpm ± SD)	Label in 2-tri (cpm ± SD)
Head	6.4 ± 0.7	0	0
Thorax	34 ± 3	0	0
Abdomen	46 ± 8	484 ± 21	109 ± 104

Dissection of the abdomen into tissue groups further narrowed down the site of pheromone biosynthesis in D. buzzatii. All of the tissues tested incorporated significant amounts of label into both free and esterified fatty acids. Of the abdominal tissues, only the ejaculatory bulb was capable of pheromone biosynthesis in vitro, with 3.3% of the recovered label (0.4% of the applied label) in the ejaculatory bulb recovered in (Z)-10-heptadecen-2-one, and 1.0% of the recovered label (0.1% of the applied label) present in 2-tridecanone (Table 7). A typical radio-GC chromatogram of ejaculatory bulbs incubated with [1-^{14}C]-acetate is presented in Figure 14. The testes and the accessory glands, which are both

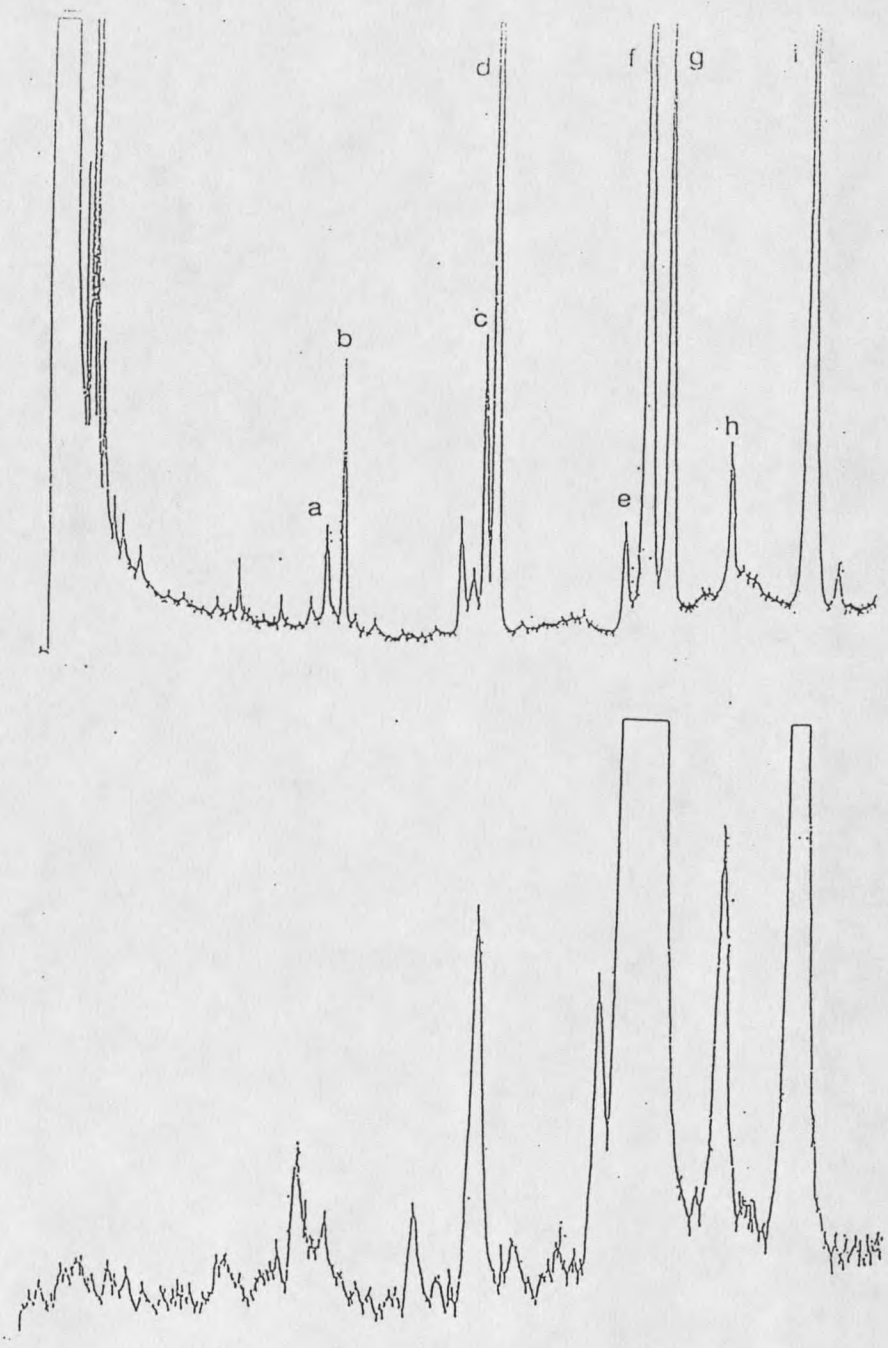


Figure 14. Radio-GC chromatogram showing the radioactive (upper) trace and mass (lower) trace for the lipid extract from ejaculatory bulbs incubated with [1-^{14}C]-acetate. a= 2-tridecanone, b=methyl laurate, c=methyl myristoleate, d=methyl myristate, e=(Z)-10-heptadecen-2-one, f=methyl palmitoleate, g=methyl oleate, h=palmitoleic acid, i=methyl oleate and methyl vaccenate.

closely associated with the ejaculatory bulb, incorporated label into fatty acyl groups, but did not incorporate label into the 2-ketones. The in vitro biosynthesis of (Z)-10-heptadecen-2-one and 2-tridecanone by whole ejaculatory bulbs was optimal at pH 6.8 in incubation buffer (Figure 15). The amounts of 2-ketones produced during a 16 hour incubation were comparable to the amounts produced by live males in the same time period. These data strongly suggest that aggregation pheromone biosynthesis is confined to the ejaculatory bulb.

Table 7. Incorporation of [1-^{14}C]-acetate into 2-ketones by the abdominal tissues of male D. buzzatii. Values are the means per male for 5 sets of 5 males each.

Abdominal Tissue	Total Recovered Label (cpm ± SD in thousands)	Label in Z-10-hepta (cpm ± SD)	Label in 2-tri (cpm ±SD)
Cuticle	57 ± 3	0	0
G. I. Tract	54 ± 2	0	0
Fat Body	46 ± 5	0	0
Testes	19 ± 4	0	0
Accessory Gland	11 ± 3	0	0
Ejaculatory Bulb	54 ± 1	1764 ± 299	536 ± 77

In addition to the 2-ketones, the ejaculatory bulbs also incorporated [1-^{14}C]-acetate into other lipids. The majority of the label was incorporated into esterified fatty acyl groups. The greatest amount of label was incorporated into esterified palmitate, palmitoleate, and oleate (Table 8). Smaller amounts of label were incorporated into esterified laurate, myristate, myristoleate, and stearate.

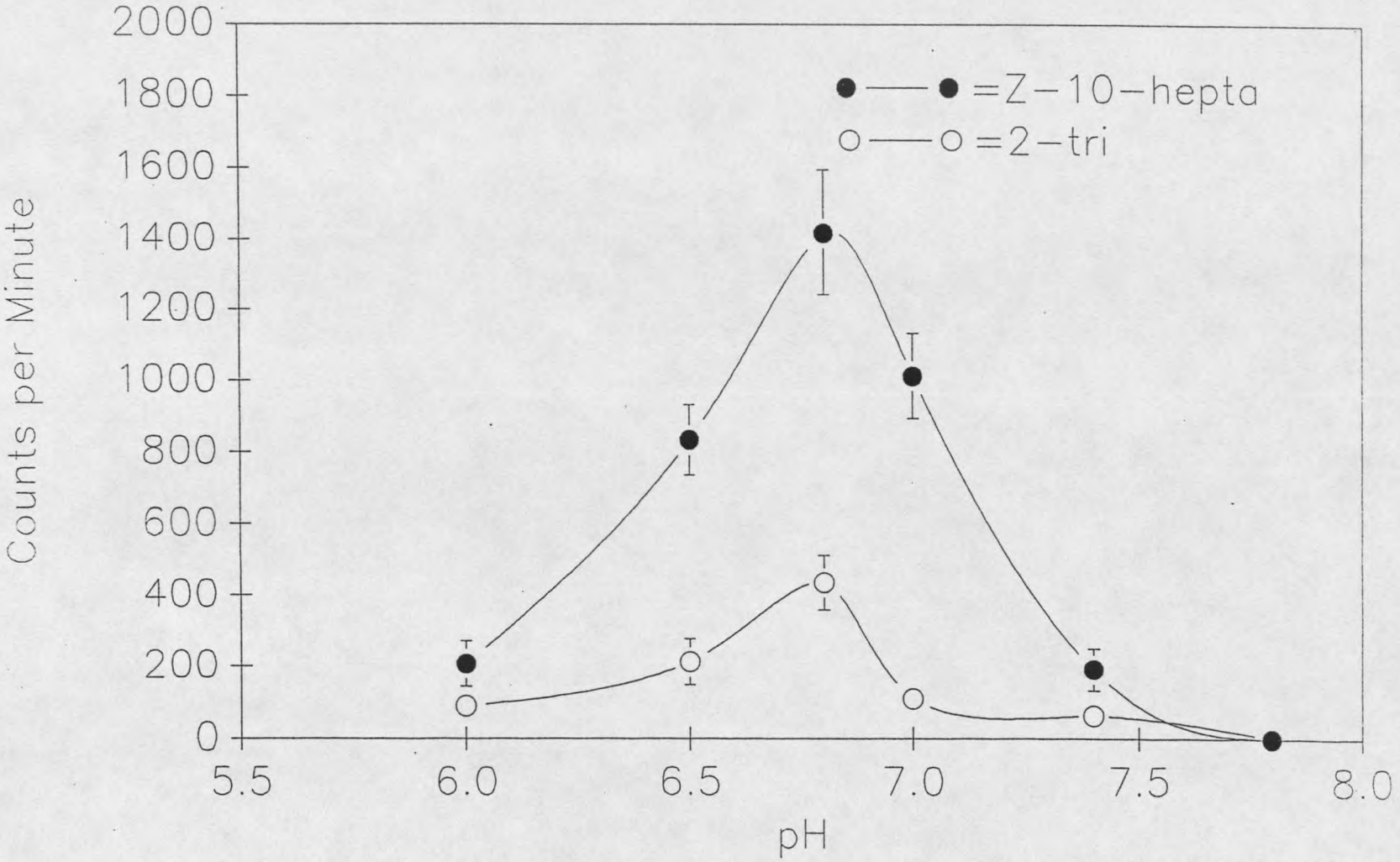


Figure 15. Change in the incorporation of [1-^{14}C]-acetate into 2-ketones by ejaculatory bulbs during a 16 hour incubation with pH. Values are means ± standard deviations for 5 sets of 5 ejaculatory bulbs each.

Table 8. Incorporation of [1-^{14}C]-acetate into 2-ketones and other lipids in the ejaculatory bulb of mature male D. buzzatii. Values are means ± standard deviations per male for 5 sets of 5 ejaculatory bulbs each.

COMPOUND	CPM
(Z)-10-hepta	1747±319
2-tri	856±226
methyl laurate	357±184
methyl myristate	767±177
methy palmitate	11,107±835
free palmitic acid	673±199
methyl palmitoleate	8,751±1017
free palmitoleic acid	730±120
methyl oleate	6,132±848
methyl stearate	347±93
free oleic acid	438±118

The incorporation of [1-^{14}C]-acetate into 2-ketones in the ejaculatory bulb with age (Table 9) parallelled the increase in the amount of the 2-ketones stored in the bulb (Schaner and Jackson, 1992). Although the ejaculatory bulb incorporated significant amounts of label into lipids at all ages tested, pheromone was not detectably labeled until four days post-eclosion. Label was most actively incorporated into pheromone from 5-6 days of age, peaking at 1162 cpm at 6 days. Between seven and nine days, incorporation into the pheromone remained at a nearly constant level of ca. 500 cpm. Likewise, label was most actively incorporated into 2-tridecanone from 5-6 days of age, peaking at 625 cpm at 6 days. Between 8 and 9 days, incorporation remained at a nearly constant level of ca. 250 cpm.

Table 9. Age dependent incorporation of [1-^{14}C]-acetate into 2-ketones by the ejaculatory bulbs from male D. buzzatii. Values are means ± standard deviations for 5 sets of 5 ejaculatory bulbs each.

Age (Days)	Total Recovered Label (cpm ± SD in thousands)	Label in Z-10-hepta (cpm ± SD)	Label in 2-tri (cpm ± SD)
1	44 ± 4	0	0
2	56 ± 4	0	0
3	40 ± 9	0	0
4	41 ± 6	323 ± 104	0
5	41 ± 7	686 ± 97	488 ± 59
6	54 ± 1	1162 ± 188	625 ± 155
7	45 ± 2	594 ± 65	433 ± 128
8	42 ± 2	484 ± 61	278 ± 79
9	47 ± 2	449 ± 68	226 ± 47

These results with radiolabeled acetate agree with the results obtained from incubations without the radiolabeled acetate. In both sets of experiments, only the abdomens from mature males were capable of 2-ketone biosynthesis in vitro. In addition, the only abdominal organ capable of 2-ketone production was the ejaculatory bulb. Taken together, these data strongly suggest that 2-ketone biosynthesis in D. buzzatii is confined to the ejaculatory bulbs of mature males.

Incubation Time

Changes in the in vitro incorporation of [1-^{14}C]-acetate with incubation time were examined with incubations conducted from 0-24 hours. In experiments of short duration (<8 hours), the overall incorporation of label into pheromone and other lipids was low (Table 10). Following a lag period, label incorporation into pheromone reached a maximum at 16 hours. Since no change in the total

label or pheromone label was seen from 16-24 hours of incubation, all subsequent incubations were carried out for 16 hours.

Table 10. Time dependent incorporation of [1-^{14}C]-acetate into 2-ketones by the ejaculatory bulbs from mature male D. buzzatii. Values are means ± standard deviations for 5 sets of 5 ejaculatory bulbs each.

Incubation Time (Hours)	Total Recovered Label (cpm ± SD in thousands)	Label in Z-10-hepta (cpm ± SD)	Label in 2-tri (cpm ± SD)
1	12 ± 1	0	0
2	18 ± 6	0	0
4	27 ± 5	264 ± 48	0
8	40 ± 4	672 ± 69	152 ± 51
12	49 ± 5	796 ± 54	450 ± 22
16	55 ± 10	925 ± 87	509 ± 99
24	53 ± 5	887 ± 96	503 ± 86

As expected, there was a lag period after label first appeared in lipids before label began to appear in the pheromone components. This is consistent with the hypothesis that a labeled precursor pool needs to be established before the pheromone can be produced. Alternatively, the amount of label present in the 2-ketones in the earliest incubations could have been below the detection limits of the instrument (60 cpm). Although label appeared in lipids in the earliest incubation (1 hour), the 2-ketones were not labelled until 4 hours into the incubation. Thereafter, the amount of label incorporated into the 2-ketones increased steadily until 16 hours, with no increase in the amount of label incorporated between 16 and 24 hours.

Subcellular Fractions

[1-^{14}C]-Acetate was incorporated into 2-ketones to some extent in all of the subcellular fractions (Table 11). The microsomal fraction, however, most actively incorporated label into the pheromone components, with 4.9% of the recovered label present in (Z)-10-heptadecen-2-one and 2.4 % present in 2-tridecanone. The remainder of the label was recovered as methyl laurate (1%), methyl myristate (2%), methyl palmitate (33%), free palmitic acid (2%), methyl palmitoleate (26%), free palmitoleic acid (2%), methyl stearate (1%), methyl oleate (18%), and free oleic acid (1%). The biosynthesis of 2-ketones by microsomes was optimal at pH 6.8 in incubation buffer (Figure 16).

Table 11. Incorporation of [1-^{14}C]-acetate into 2-ketones by the subcellular fractions from D. buzzatii ejaculatory bulbs. Values are means ± standard deviations for 5 sets of 5 ejaculatory bulbs each.

Subcellular Fraction	Recovered Label (cpm ± SD in thousands)	Label in Z-10-hepta (cpm ± SD)	Label in 2-tri (cpm ± SD)
Cellular Debris	36 ± 3	312 ± 271	174 ±96
Mitochondria	41 ± 9	756 ± 190	351 ± 73
Microsomes	33 ± 2	1604 ± 354	782 ± 110
Soluble	34 ± 5	340 ± 308	197 ± 59

These results are consistent with previous studies of pheromone biosynthesis in other insects. Both the microsomal and mitochondrial fractions from houseflies converted (Z)-9-tricosene to the epoxide and ketone pheromone components, with the microsomes having the highest activity (Ahmad et al., 1987). Similarly, subcellular fractionation of the pheromone gland from the spruce

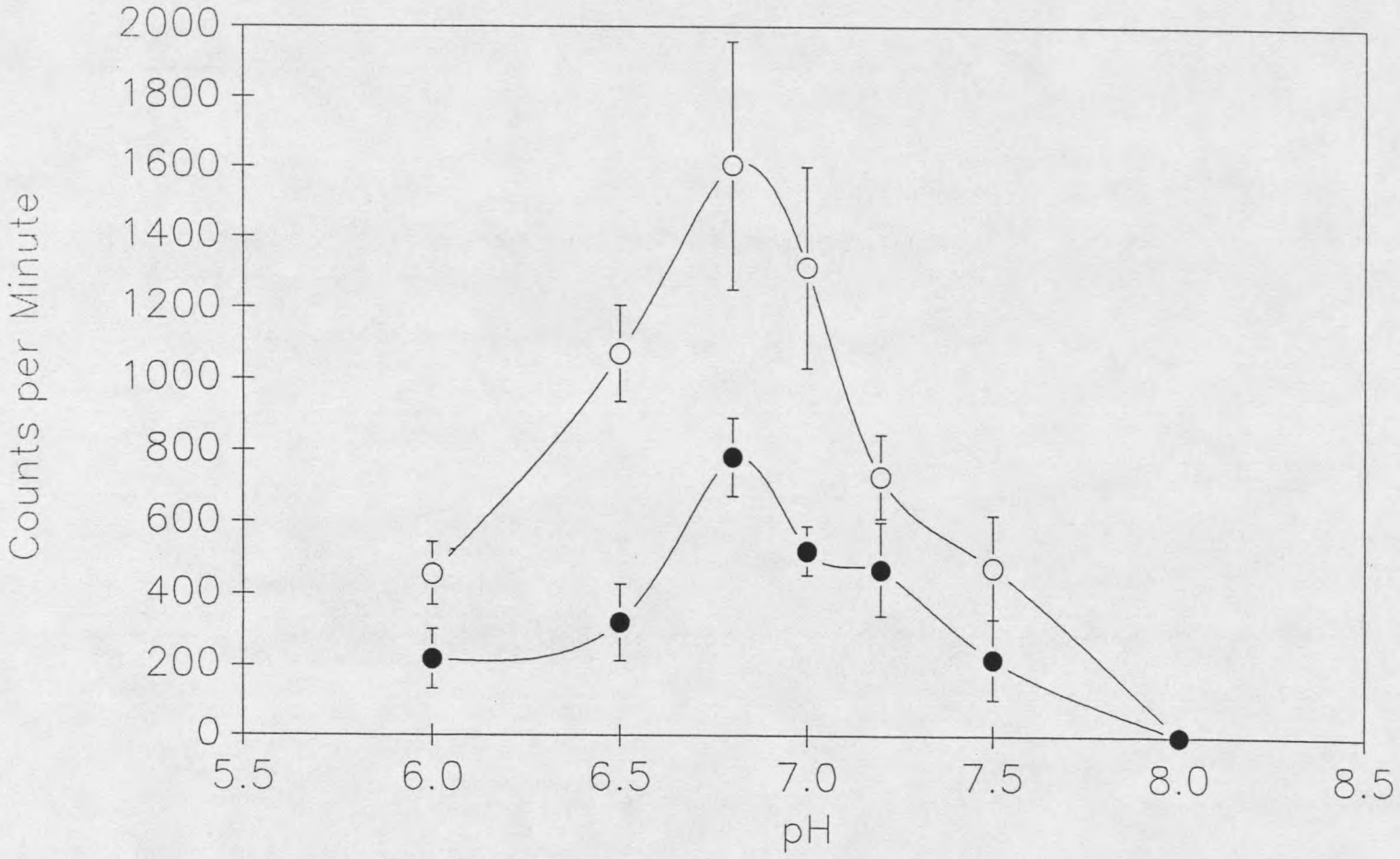


Figure 16. Change in the incorporation of [1-^{14}C]-acetate into 2-ketones by the microsomal fraction from ejaculatory bulbs during a 16 hour incubation with pH. Values are means $\pm$ standard deviations for 5 sets of 5 males each.

budworm indicated that the biosynthetic activities involved in converting the fatty acid into the acetate ester are located in the microsomes (Morse and Meighen, 1986).

Once the microsomal fraction from mature male ejaculatory bulbs was determined to be able to incorporate 1-[^{14}C]-acetate into the 2-ketones, other putative precursors could be investigated. The development of a cell-free preparation for 2-ketone biosynthesis allowed for the incorporation of fatty acyl-CoA substrates that have much higher water solubility and a greater chance at association with the enzymes. The removal of the plasma membrane provides one less barrier to the incorporation of precursors and also allows coenzyme specificity to be determined.

Lipid Analysis

We hypothesized that the 2-ketone precursors may originate from esterified fatty acids in the general esterified fatty acid pool or in a particular subset of the esterified fatty acid pool, as is seen in several lepidopteran species (diacylglycerols or a specific phospholipid, Bjostad et al., 1981; Bjostad and Roelofs, 1984). Hexane-isopropanol extraction of the ejaculatory bulb provided a sample of total lipid from which more detailed analyses could be made. The total lipid extract was separated into individual lipid groups.

In order to identify possible fatty acyl precursors to the 2-ketones, the ejaculatory bulbs from males of various ages were analyzed. The ejaculatory bulbs contained esterified fatty acids from 12 to 18 carbons in length (Table 12). The most abundant fatty acyl groups were 16:1, 16:0, and 18:1, accounting for ca. 67% of the total esterified fatty acids. Also, 12:0 accounted for ca. 3% and 14:0

accounted for 22% of the total esterified fatty acyl groups. In addition to the esterified fatty acid, the ejaculatory bulbs also contained small amounts of free fatty acids. The change in the esterified fatty acids present in the ejaculatory bulb of male D. buzzatii with age is shown in table 12. Since other insect pheromones have been shown to be produced from fatty acid precursors, the concomitant appearance of certain fatty acyl moieties with the 2-ketones would provide strong circumstantial evidence for fatty acid precursors. However, the esterified fatty acids of D. buzzatii ejaculatory bulbs did not change significantly with age with two exceptions. The amounts of esterified palmitate and oleate increased dramatically at four days of age before returning to near day three levels. The amounts of esterified fatty acids present in the ejaculatory bulb could not be directly correlated with the amounts or production of the 2-ketones.

Although the proportions of the esterified fatty acids remained relatively constant, there was one change in the profiles as the flies aged (Table 12). Beginning at 2-3 days of age a second 18:1 fatty acid was detected in ejaculatory bulb extracts. This fatty acid was identified as vaccenic acid (18:1w7) based on GC/MS of the dimethyl disulfide adduct. The amount of vaccenic acid present in the ejaculatory bulb peaked at 4-5 days of age which is also the age of maximum (Z)-10-heptadecen-2-one production, suggesting that vaccenic acid may be involved in the biosynthesis of (Z)-10-heptadecen-2-one, possibly via a chain shortening process.

The esterified fatty acyl groups present in various lipid fractions were determined by base methanolysis of different glycerolipid classes. The esterified fatty acyl groups appear to be evenly distributed among the neutral lipid,

glycolipid, and polar lipid fractions (Table 13).

Table 12. Amounts of esterified fatty acids (ng) present in the ejaculatory bulbs from mature male D. buzzatii of different ages. Values are the mean per male for 5 sets of 5 ejaculatory bulbs each.

AGE	12:0	14:0	14:1	16:0	16:1	18:0	18:1w9	18:1w7	18:2
1	93	689	167	667	907	100	674	0	226
2	41	800	248	737	1129	78	1022	7	137
3	74	485	93	1303	518	199	400	59	159
4	111	344	48	792	322	215	837	467	474
5	48	689	159	748	900	148	685	389	241
6	69	715	210	863	948	122	801	41	194
7	87	651	188	954	1012	104	892	74	219
8	72	708	207	880	967	167	917	68	247

Table 13. Esterified fatty acids present in lipid classes from the ejaculatory bulbs of mature male D. buzzatii. Values are mean ng ± standard deviations per ejaculatory bulb for 5 sets of 5 bulbs each.

LIPID	12:0	14:0	16:1	18:1
NEUTRAL	182±67	198±43	110±28	0
GLYCO	122±24	227±68	115±17	8±4
POLAR	461±89	250±36	102±22	6±5

In addition to esterified fatty acids, the ejaculatory bulb also contained small amounts of free fatty acids. The most abundant free fatty acids were myristic acid (ca. 80 ng), palmitic acid (ca. 80 ng), palmitoleic acid (ca. 200 ng), and oleic acid (ca. 150 ng) [Table 14]. However, the amounts of free fatty acids in the ejaculatory bulb could not be correlated with the biosynthesis of the 2-ketones.

Table 14. Change in the amounts (ng) of free fatty acids present in the ejaculatory bulb of male D. buzzatii with age. Values are the means per male for 5 sets of 5 ejaculatory bulbs each.

Age	12:0	14:1	14:0	16:1	16:0	18:1	18:0
1	0	0	29	107	48	68	0
2	0	0	56	212	29	103	0
3	14	0	83	263	53	149	2
4	12	8	71	208	84	189	0
5	9	6	82	247	77	122	0
6	9	0	44	219	91	143	0
7	4	3	73	188	65	158	0
8	0	0	50	204	58	150	0
9	2	0	56	197	63	121	0

Stable Isotope Experiments

The use of multiple-labeled stable isotopes as markers for biosynthetic studies has some advantages over the use of radiolabeled compounds. First, the likelihood of misleading results because of the recycling of acetyl-CoA from degradation of the putative precursor is greatly reduced. A second advantage of mass-labeled compounds is the ability to distinguish alternative biosynthetic pathways in the same experiment.

We proposed that the 2-ketones in D. buzzatii were formed by a branching of the pathways that produce beta-keto-acids to the appropriate 2-ketone by loss of the carboxyl group as CO_2. Although the exact mechanism of chain shortening is not known, there is a loss of two carbons which suggests a type of beta-oxidation. A presumed intermediate in the chain shortening would be a beta-keto

acyl-CoA, which if the free acid were formed could decarboxylate to produce the 2-ketone.

Failure to reduce the beta-carbonyl group during an elongation reaction of fatty acids allows for the proposal of an elongation pathway. Medium chain fatty acids from the ejaculatory bulb could be elongated by addition of two carbons (as acetyl-CoA or malonyl-CoA) without carbonyl reduction to give a beta-keto acyl-CoA. If the free beta-keto fatty acid were formed, it would decarboxylate to produce the 2-ketone.

Common to both the elongation and chain shortening pathways is the production and decarboxylation of a beta-keto acid to produce the 2-ketones. In the elongation pathway, lauric acid is the proposed precursor to 2-tridecanone and palmitoleic is the proposed precursor to (Z)-10-heptadecen-2-one. Similarly, in the chain shortening pathway, myristic acid is the precursor to 2-tridecanone and vaccenic acid is the precursor to (Z)-10-heptadecen-2-one.

To test these proposed pathways, specific ^{2}H and ^{13}C-labeled fatty acyl-CoA esters were synthesized. The pathways for the biosynthesis of 2-tridecanone were investigated with [$^{2}H_{23}$]-lauroyl-CoA and [$^{2}H_{27}$]-myristoyl-CoA. The elongation of [$^{2}H_{23}$]-lauroyl-CoA to produce 2-tridecanone would produce 2-tridecanone containing 23 additional mass units, while chain shortening of [$^{2}H_{27}$]-myristoyl-CoA would produce 2-tridecanone containing 25 additional mass units (Figures 17 and 18).

The pathways for the biosynthesis of (Z)-10-heptadecen-2-one were examined with [1,2-^{13}C]-palmitoleoyl-CoA and [2,3,4-^{13}C]-vaccenoyl-CoA. The elongation of [1,2-^{13}C]-palmitoleoyl-CoA would produce (Z)-10-heptadecen-2-one

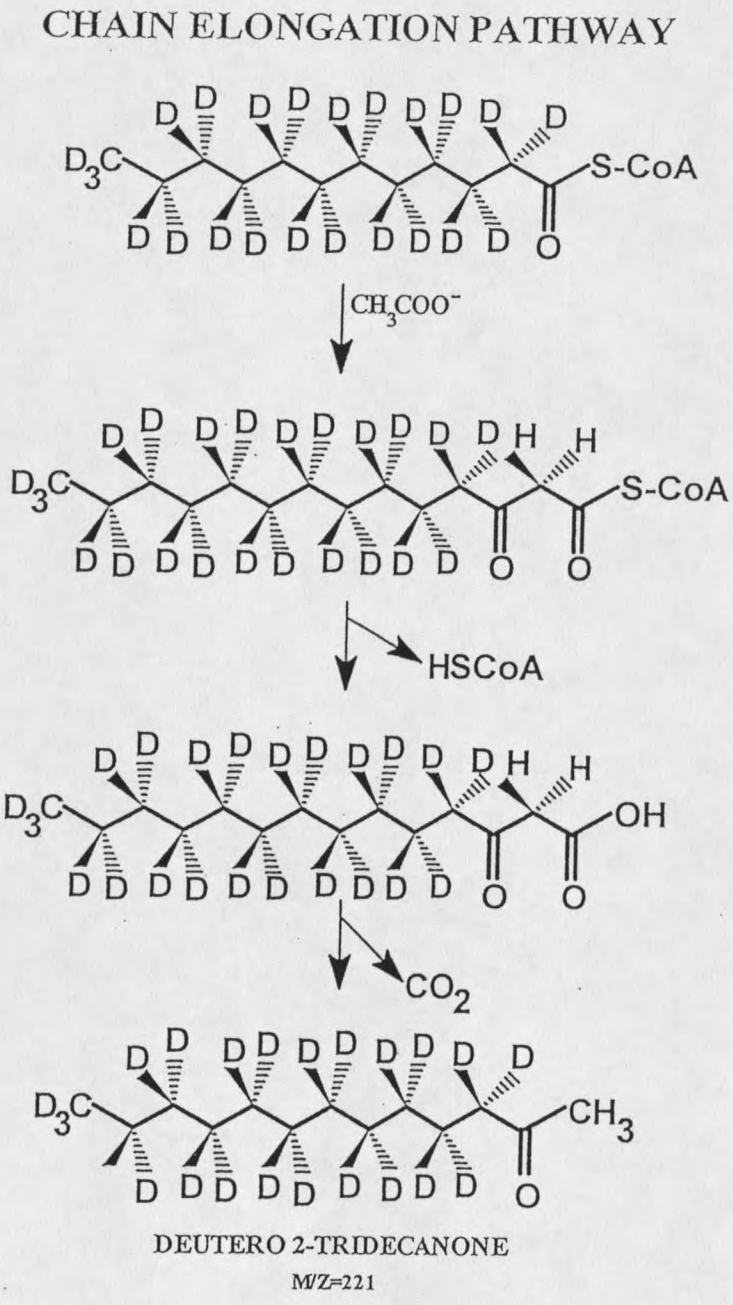


Figure 17. Production of labeled 2-tridecanone via the proposed chain elongation pathway with [$^2H_{23}$]-lauroyl-CoA.

CHAIN SHORTENING PATHWAY

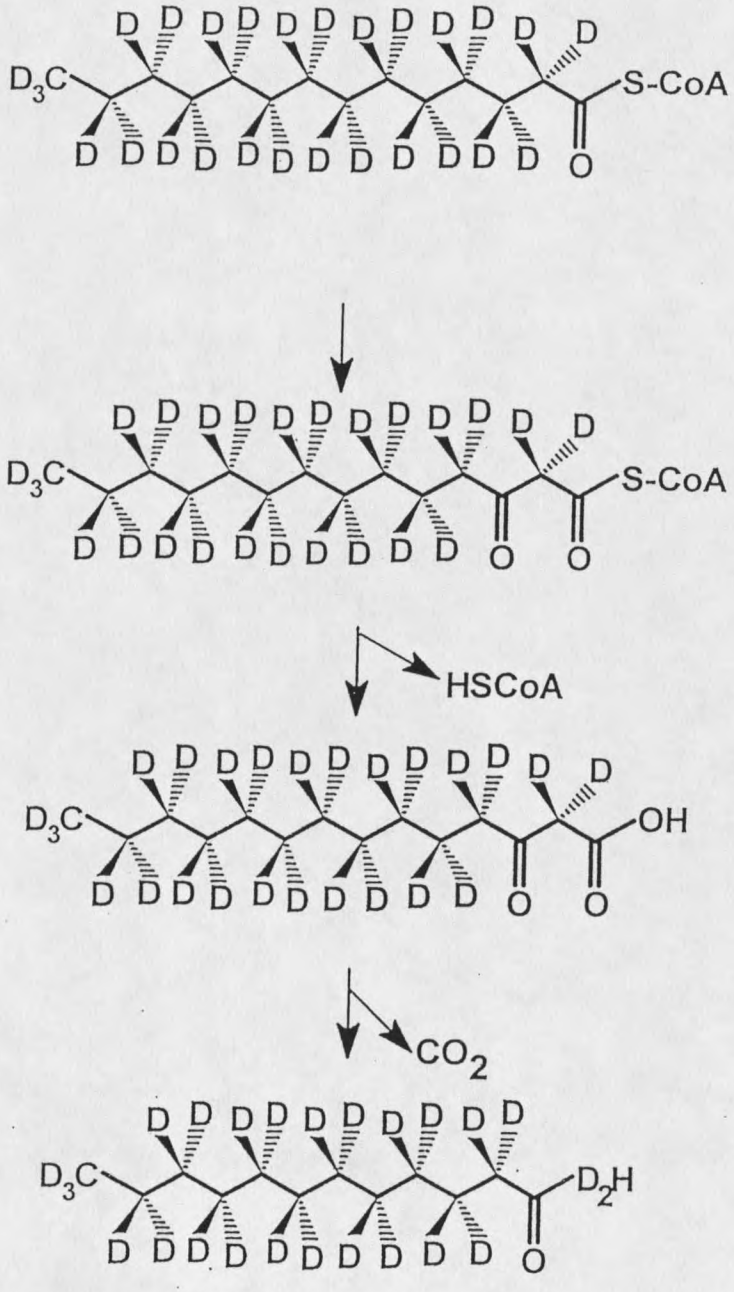


Figure 18. Production of labeled 2-tridecanone via the proposed chain shortening pathway with [$^2H_{27}$]-myristoyl-CoA.

with 2 additional mass units, while the chain shortening of [2,3,4-^{13}C]-vaccenoyl-CoA would produce (Z)-10-heptadecen-2-one with 3 additional mass units (Figures 19 and 20).

[^{2}H]-Labeled Experiments

The recycling problem for radiolabeled compounds is less troublesome when multiple-labeled stable isotopomers are used instead. For example, although degradation of [$^{2}H_{27}$]-tetradecanoyl-CoA would produce [$^{2}H_{2}$]-acetyl-CoA, with each round of beta-oxidation two of the deuteriums are lost. Therefore, the ^{2}H-acetyl-CoA could not be recycled to produce compounds containing 27 additional mass units. Random incorporation into the chain is therefore detectable. In addition, by analyzing the microsomal incubations in the total ion mode, it can be determined where the [^{2}H]- label is present in the molecules.

The purity of the [^{2}H]-labeled Coenzyme A thioesters was determined by GC and GC/MS of the methyl esters formed by reaction of the purified products with 0.5M KOH in methanol. This mild alkaline methanolysis forms methyl esters only from the esterified fatty acids present in the extract. GC and GC/MS analysis of the resulting methyl ester in the presence of heptadecanoic acid methyl ester indicated that the synthesized CoA esters were obtained in overall yields of 52-71% (relative to the amount of fatty acid used) and 83-89 % purity.

The results of incubating the microsomal fraction of ejaculatory bulbs from mature male D. buzzatii with [$^{2}H_{23}$]-lauroyl-CoA are presented in table 15. Microsomal fractions incubated with [^{2}H]-labeled lauroyl-CoA were able to incorporate this compound into the aggregation inhibiting compound 2-tridecanone. This incorporation is apparent in comparison of the M^{+}+23 GC

CHAIN ELONGATION PATHWAY

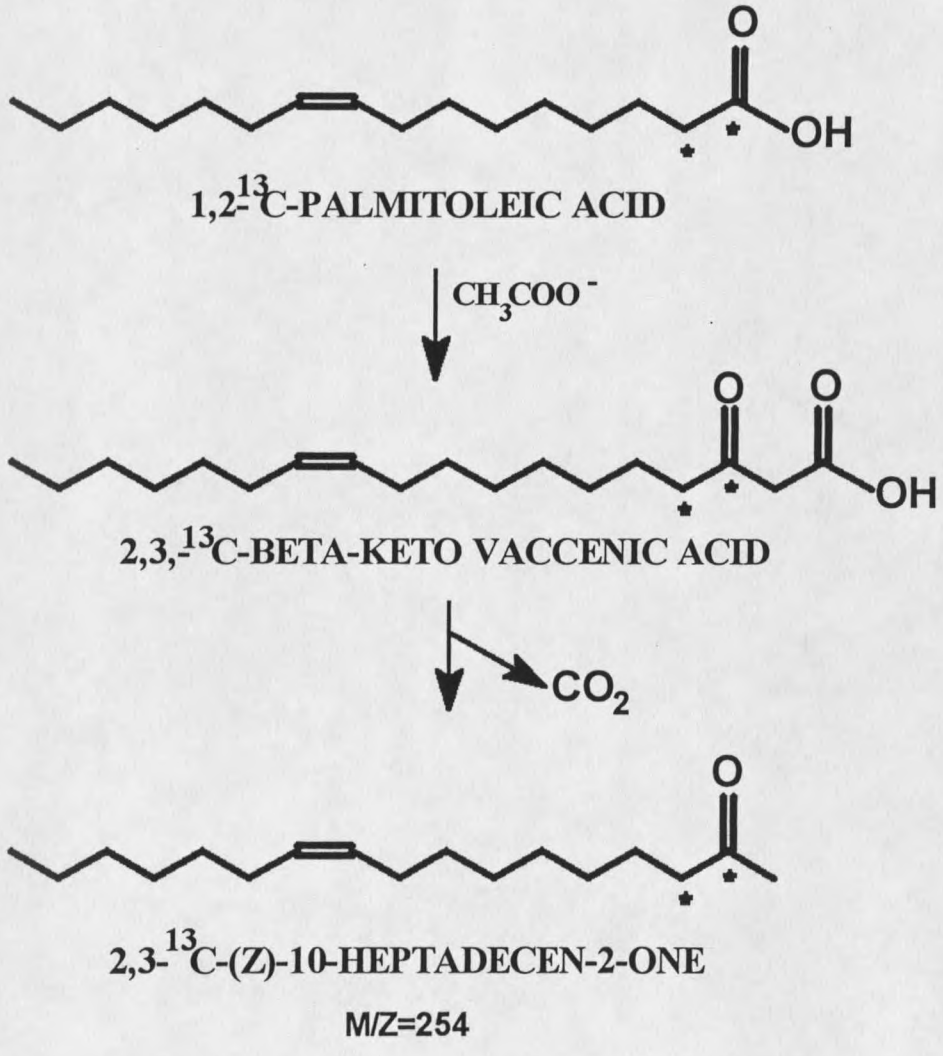


Figure 19. Production of labeled (Z)-10-heptadecen-2-one via the proposed chain elongation pathway with [1,2-^{13}C]-palmitoleoyl-CoA.

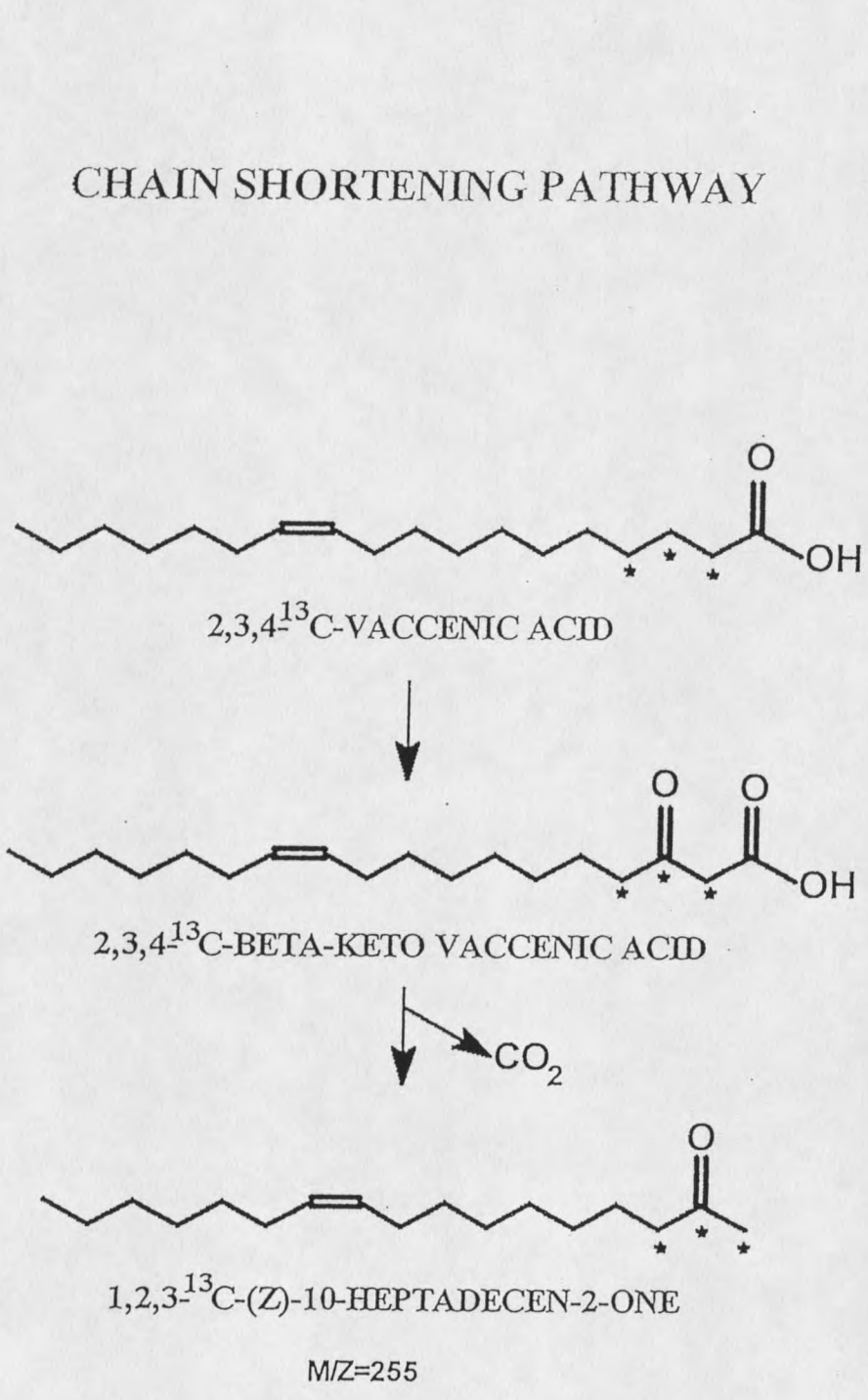


Figure 20. Production of labeled (Z)-10-heptadecen-2-one via the proposed chain shortening pathway with [2,3,4-^{13}C]-vaccenoyl-CoA.

trace for control microsomes with the trace for microsomes incubated with [$^2H_{23}$]-lauroyl-CoA (Figures 21 and 22). The only peak detected in the chromatorgram from control microsomes was the M^+ peak for the methyl heptadecanoate standard (Figure 21). The $[M+23]^+$ peaks were not detected in the control incubations. The compounds into which [$^2H_{23}$]-lauroyl-CoA was incorporated

Table 15. Incorporation[$^2H_{23}$]-lauroyl-CoA into 2-ketones and fatty acyl groups in the microsomal fraction from D. buzzatii ejaculatory bulbs. Values are the means ± standard deviations of 5 groups of 5 male equivalents of the M^++23 peak as the proportion of the M^+ peak for the internal standard, methyl heptadecanoate.

COMPOUND	M^++23 FOR CONTROL MICROSOMES	M^++23 FOR MICROSOMES INCUBATED WITH $^2H_{23}$-LAUROYL-CoA
12:0 ME	0	1207±318
14:0 ME	0	422±113
16:1 ME	0	0
16:0 ME	0	9±4
18:1 ME	0	0
18:0 ME	0	0
2-TRIDECANONE	0	238±77
(Z)-10-HEPTA	0	0
FREE 16:1	0	0
FREE 18:1	0	0
FREE 12:0	0	418±129
FREE 14:0	0	17±2

were separated from the unlabeled compounds by gas chromatography (Figure 23). The majority of the label (52%) was recovered as esterified lauric acid. In

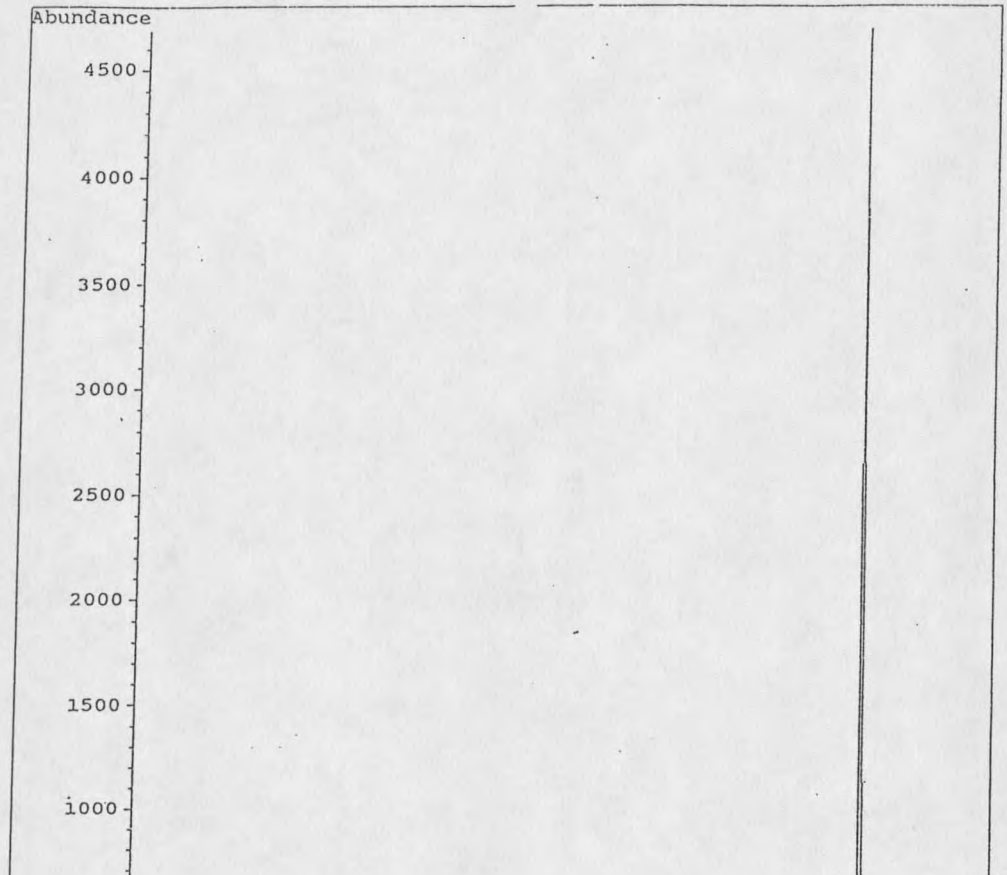


Figure 21. Selected ion monitoring chromatogram of the lipid extract from control microsomes from ejaculatory bulbs, monitored for $M^{+}+ 23$.

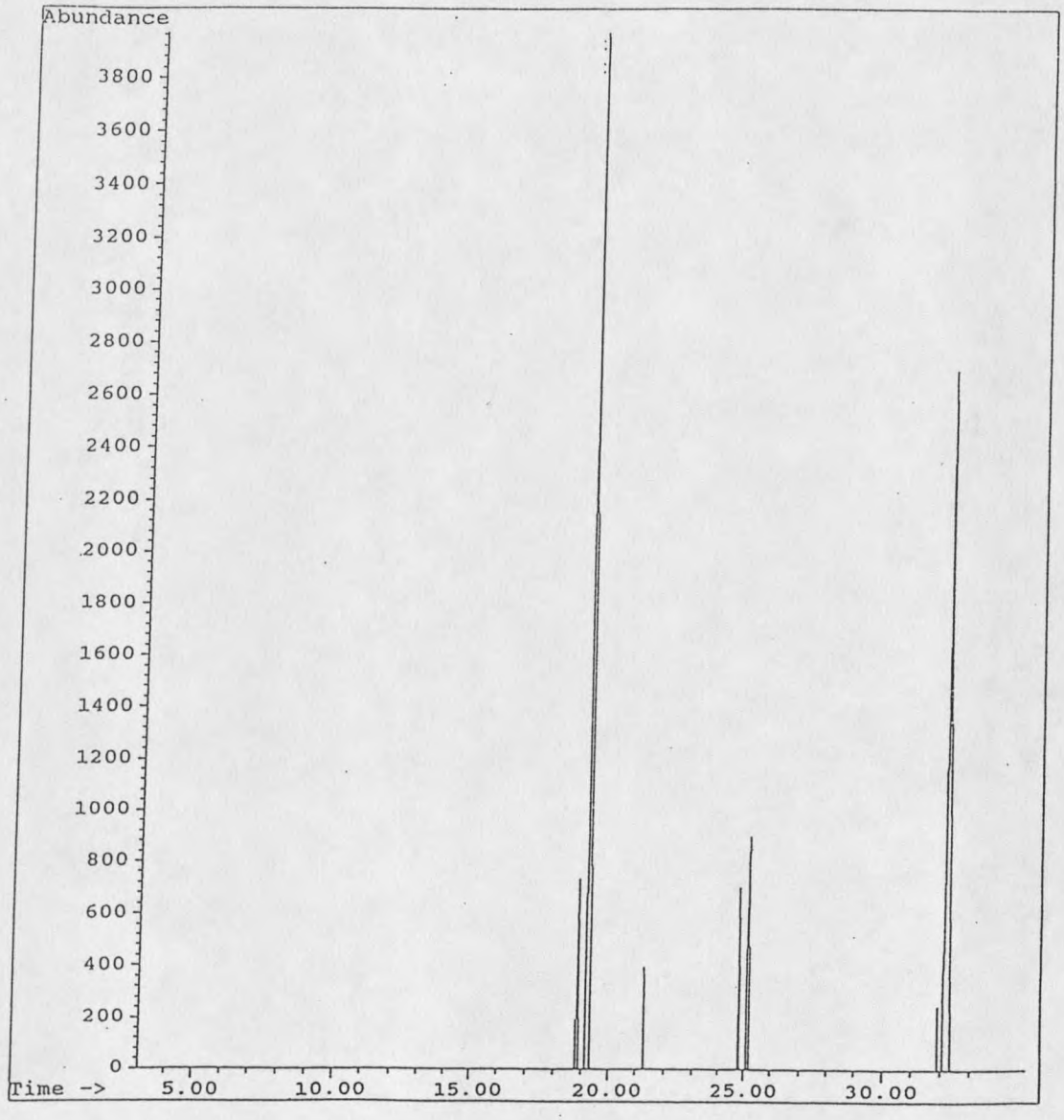


Figure 22. Selected ion monitoring chromatogram of the lipid extract from the microsomal fraction from ejaculatory bulbs incubated with [$^{2}H_{23}$]-lauroyl-CoA.

addition to 2-tridecanone, the mass-labeled thioester was also incorporated into both free and esterified myristic acid. Esterified palmitic acid also showed the incorporation of a small amount of the [^{2}H]-label.

The conversion of[^{2}H]-labeled lauroyl-CoA into[^{2}H]-labeled myristic and palmitic acids also supports the existence of a classical fatty acid chain elongation in the microsomal fraction of D. buzzatii ejaculatory bulbs. Eighteen percent of the mass labeled laurate was elongated to esterified myristate, and ca.0.5% was further elongated to esterified palmitate. In addition to the esterified fatty acids, 18% of the label was recovered in free lauric acid and 0.7% was recovered in free myristic acid. The majority of the recovered label (53%) was recovered as methyl laurate. This indicates that the label either remained as the CoA thioester or was incorporated into another form of esterified fatty acid. Ten percent of recovered label was incorporated into 2-tridecanone by the microsomal fraction. These data indicate that 2-tridecanone is formed via a type of chain elongation reaction.

The identity of [$^2H_{23}$]-2-tridecanone was confirmed in several ways. First, in the SIM mode, the presence of mass 221 (M^++23 for 2-tridecanone) in microsomes incubated with [$^2H_{23}$]-lauroyl-CoA indicated the incorporation of 23 additional mass units into 2-tridecanone (Figure 22). The mass of 221 was not present in the control microsomes or in heat deactivated microsomes which were incubated with [$^2H_{23}$]-lauroyl-CoA. Additionally, in the total ion mode, the complete mass spectrum of the[^{2}H]-labeled compounds could be determined (Figure 23). The mass spectra of both labeled and unlabeled 2-tridecanone are presented in Figures 24 and 25. The mass spectrum of deutero-2-tridecanone from microsomal incubations was also compared to the spectrum of synthetic

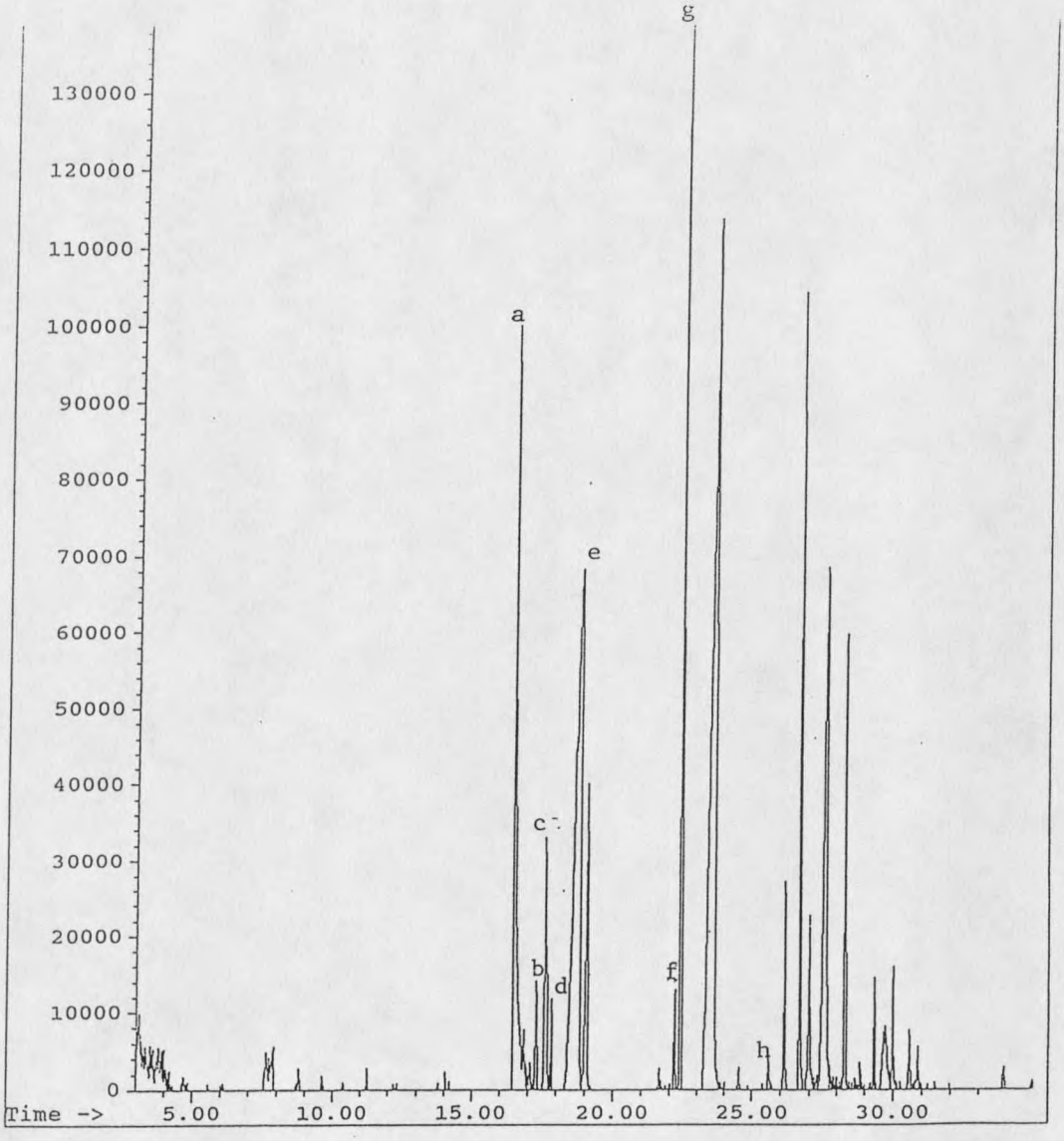


Figure 23. Total ion chromatogram of the lipid extract from the microsomal fraction from ejaculatory bulbs incubated with [$^2H_{23}$]-lauroyl-CoA. a= ^{2}H-labeled methyl laurate, b= ^{2}H-labeled 2-tridecanone, c= methyl laurate, d= 2-tridecanone, e= ^{2}H-labeled lauric acid, f= ^{2}H-labeled methyl myristate, g= methyl myristate, h= ^{2}H-labeled methyl palmitate.

deutero-2-tridecanone, which also had the same retention time. The mass spectra of synthetic [$^2H_{23}$]-2-tridecanone is presented in Figure 26. The M^+ ion at m/z=221 represents 2-tridecanone with 23 additional mass units. In addition, the key fragment ions at m/z=61, representing $C_3{}^2H_3H_3O$, and 43, representing C_2H_3O, further identify this synthetic compound as deuterated 2-tridecanone. Comparing the mass spectra of the 2-tridecanone peak from microsomes incubated with [$^2H_{23}$]-lauroyl-CoA to the mass spectra of synthetic [^2H]-2-tridecanone (Figures 24 and 26) indicates that the compound produced by the microsomes is the same as the synthetic compound.

Microsomal fractions incubated with [$^2H_{27}$]-myristoyl-CoA did not incorporate deuteriums into 2-tridecanone (Table 16). If [$^2H_{27}$]-myristoyl-CoA were incorporated into 2-tridecanone, the heavy atom labeled 2-ketone would have 25 additional mass units. Neither the total ion spectra nor the SIM spectra of microsomes incubated with [$^2H_{27}$]-myristoyl-CoA indicated the presence of a compound with a mass of 223 (Figures 27 and 28). The majority of the recovered label (73%) was present as esterified myristic acid. Seven percent was elongated to esterified palmitic acid, and 20% was recovered as free myristic acid. These data strongly suggest that myristate is not chain shortened to produce 2-tridecanone in the microsomal fraction from mature male D. buzzatii ejaculatory bulbs.

Incubation of the microsomal fraction from ejaculatory bulbs from mature male D. buzzatii with putative [^2H]-labeled fatty acyl precursors indicated that 2-tridecanone is formed via a type of chain elongation reaction rather than via a chain shortening reaction. Further support of the chain elongation reaction is

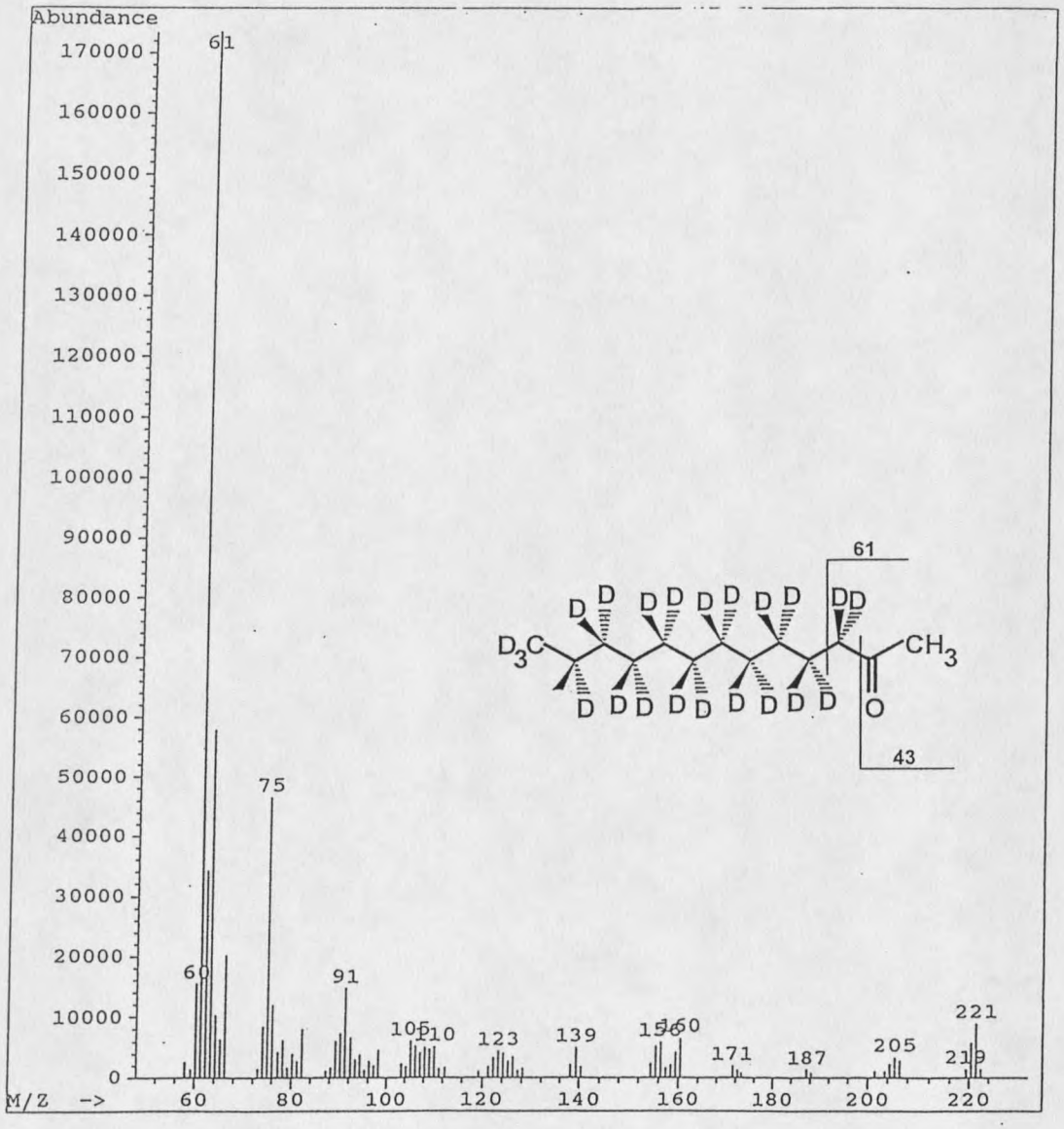


Figure 24. Mass spectrum of [$^2H_{23}$]-labeled 2-tridecanone produced by D. buzzatii.

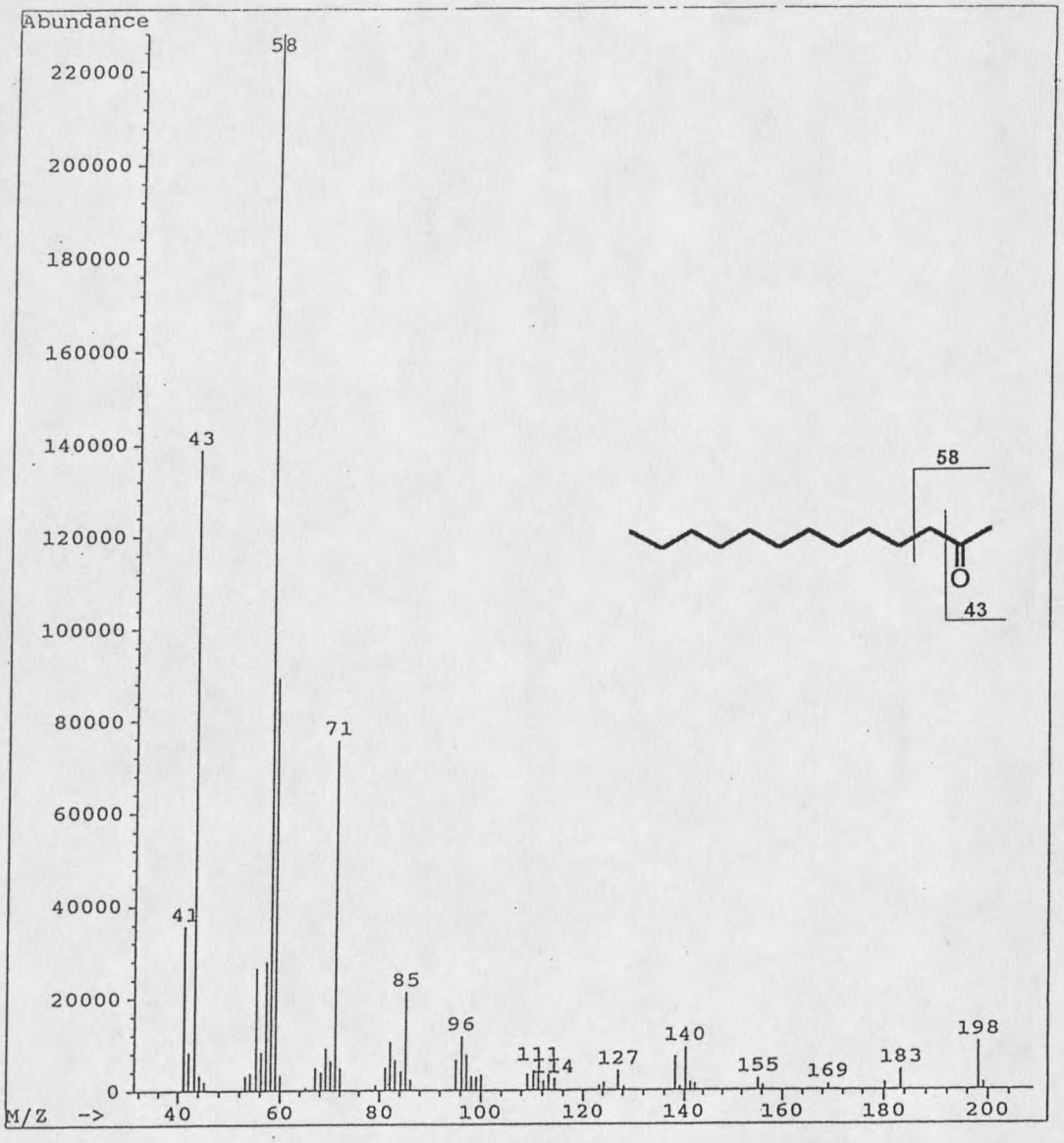


Figure 25. Mass spectrum of 2-tridecanone from D. buzzatii.

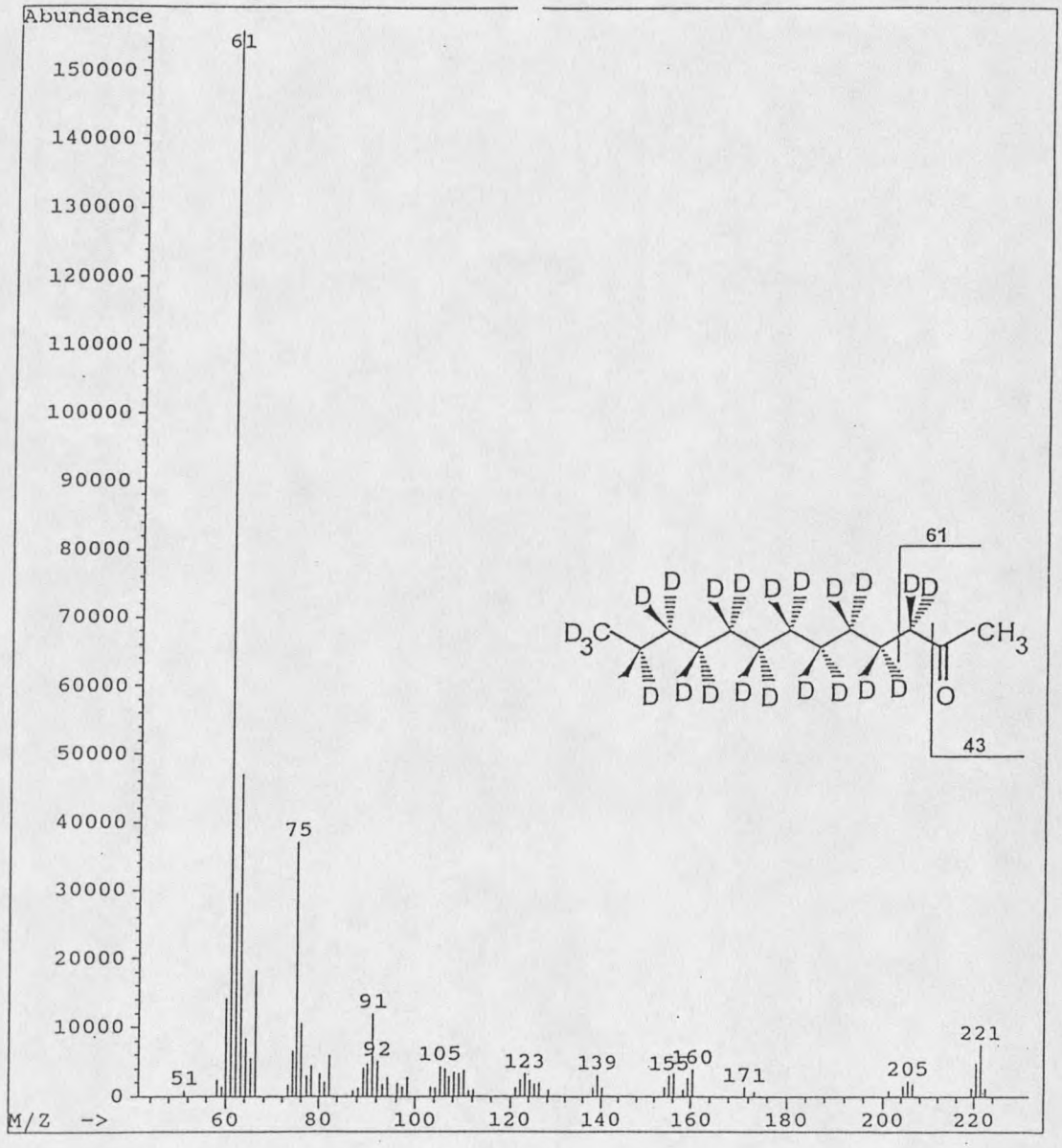


Figure 26. Mass spectrum of synthetic [$^2H_{23}$]-labeled 2-tridecanone.

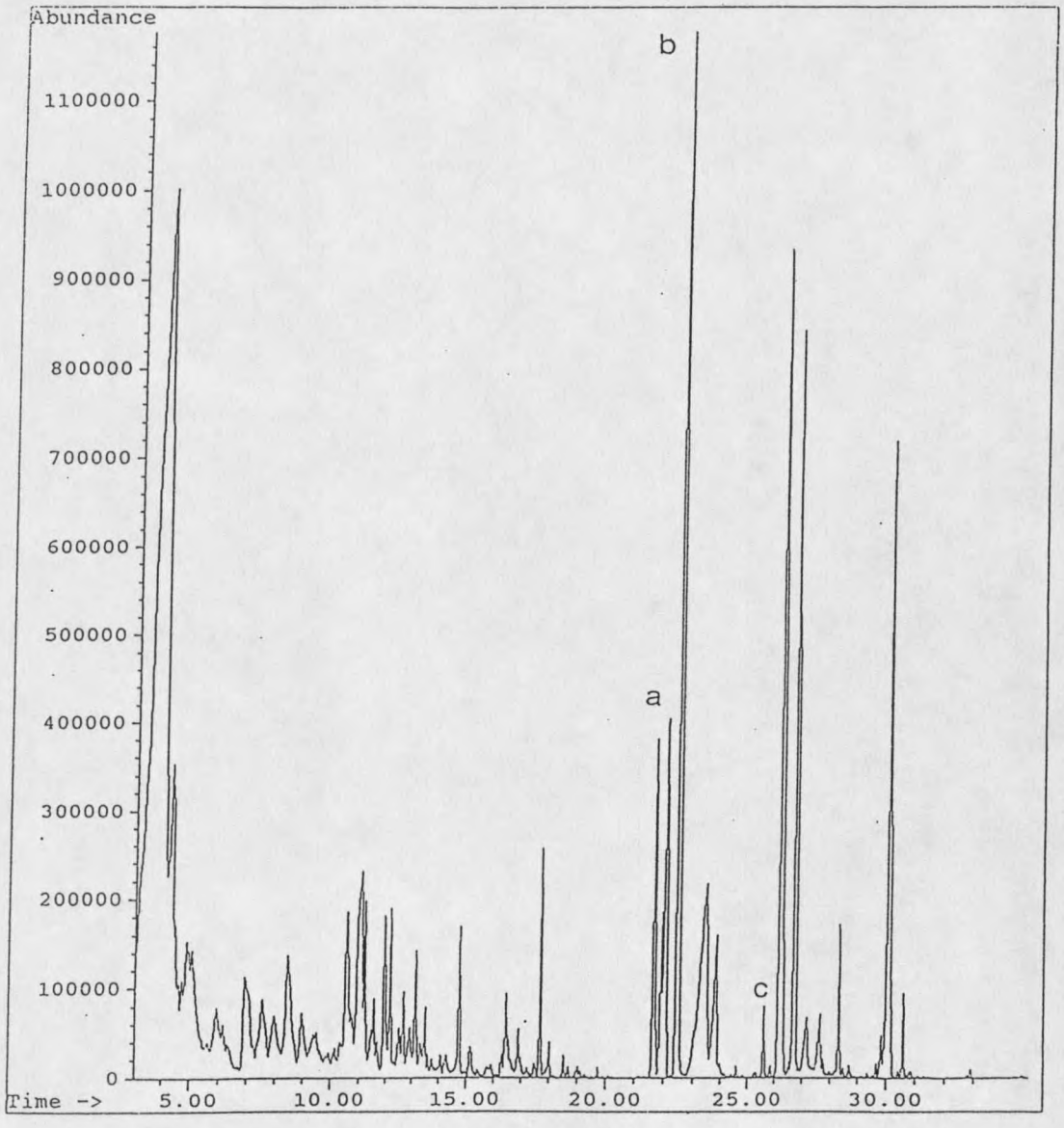


Figure 27. Total ion chromatogram of the lipid extract from the microsomal fraction from ejaculatory bulbs incubated with [$^2H_{27}$]-myristoyl-CoA. a= ^2H-labeled methyl myristate, b= methyl myristate, c= ^2H-labeled methyl palmitate.

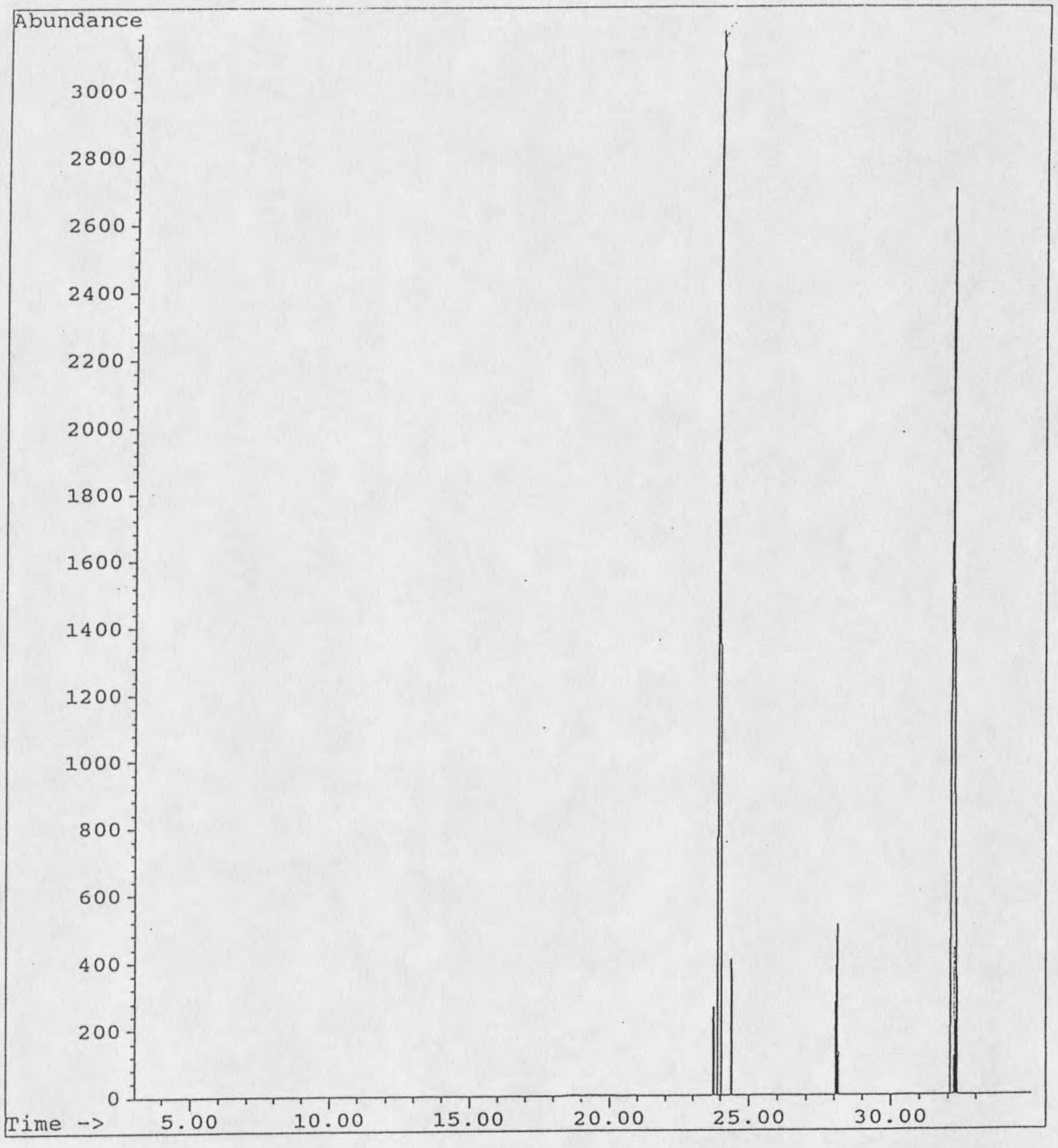


Figure 28. Selection ion monitoring chromatogram of the lipid extract from the microsomal fraction from ejaculatory bulbs incubated with [$^{2}H_{27}$]-myristoyl-CoA.

provided by the fact that the mass labeled fatty acids were also elongated to produce mass labeled fatty acids two carbons and four carbons longer. Since microsomal elongation has been shown to be present in a number of insects (Gonzales-Buitrago et al., 1979; Vaz et al., 1988), it is reasonable that D. buzzatii also possess a microsomal elongation system that could be used to produce the 2-ketone pheromone components.

Table 16. Incorporation[$^2H_{27}$]-myristoyl-CoA into 2-ketones and fatty acyl groups in the microsomal fraction from D. buzzatii ejaculatory bulbs. Values are the means $\pm$ standard deviations for 5 sets of 5 male equivalents of the M^++27 peak as a proportion of the M^+ peak for methyl heptadecanoate.

COMPOUND	M^++27 FOR CONTROL MICROSOMES	M^++27 FOR MICROSOMES INCUBATED WITH $^2H_{27}$-MYRISTOYL-CoA
12:0 ME	0	0
14:0 ME	0	1404$\pm$389
16:1 ME	0	0
16:0 ME	0	138$\pm$27
18:1 ME	0	0
18:0 ME	0	0
2-TRIDECANONE	0	0
(Z)-10-HEPTA	0	0
FREE 16:1	0	0
FREE 18:1	0	0
FREE 12:0	0	0
FREE 14:0	0	387$\pm$46

[^{13}C]-Labeled Experiments

The purity of the [^{13}C]-labeled Coenzyme A thioesters was determined by GC and GC/MS of the methyl esters formed by reaction of the purified products with 0.5M KOH in methanol. This mild alkaline methanolysis forms methyl esters only from the esterified fatty acids present in the extract. GC and GC/MS analysis of the resulting methyl ester in the presence of heptadecanoic acid methyl ester indicated that the synthesized CoA esters were obtained in overall yields of 65-77% (relative to the amount of fatty acid used) and 86-94 % purity.

The microsomal fraction from mature male ejaculatory bulbs also incorporated [1,2-^{13}C]-palmitoleoyl-CoA into (Z)-10-heptadecen-2-one (Table 17). The majority of the label was recovered in free and esterified palmitoleic acid. In addition, ca. 4% of the label was incorporated into esterified vaccenic acid. The selected ion monitoring chromatogram showing the incorpration of two additional mass units into (Z)-10-heptadecen-2-one as presented in figure 29.

Further, the use of total ion chromatograms allowed the position of the ^{13}C atoms in the molecule to be determined. The total ion chromatograms from microsomes incubated with [1,2-^{13}C]-labeled palmitoleoyl-CoA show that the label is incorporated into the two and three carbon positions of the chain in (Z)-10-heptadecen-2-one (Figure 30). Via the McLafferty rearrangement, one of the most prominent fragment ions produced from the EI mass spectra of 2-ketones is at m/z=58, corresponding to the $C_3H_6O^+$ ion (see figure 8). The 2-ketones produced from [1,2-^{13}C]-palmitoleoyl-CoA would contain ^{13}C in the 2 and 3 positions of (Z)-10-heptadecen-2-one. The McLafferty rearrangement of this heavy atom labeled 2-ketone produces a fragment ion of m/z=60, corresponding

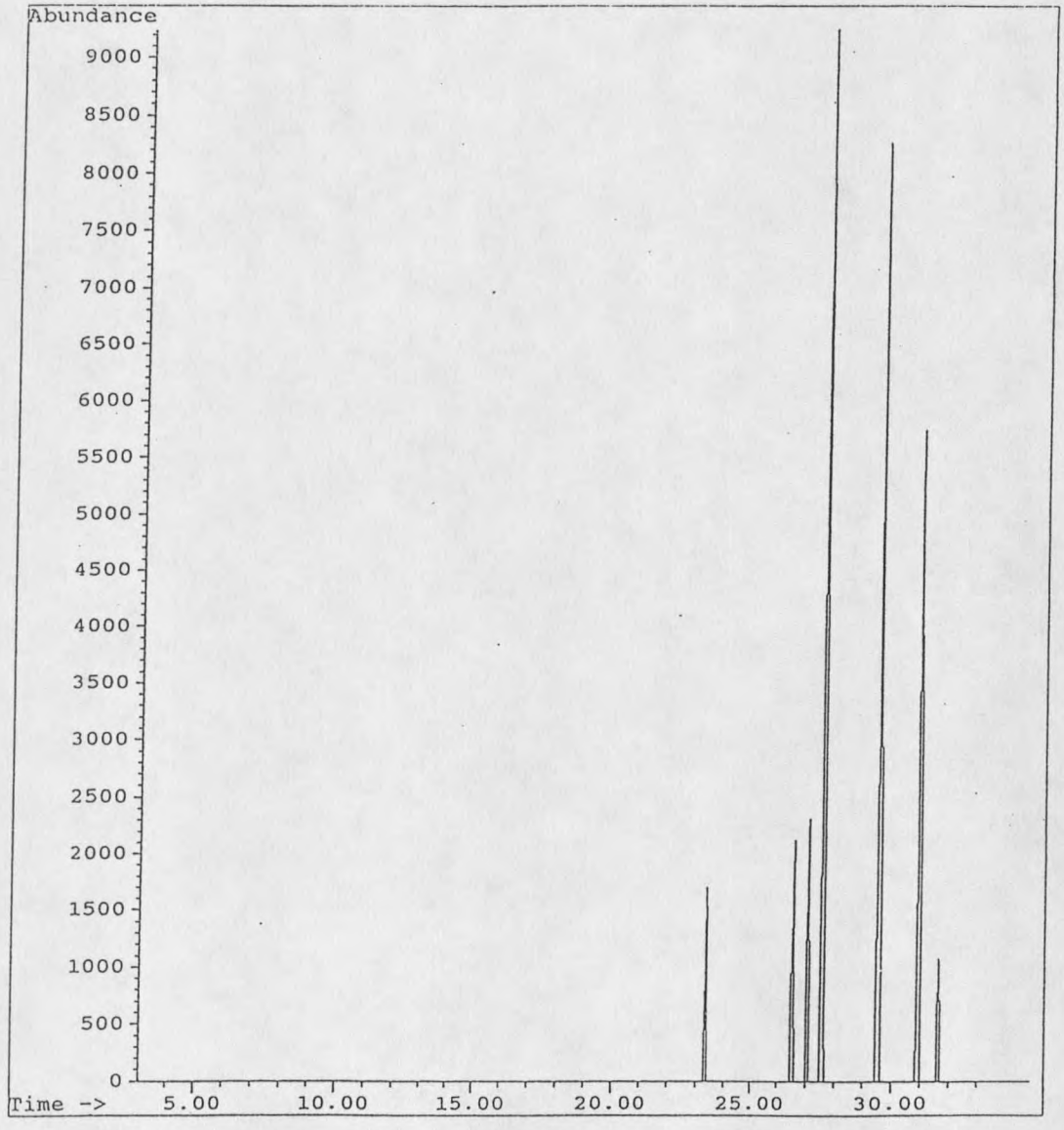


Figure 29. Selected ion monitoring chromatogram of the lipid extract from the microsomal fraction from ejaculatory bulbs incubated with [1,2-^{13}C]-palmitoleoyl-CoA.

to $C^{13}C_2H_6O$. In addition, another prominent ion in the spectra of 2-ketones is at m/z=43, corresponding to both the C_3H_7 and the C_2H_3O ions. However, if the 2-ketone incorporated a ^{13}C atom into the 2 position, this would result in an ion containing one additional mass unit. Therefore, the stable isotope labeled (Z)-10-heptadecen-2-one would be expected to have a prominent ion at m/z=44. In this manner, the position of the ^{13}C atoms in the product can be determined. From the existence of the molecular ion peak at m/z=254, the McLafferty rearrangement ion at m/z=60, and the m/z=44 ion in the peak that elutes at the retention time of (Z)-10-heptadecen-2-one, it is obvious that palmitoleoyl-CoA is elongated to produce (Z)-10-heptadecen-2-one (Figure 30).

Table 17. Incorporation [1,2-^{13}C]-palmitoleoyl-CoA into 2-ketones and fatty acyl groups in the microsomal fraction from D. buzzatii ejaculatory bulbs. Values are the means ± standard deviations for 5 sets of 5 male equivalents each as proportion of methyl heptadecanaoate.

COMPOUND	M^++2 FOR CONTROL MICROSOMES	M^++2 FOR MICROSOMES INCUBATED WITH 1,2-^{13}C-PALMITOLEOYL-CoA
12:0 ME	21±11	27±8
14:0 ME	100±35	111±29
16:1 ME	43±8	239±44
16:0 ME	46±14	64±17
18:1 ME	65±21	162±38
18:0 ME	72±19	34±4
2-TRIDECANONE	66±15	78±21
(Z)-10-HEPTA	71±27	741±109
FREE 16:1	40±9	864±167
FREE 18:1	61±28	85±16

However, [2,3,4-^{13}C]-vaccenoyl-CoA was not incorporated into (Z)-10-

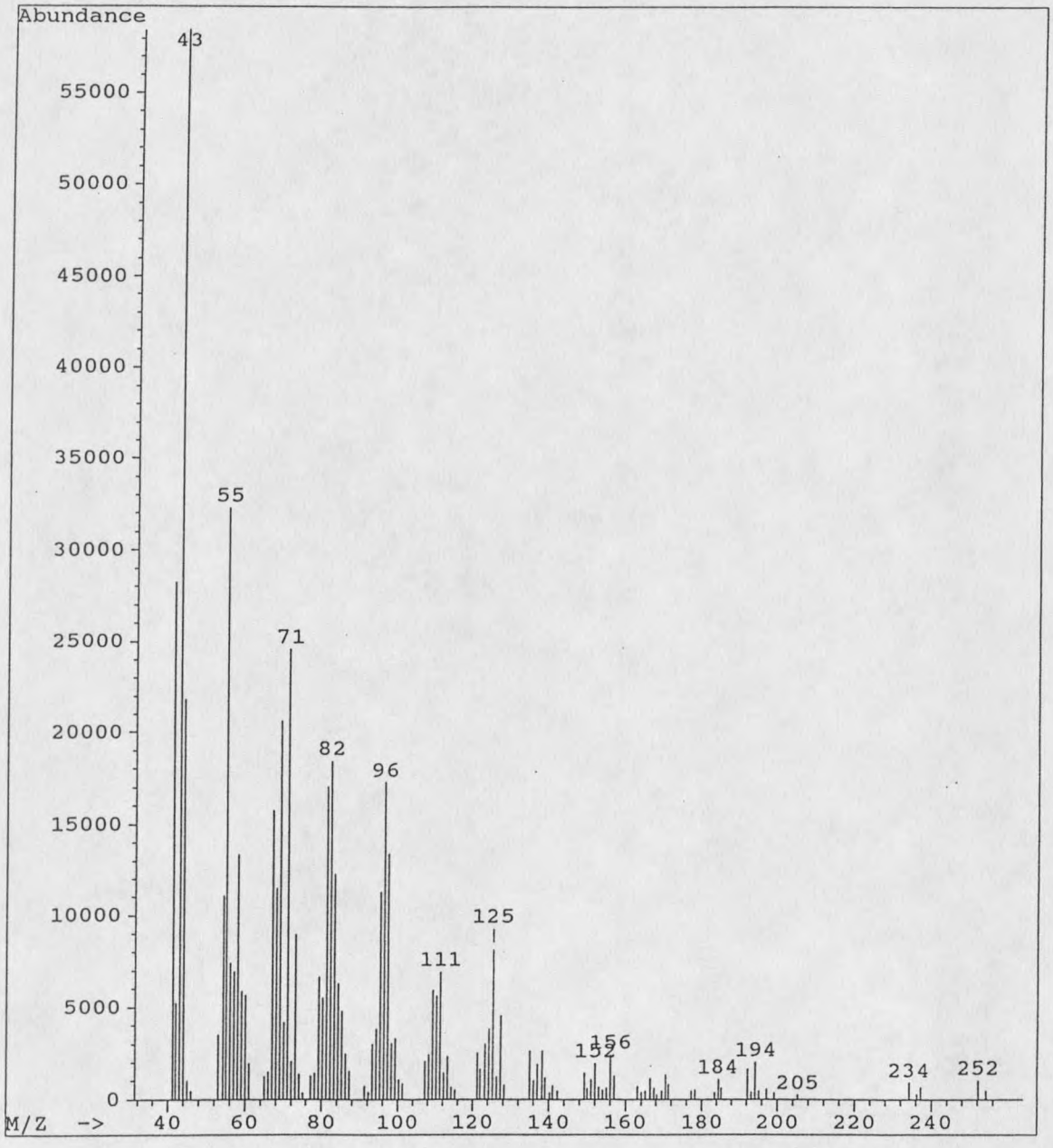


Figure 30. Mass spectrum of the (Z)-10-heptadecen-2-one peak from the microsomal fraction from ejaculatory bulbs incubated with [1,2-^{13}C]-palmitoleoyl-CoA.

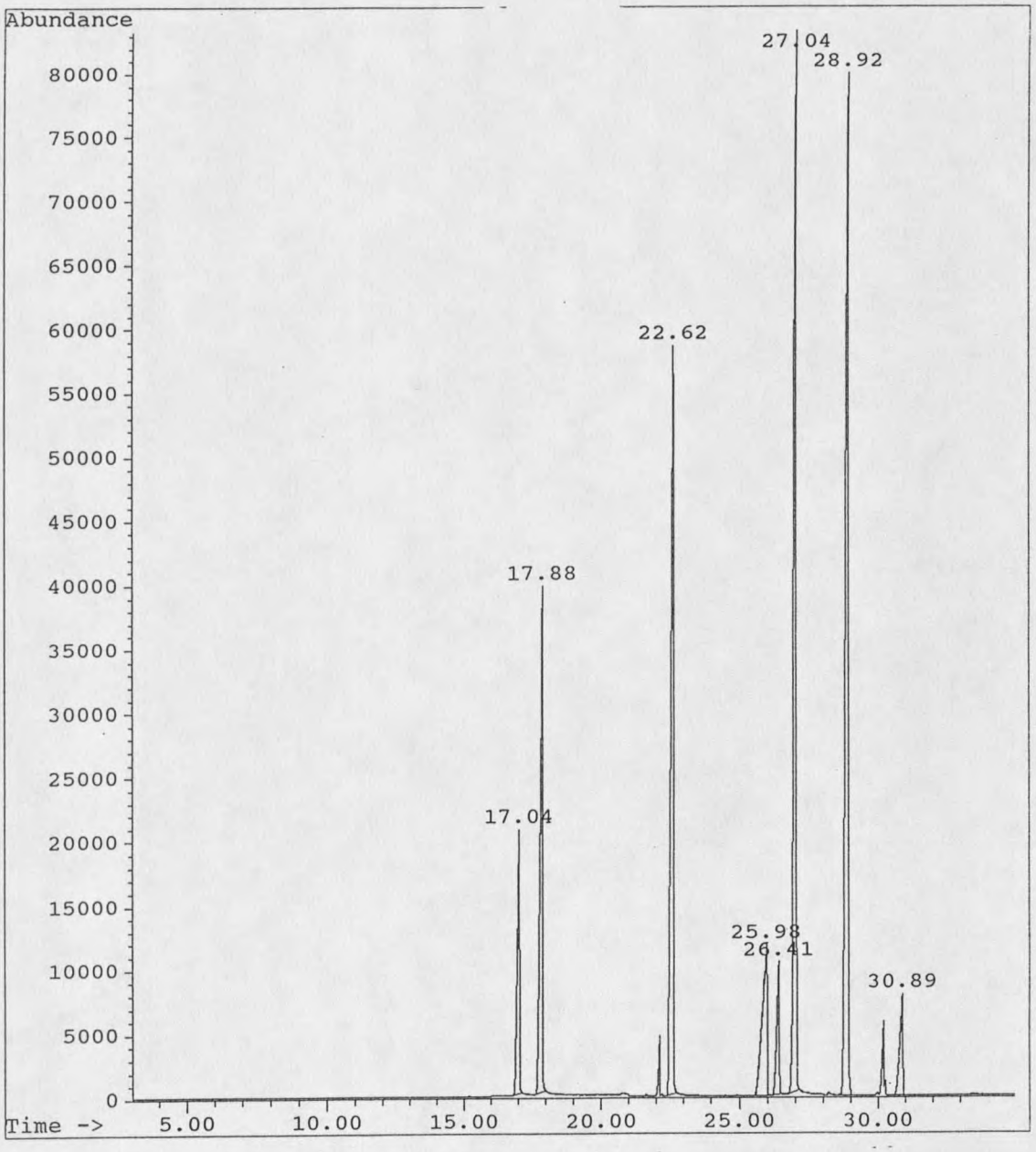


Figure 31. Total ion chromatogram of the lipid extract from the microsomal fraction from ejaculatory bulbs incubated with [2,3,4-^{13}C]-vaccenoyl-CoA.

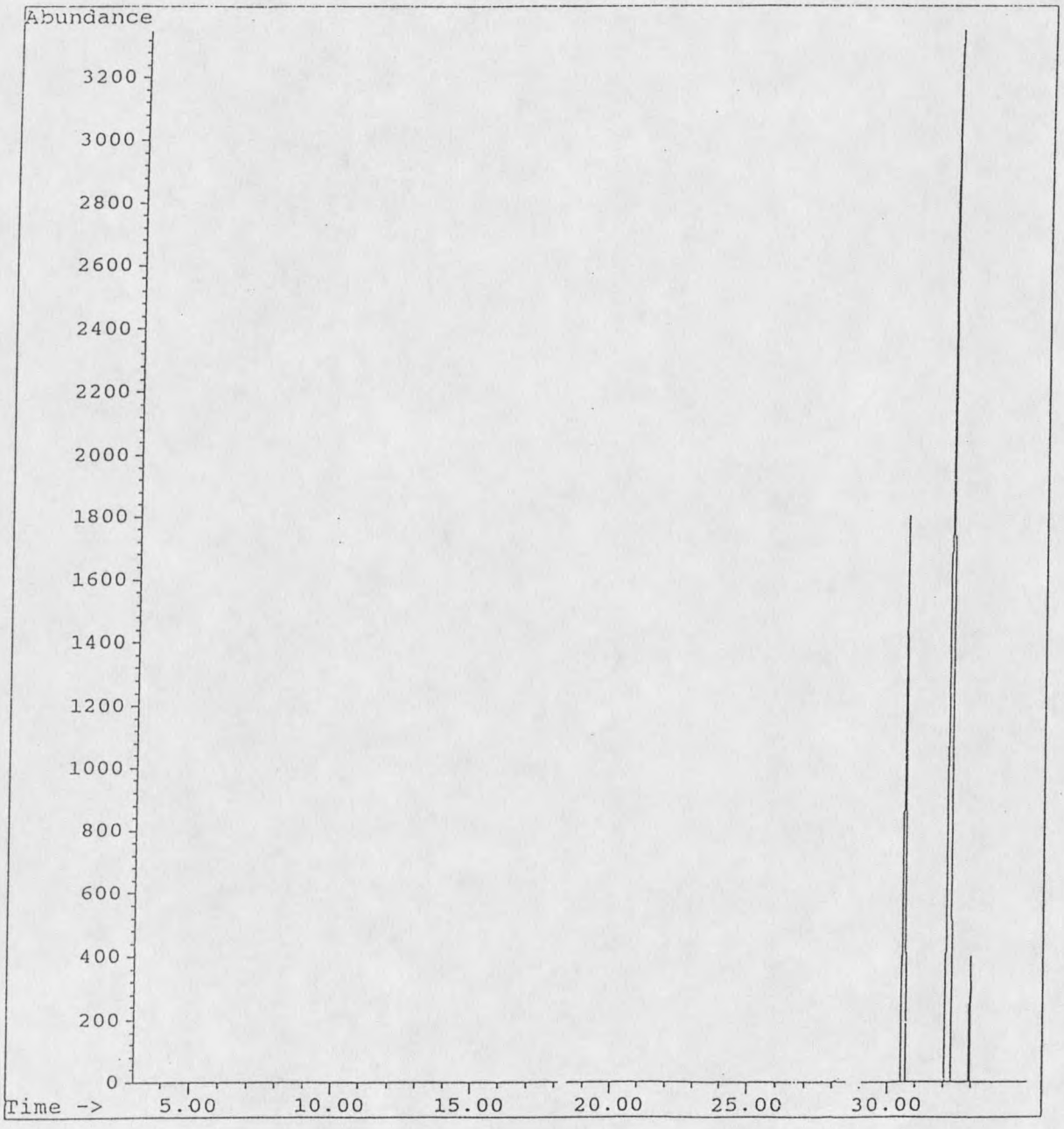


Figure 32. Selected ion monitoring chromatogram of the lipid extract from the microsomal fraction from ejaculatory bulbs incubated with [2,3,4-^{13}C]-vaccenoyl-CoA.

heptadecen-2-one. Both the total ion chromatogram and the selected ion monitoring chromatogram indicate that the label was only recovered in free and esterified vaccenic acid (Figures 31 and 32, Table 18). This evidence strongly suggests that a chain shortening pathway is not involved in the biosynthesis of 2-ketones in D. buzzatii.

Acetyl-CoA Carboxylase Inhibition

Although these results indicated that the 2-ketones in D. buzzatii were produced by the elongation of a fatty acyl precursor, it was still unclear whether they were elongated via a fatty acid synthesis or fatty acid elongation reaction. However, the incubation of microsomes with [1-^{14}C]-acetate in the presence of the acetyl CoA carboxylase inhibitor avidin indicates that the 2-ketones are not

Table 18. Incorporation of [2,3,4-^{13}C]-vaccenoyl-CoA into lipids in the microsomal fraction from D. buzzatii ejaculatory bulbs. Values are the means $\pm$ standard deviations for 5 sets of 5 male equivalents each of the M^++3 peak as proportion of the M^+ peak for methyl heptadecanoate.

COMPOUND	M^++3 FOR CONTROL MICROSOMES	M^++3 FOR MICROSOMES INCUBATED WITH 2,3,4-^{13}C-VACCENOYL-CoA
12:0 ME	4	2
14:0 ME	2	3
16:1 ME	1	5
16:0 ME	3	2
18:1 ME	5	1561
18:0 ME	4	6
2-TRIDECANONE	3	3
(Z)-10-HEPTA	3	5
FREE 16:1	4	4
FREE 18:1	7	472

synthesized via a microsomal fatty acid synthetase (Table 19). In the presence of avidin, acetate cannot be converted into malonate which is required for microsomal chain elongation. When microsomes were incubated with [1-^{14}C]-acetate in the presence of avidin, radiolabel was not incorporated into the 2-ketones, while in the absence of avidin radiolabel was actively incorporated into the 2-ketones. Since the microsomal elongation requires malonate, the lack of incorporation of label in the presence of avidin indicates the involvement of a microsomal chain elongation mechanism in the production of 2-ketones.

Further support for the involvement of a microsomal elongation system was obtained from experiments using stable isotopes. When microsomes from ejaculatory bulbs were incubated with acetyl-CoA and [$^{2}H_{23}$]-lauroyl in the

Table 19. Incorporation of [1-^{14}C]-acetate into 2-ketones by the microsomal fraction from D. buzzatii ejaculatory bulbs in the presence and absence of the acetyl-CoA carboxylase inhibitor avidin. Values are the mean counts per minute $\pm$ the standard deviations for 5 sets of 5 male equivalents each.

TREATMENT	(Z)-10-HEPTA	2-TRIDECANONE
CONTROL	1604$\pm$318	782$\pm$67
AVIDIN	118$\pm$32	---

presence of avidin, deuterated 2-tridecanone was not produced, while in the absence of avidin the deuterated fatty acyl-CoA was converted into the 2-ketone. Likewise, microsomes incubated with [1,2-^{13}C]-palmitoleoyl-CoA in the presence of avidin (Z)-10-heptadecen-2-one was not labeled, while in the absence of avidin the ^{13}C-labeled 2-ketone was produced.

Further experiments with microsomes that were washed with incubation buffer several times and recentrifuged indicate that a soluble acetyl-CoA carboxylase was present in the original microsomal fractions. The original

microsomal fraction was capable of incorporating both stable-isotope labeled fatty acyl-CoA esters into the corresponding 2-ketones in the presence of acetyl-CoA. However, microsomes that were washed and recentrifuged twice could not incorporate label from either of the stable-isotope labeled fatty acyl-CoA esters into the 2-ketones when incubated with acetyl-CoA. In addition, the same microsomes incorporated the label into the 2-ketones when the incubation media contained malonyl-CoA (Tables 20 and 21). These data strongly support the role of a microsomal chain elongation system in the biosynthesis of 2-ketones in Drosophila buzzatii.

Table 20. Incorporation of [^{14}C]-vaccenoyl-CoA into 2-tridecanone by the microsomal fraction of D. buzzatii ejaculatory bulbs in the presence and absence of the acetyl-CoA carboxylase inhibitor avidin. Values are the means $\pm$ the standard deviations of the M^++2 peak as a proportion of the M^+ for methyl heptadecanoate X 1000 (PMH X 1000)for 5 sets of 5 male equivalents each.

TREATMENT	Z-10-HEPTA AS PMH X 1000
AcCoA	741
AcCoA + AVIDIN	0
MalCoA	802
WASHED + AcCoA	0
WASHED + MalCoA	688

Table 21. Incorporation of [$^2H_{23}$]-lauroyl-CoA into 2-tridecanone by the microsomal fraction of D. buzzatii ejaculatory bulbs in the presence and absence of the acetyl-CoA carboxylase inhibitor avidin. Values are the means $\pm$ the standard deviations of the M^++23 peak as a proportion of the M^+ peak for methyl heptadecanoate for 5 sets of 5 male equivalents each.

TREATMENT	2-TRI AS PMH X 1000
AcCoA	238
AcCoA + AVIDIN	0
MalCoA	322
WASHED + AcCoA	0
WASHED + MalCoA	206

SUMMARY

Although pheromones have been identified and characterized in many insect species, the study of the biosynthesis of these important compounds has lagged far behind the study of their biology. Aggregation pheromones were first discovered in Drosophila eight years ago, yet the data presented here represent the first studies on the biosynthesis of these compounds.

Since previous experiments on the identification of the aggregation pheromone components in D. buzzatii indicated that the 2-ketones were stored in the ejaculatory bulbs of males, we believed that the ejaculatory bulb was also the site of 2-ketone biosynthesis. It appeared to us that fatty acyl precursors would be involved in the biosynthesis of the 2-ketones because of their structural similarities. The putative precursor fatty acids for both the proposed elongation and shortening pathways were present in the ejaculatory bulb, yet the change in the fatty acids with age could not be correlated with the production of 2-ketones.

Initial experiments with radiolabeled acetate indicated that the 2-ketones in D. buzzatii were produced in the ejaculatory bulbs of mature males. The incorporation of radiolabeled acetate into the 2-ketones was pH dependent and ATP increased the amount of label incorporated. In addition to the 2-ketones, radiolabeled acetate was also incorporated into both free and esterified fatty acyl groups in the ejaculatory bulbs.

Furthermore, the majority of the biosynthetic activity was found to be localized in the microsomal fraction from the ejaculatory bulbs. The development of a cell-free

preparation for 2-ketone biosynthesis was a considerable advantage for further investigations. The removal of the plasma membrane provided one less barrier to the incorporation of putative substrates. Also, since the mitochondria were eliminated, greater substrate integrity was possible because the B-oxidation enzymes were removed. In addition, cell-free preparations allowed us to utilize fatty-acyl-CoA substrates which are water soluble and have a greater chance of association with the enzymes.

The use of ^{2}H-labeled and ^{13}C-labeled fatty acyl-CoA esters allowed us to determine the biosynthetic pathways to 2-ketones in D. buzzatii. The incorporation of [$^{2}H_{23}$]-lauroyl-CoA into 2-tridecanone by the microsomal fraction from ejaculatory bulbs indicated that a chain elongation pathway was present. This data, along with the lack of incorporation of [$^{2}H_{27}$]-myristoyl-CoA, which indicated that a chain shortening pathway was not operating, provided strong evidence that 2-tridecanone was produced via the chain elongation pathway. Likewise, the incorporation of [1,2-^{13}C]-palmitoleoyl-CoA into (Z)-10-heptadecen-2-one and the lack of incorporation of [2,3,4-^{13}C]-vaccenoyl-CoA, indicated that this 2-ketone was also produced via a chain elongation reaction.

Although the use of the stable isotope labeled fatty acyl-CoA esters indicated that the 2-ketones were formed via an elongation reaction, two forms of elongation were possible. A microsomal fatty acid elongation system which has been found in a number of organisms, or a microsomal fatty acid synthetase system which has been shown to be involved in pheromone biosynthesis in the German cockroach. To

distinguish between these two systems, the acetyl-CoA carboxylase inhibitor avidin was used. Avidin inhibits the conversion to acetyl-CoA to malonyl-CoA. Malonyl-CoA is required by the microsomal fatty acid elongation system while the microsomal fatty acid synthetase utilizes acetyl-CoA for elongation. Both [$^{2}H_{23}$]-lauroyl-CoA and [1,2-^{13}C]-palmitoleoyl-CoA were elongated to the corresponding 2-ketones in the absence of avidin when provided with acetyl-CoA. However, in the presence of avidin the 2-ketones were not produced. These data strongly support the microsomal fatty acid elongation pathway for 2-ketone biosynthesis.

Further experiments with microsomes that were washed with buffer and recentrifuged provide additional support for the elongation pathway. After being washed, the microsomal fraction did not incorporate the labeled fatty acyl-CoA esters into the 2-ketones when incubated with acetyl-CoA. However, when incubated with malonyl-CoA, the microsomes did incorporated label into the 2-ketones.

The use of [1-^{14}C]-labeled acetate, specific stable isotope labeled fatty acyl-CoA esters, and the acetyl-CoA carboxylase inhibitor avidin in these experiments strongly support the involvement of a microsomal fatty acid elongation system in the biosynthesis of 2-ketones in D. buzzatii

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