INSECT LIPIDS

Chemistry, Biochemistry & Biology

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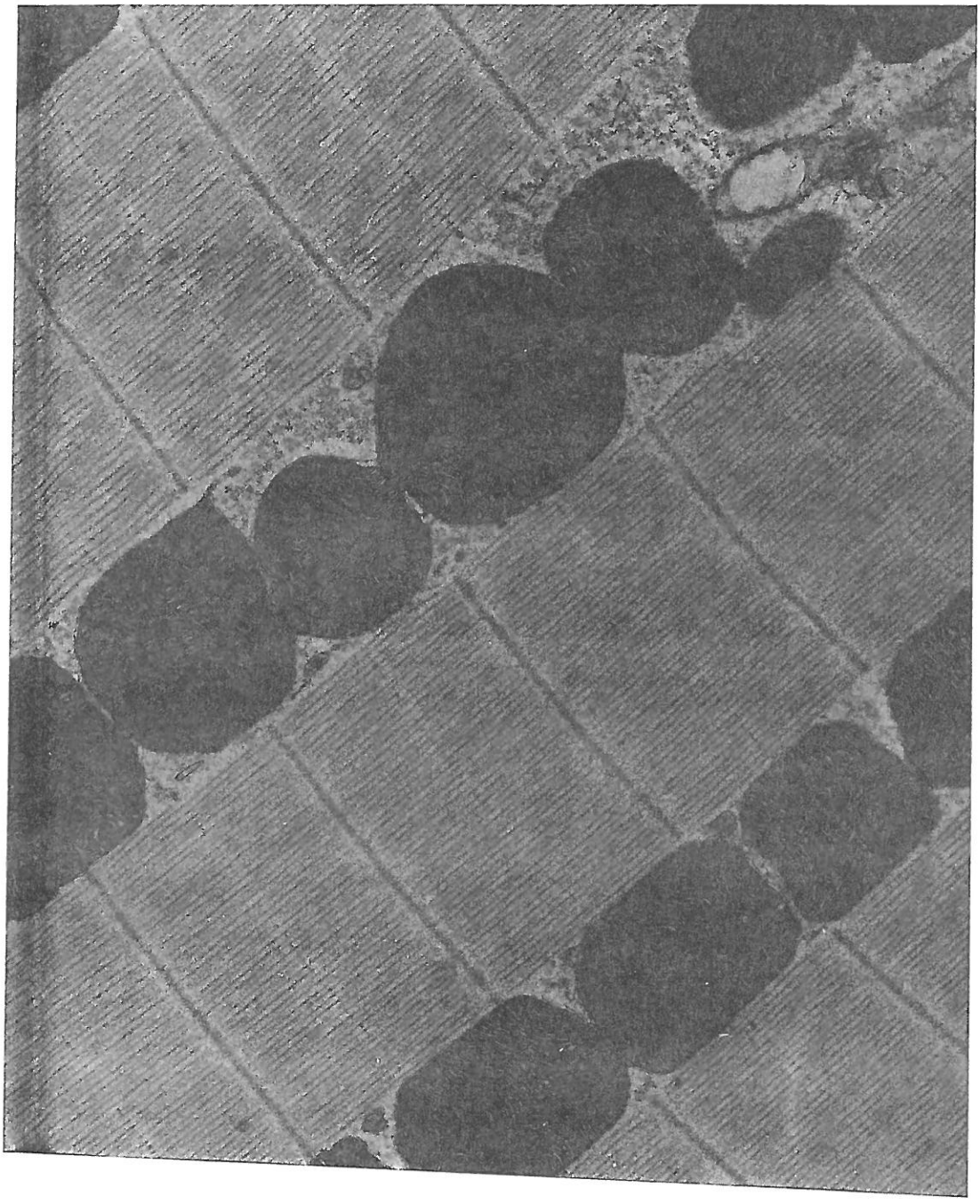

Hydrocarbon and Hydrocarbon Derived Sex Pheromones in Insects: Biochemistry and Endocrine Regulation

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INSECT LIPIDS: CHEMISTRY, BIOCHEMISTRY AND BIOLOGY

1 Abstract

Many insect species use cuticular lipid components in chemical communication. Cuticular hydrocarbons and their derivatives serve as close range and contact sex pheromones, aphrodisiacs, anti-aphrodisiacs, aggregation pheromones, kairomones, nestmate recognition cues and function in chemical mimicry. Recent work with several species of insects has led to new insights into how the production of hydrocarbons and their derivatives for use in chemical communication is regulated. The close range and contact sex pheromones of the housefly, *Musca domestica*, and the German cockroach, *Blattella germanica*, consist of modified cuticular lipids. In the housefly, the chain length of the synthesized alkenes switch from 27 carbons and longer in immature females, to 23 carbons as the female becomes vitellogenic. This 23-carbon alkene, (*Z*)-9-tricosene, is the major sex pheromone component of female houseflies. Removal of the ovaries within six hours of emergence prevents sex pheromone production, whereas reimplantation of ovaries or treatment with 20-hydroxyecdysone (20-HE) (all abbreviations defined in Table 1) restores sex pheromone production. Recent work has shown that 20-HE exerts its action by causing a change in the chain length specificity of the elongation reactions and not in the reactions that reductively convert very-long chain acyl-CoAs to hydrocarbons.

The contact sex pheromone of the German cockroach consists of the oxygenated derivatives of 3,11-dimethylnonacosane and 3,11-dimethylheptacosane. The methyl-branched hydrocarbons arise from the insertion of methylmalonyl-CoA, derived from branched-chain amino acids or succinate, in place of malonyl-CoA early in chain synthesis by a microsomal fatty acid synthase (FAS). The methyl-branched fatty acids present in the integument of the insect have the same positional isomers as do the major methyl-branched hydrocarbons. The C29 methyl-branched alkane consists of 3 isomers with 3,7-, 3,9- and 3,11-dimethyl branching patterns. Only the 3,11-dimethylnonacosane is specifically hydroxylated

Table 1
Abbreviations

JH	juvenile hormone
JHA	juvenile hormone analog
20-HE	20-hydroxyecdysone
Z9-23:Hy	(*Z*)-9-tricosene
Z9-27:Hy	(*Z*)-9-heptacosene
C23 epoxide	9,10-epoxytricosane
C23 ketone	(*Z*)-14-tricosen-10-one
PBAN	pheromone biosynthesis activating neuropeptide
18:1-CoA	oleoyl-CoA
24:1-CoA	15-tetracosenoyl-CoA
CA	corpora allata
CC	corpora cardiaca
PMSO	polysubstrate monooxygenase
FAS	fatty acid synthase

in the 2-position and then oxidized to the corresponding methyl ketone, the major sex pheromone of the female insect. The hydroxylation reaction is the sex-specific step that is regulated by juvenile hormone. This chapter summarizes the role of insect cuticular lipids in chemical communication and discusses the biology, biochemistry and endocrine regulation of sex pheromone production in the housefly and German cockroach.

2 Introduction

2.1 *Hydrocarbon and Hydrocarbon Derived Compounds in Chemical Communication*

The primary function of the cuticular lipids of insects is to restrict water loss to prevent desiccation (Hadley, 1984; Noble-Nesbitt, 1991). However, it has become recognized over the last decade that cuticular lipids, particularly the hydrocarbons, function widely in chemical communication (Howard and Blomquist, 1982; Nelson and Blomquist, 1993). Semiochemical (chemical signal) functions attributed to hydrocarbons include serving as sex attractants and aphrodisiacs, anti-aphrodisiacs, species, caste and kin recognition cues, aggregation pheromones, kairomones and undoubtedly other still undescribed functions. Cuticular lipids have been widely implicated as dipteran sex pheromones (Blomquist *et al.*, 1987b). Indeed, one-half of the 36 species of insects listed in Table 2, in which

Table 2

Cuticular lipids which function in chemical communication in insect.

Species	Function	Chemical(s) involved	References
Diptera			
Drosophila melanogaster	sex pheromone	*(Z,Z)*-7,11-heptacosadiene	(Antony and Jallon, 1982) (Antony *et al.*,1985)
D. melanogaster Canton-S	Homosexual courtship stimulation	*(Z)*-11- & *(Z)*-13-tri-triacontenes	(Schaner *et al.*,1989)
D. melanogaster Canton-S	Anti-aphrodisiac	7,11-heptacosadiene	(Scott *et al.*, 1988)
D. melanogaster Canton-S	Anti-aphrodisiac	7-tricosene	(Scott, 1986)
D. melanogaster Canton-S	Anti-aphrodisiac	7-pentacosene	(Scott and Jackson, 1988)
Drosophila americana americana	male aggregation	*(Z)*-9-heneicosene	(Bartelt *et al.*, 1986)
Drosophila americana taxana	male aggregation	*(Z)*-9-heneicosene	(Bartelt *et al.*, 1986)
Drosophila novamexicana	male aggregation	*(Z)*-9-heneicosene	(Bartelt *et al.*; 1986)
Drosophila simulans	sex pheromone	7-tricosene	(Jallon, 1984)
Drosophila virillis	male aggregation	*(Z)*-10-heneicosene	(Bartelt and Jackson, 1984)
Fannia canicularis	sex pheromone	*(Z)*-9-pentacosene	(Uebel *et al.*, 1977)
Fannia femorallis	sex pheromone	*(Z)*-11-hentriacontene	(Uebel *et al.*, 1978b)
Fannia pusio	sex pheromone	*(Z)*-11-hentriacontene	(Uebel *et al.*, 1978a)
Glossinia austeni	contact sex pheromone	15,19-dimethyltritriacontane	(Huyton *et al.*, 1980)
Glossina morsitans	contact sex pheromone	dimethyl-C37 alkane 15,19,23-trimethylhepta-triacontane	(Carlson *et al.*, 1978)

Glossinia morsitans	male anti-aphrodisiac	19,23-dimethyltri-triacont-1-ene	(Carlson and Schlein, 1991)
Glossina pallidepes	contact sex pheromone	13,23-dimethylpent-atriacontane	(Carlson *et al.*, 1984)
Lycoriella mali	sex pheromone	*n*-alkanes, C15 to C26	(Kostelc *et al.*, 1975)
Lixophaga diatraeae	larviposition kairomone	*n*-alkanes	(Thompson *et al.*, 1983)
Microdon piperi	chemical mimicry of *Camponotus modoc*	mono- & dimethyl-*(Z)*-4-alkenes	(Howard *et al.*, 1990)
Musca autumnalis	sex pheromone	*(Z)*-13- & *(Z)*-14-nona-cosene and *(Z)*-13-heptacosene	(Uebel *et al.*, 1975b)
Musca domestica	sex pheromone	*(Z)*-9-tricosene	(Carlson *et al.*, 1971)
	arrestant	methylalkanes	(Uebel *et al.*, 1976)
	sex recognition	C23 epoxide & ketone	(Uebel *et al.*, 1978a) (Adams and Holt, 1987)
	male sex pheromone	9-heptacosene, 9-nona-cosene & 11-nonacosene	(Rao *et al.*, 1990)
Phormia regina	species recognition	surface hydrocarbons	(Stoffolano *et al.*, unpub.)
Stomoxys calcitrans	mating stimulant	methyl-branched & unsat-urated hydrocarbons, C31-C33	(Uebel *et al.*, 1975a)
Hymenoptera			
Apis mellifera	kairomone for mite *Acarapis woodi*	alkanes/alkenes	(Phelan *et al.*, 1991)
	kin recognition	alkanes/alkenes	(Page *et al.*, 1991)
Camponotus floridanus	nestmate recognition	hydrocarbons	(Morel *et al.*, 1988)
Camponotus vagus	nestmate recognition	dimethylalkanes	(Bonavita-Cougourdan *et al.*, 1987)

Table 2 (*cont.*)

Species	Function	Chemical(s) involved	References
Formica selysi and *Manica rubida*	species recognition inhabit same nest	surface hydrocarbons	(Bagnères, *et al.*, 1991)
Pikonema alaskensis	sex pheromones	*(Z,Z)*-9,19-alkadienes C28-C37	(Bartelt *et al.*, 1982)
Pseudomyrmex ferrugineus	chemical mimicry for social wasp	surface hydrocarbons	(Espelie and Hermann, 1988)
Pseudomyrmex spp.	nestmate recognition	surface hydrocarbons	(Mintzer, 1989)
Solenopsis invicta	chemical mimicry for wasp *Oransema sp*	surface hydrocarbons	(Vander Meer *et al.*, 1989a)
	nestmate recognition	surface hydrocarbons	(Vander Meer *et al.*, 1989b)
Isoptera			
Reticulitermes (lucifugus) grassei and *R. (l.) banyulensis*	species recognition	surface hydrocarbons	(Bagnères *et al.*, 1991b)
Zootermopsis spp.	Species recognition prevent agonistic behavior	surface hydrocarbons	(Haverty and Thorne, 1989)
Lepidoptera			
Colias eurytheme	aphrodisiac	13-methylheptacosane	(Grula *et al.*, 1980)
Heliothis virescens	kairomone for *Cadiochiles nigriceps*	methylalkanes C31-C33	(Vinson *et al.*, 1975)
Helicoverpa zea	kairomone for *Trichogramma spp.*	C23 hydrocarbon	(Jones *et al.*, 1973) (Lewis *et al.*, 1975)

Heliothis zea	kairomone for *Microplitis croceipes*	13-methylhentriacontane	(Jones *et al.*, 1971)
Ostrinia nubilalis	kairomone for *Trichogramma nubilale*	13,17-dimethylnonatriacontane	(Shu *et al.*, 1990)
Coleoptera			
Aleochara curtula	sex pheromone	*(Z)*-7-heneicosene & *(Z)*-7-tricosene	(Peschke and Metzler, 1987) (Peschke, 1987)
Callosobruchus chinensis	copulation release pheromone	mono- & dimethylalkanes C26-C33 & acid	(Tanaka *et al.*, 1981)
Myrmicaphodius excavaticollis	chemical mimicry	acquires surface hydrocarbons from host ant	(Vander Meer and Wojcik, 1982)
Trichopsenius frosti	chemical mimicry	surface hydrocarbon pattern	(Howard *et al.*, 1980)
Dictyoptera			
Blattella germanica	contact sex pheromone	3,11-dimethylnonacosa-2-one and other derivatives	(Nishida and Fukami, 1983)
	contact sex pheromone	3,11-dimethylheptacosa-2-one	(Schal *et al.*, 1990)
Nauphoeta cinera	contact sex pheromone	surface hydrocarbons	(Takahashi and Fukui, 1980)
	intermale recognition (nauphoetin)	octadecyl *(Z)*-9-tetracosenoate	(Takahashi and Fukui, 1983)

evidence has been obtained that cuticular lipids or their derivatives serve in chemical communication, are dipterans. The largest bioactive hydrocarbons were found in the surface lipid of tsetse. The 40-carbon symmetrical trimethyl-branched alkane, 15,19,23-trimethylheptatriacontane, in female *Glossina morsitans morsitans*, stimulated copulation by males (Carlson *et al.*, 1978). Monoenes and dienes between 20 and 30 carbons compose many of the sex pheromones of *Drosophila* species and other Diptera.

A number of specialized glands also produce hydrocarbons in insects. Thus it is not always easy to determine with certainty whether the chemical of interest is a glandular secretion or is derived from cuticular lipids. We have included in Table 2 those insect species that use long-chain hydrocarbons, often unsaturated or methyl-branched, or their derivatives, in chemical communication. We omitted those species that use hydrocarbons of under 20 carbons, which are unlikely to be closely related to or derived from cuticular lipids. In the housefly and the German cockroach, biosynthetic relationships between cuticular lipid components and sex pheromones are such that it is quite certain that the pheromones are produced from a "modified" biosynthetic pathway which also produces cuticular lipids, or they are directly derived from a cuticular lipid component.

Earlier work established that certain insect species, such as the housefly, tsetse and the German cockroach used specific compounds or mixtures of a few compounds as either close range or contact sex pheromones. Likewise, certain parasitic insects use single components as kairomones to locate hosts. More recent work has shown that some species use more subtle variations in the cuticular lipid composition as species and nestmate recognition cues. Considering the diversity and number of cuticular components on the surface of insects, it is not surprising that certain species have evolved to use them as chemical cues. Indeed, the number of recent papers dealing with cuticular hydrocarbons as chemotaxonomic characters demonstrates that most insects have species-specific chemical compositions (Lockey, 1988; Nelson and Blomquist, 1993). It is expected that over the next few years many more insect species will be shown to use cuticular components in chemical communication.

2.2 *Endocrine Regulation of Sex Pheromone Production*

Females of some insect species undergo repeated reproductive cycles and mate only during specific periods within each cycle. Thus, it was suggested that sex pheromone production might be humorally mediated. Engelmann (1960) first suggested that products from the CA mediated sexual receptivity in the female cockroach *Leucophaea maderae*. Subsequent

studies with the Cuban cockroach, *Byrostria fumigata* (Barth, 1961) showed that females required functional CA to produce sex pheromone. Furthermore, it was shown that JH mediated sex pheromone production in other insects, including the cockroaches *Pycnoscelus indicus* (Barth, 1965), *Periplaneta americana* (Barth, 1965) and *Blaberus discoidalis* (Barth and Lester, 1973), and the beetles *Tenebrio molitor* (Menon, 1970, 1976), *Ips paraconfusus* (Borden *et al.*, 1969; Hughes and Renwick, 1977) and other beetles (Vanderwel and Oehlschlager, 1987). Because JH induced vitellogenesis and regulated ovarian development in many insect species, the observation that JH also regulated sex pheromone production suggested that the same hormone was used to coordinate different reproductive events (Barth and Bell, 1970; Barth and Lester, 1973).

To date, three hormones have been shown to regulate sex pheromone production in insects. In some Lepidoptera, a pheromone biosynthesis-activating neuropeptide (PBAN) induces sex pheromone biosynthesis (Raina *et al.*, 1989). In the housefly, ecdysteroids produced by the maturing ovary induce sex pheromone production (Blomquist *et al.*, 1987a). In some Blattaria (Barth, 1965; Smith and Schal, 1990; Schal and Smith, 1990), Coleoptera (Vanderwel and Oehlschlager, 1987) and Lepidoptera (Cusson and McNeil, 1989), JH regulates sex pheromone production. Until recently, it was not known which enzyme(s) in the pheromone biosynthetic pathways was regulated by hormones. In this chapter, the evidence showing that ecdysteroids regulate the chain length of alkenes is reviewed. It follows that sex pheromone production in the housefly is regulated by the chain length specificity of fatty acyl-CoA reactions. Likewise, evidence showing that JH induces a specific hydroxylase in the female German cockroach is discussed. In both cases, the same hormone that plays a central role in regulating vitellogenesis, and hence egg maturation, also induces sex pheromone production. Thus, the mating event is coordinated with egg maturation by the use of a common hormone to control both processes.

3 The Housefly Sex Pheromone: Biology, Biochemistry and Endocrine Regulation

3.1 *Housefly Sex Pheromone*

A sex pheromone was first demonstrated in the housefly by Rogoff *et al.* (1964). It was identified as (*Z*)-9-tricosene (Z9-23:Hy) and given the trivial name muscalure by Carlson *et al.* (1971). Subsequently, (*Z*)-9,10-epoxytricosane (C23 epoxide) and (*Z*)-14-tricosen-10-one (C23 ketone) (Uebel *et al.*, 1978a) and methylalkanes (Uebel *et al.*, 1978a; Rogoff *et al.*,

1980) (Fig. 1) were shown to enhance the activity of Z9-23:Hy. A detailed study by Adams and Holt (1987) showed that these pheromone components had different roles in male courtship behavior. Z9-23:Hy increased male mating strike activity toward females and other males. The non-hydrocarbon fraction, including both the epoxide and the ketone, decreased the number of homosexual mating strikes when muscalure was present. Thus, the non-hydrocarbon fraction contained sex recognition factors. Both the methylalkanes and the non-hydrocarbon fraction increased the number of copulatory attempts made by the test males (Adams and Holt, 1987). This was not observed with Z9-23:Hy. Finally, the methylalkane fraction acted as an arrestant and increased the amount of time that a male spent with a treated model.

3.2 *Correlation of Pheromone Production with Oogenesis*

Whereas newly emerged female houseflies do not have detectable amounts of any of the C23 sex pheromone components (alkene, epoxide or ketone), by day 4 they have large quantities of these as well as increased levels of methylalkanes. To examine the timing of sex pheromone production, both the amounts and relative rates of biosynthesis of sex pheromone components were monitored during oogenesis (Dillwith *et al.*, 1983). Females with previtellogenic ovaries did not produce detectable amounts of any of the C23 sex pheromone components. Z9-23:Hy, C23 epoxide and C23 ketone first appeared on females with early vitellogenic (stage 4) ovaries and increased to a maximum by ovarian stage 8.

These data were consistent with observations correlating female attractancy in an olfactometer to the stage of oocyte development. Females with stage 2 or 3 ovaries (previtellogenic) did not attract males, but females with ovaries at stages 4 through 10 readily attracted males (Adams *et al.*, 1984a). Thus, the behavioral data correlated with the timing of sex pheromone production determined by chemical analyses.

The sex pheromone of the housefly consists of modified cuticular lipid components. Newly emerged males and females have similar cuticular lipid profiles: the major hydrocarbon components are (*Z*)-9-alkenes of 27 carbons and longer. During egg stages 4 through 8 both the percentage of alkenes and the percentage of Z9-27:Hy in the alkene fraction decrease while the amount of methyl-branched alkanes and Z9-23:Hy increase. Since Z9-23:Hy is readily converted to both the C23 epoxide and ketone by both sexes at all ages (Blomquist *et al.*, 1984a; Ahmad *et al.*, 1987), the increase in Z9-23:Hy is mirrored by a concomitant increase in the C23 epoxide and ketone.

The incorporation of radioactivity from labeled acetate into the C23 sex pheromone components followed a pattern consistent with the pattern

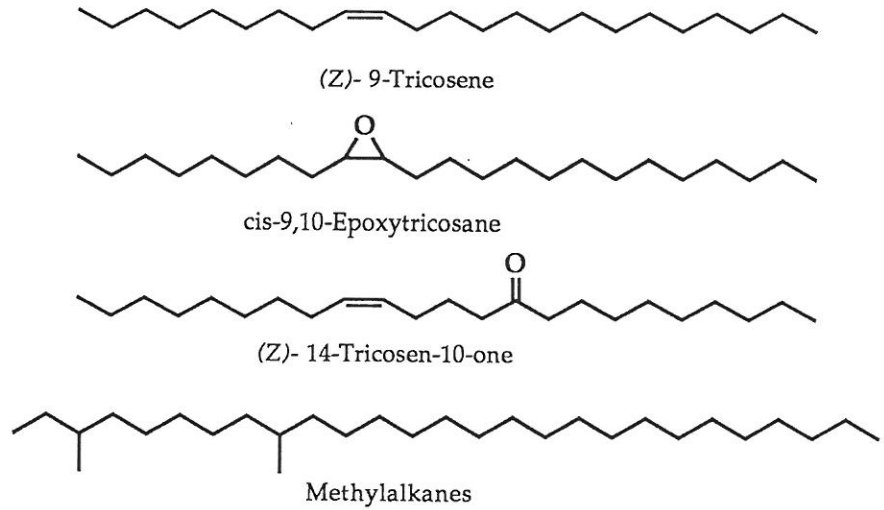

Fig. 1. Components of the sex pheromone of the female housefly.

described above and emphasized the role that the maturing ovary plays in initiating sex pheromone production (Dillwith *et al.*, 1983). Thus, both mass studies and radiotracer experiments documented the association of ovarian maturation with sex pheromone production.

3.3 *Endocrine Regulation of Sex Pheromone Production*

Since pheromone production was correlated with ovarian development, it was possible that both processes were regulated by a common factor. In some cockroaches and beetles, for example, JH induces vitellogenesis and sex pheromone production. Alternatively, a product of the developing ovary could initiate pheromone production. To determine which occurs in the housefly, the effects on sex pheromone production of removing either the corpus allatum-corpus cardiacum (CA-CC) complex or ovaries were examined. Female houseflies that had the CA-CC complex removed within 6 hr of eclosion produced Z9-23:Hy, C23 epoxide and C23 ketone, and developed ovaries to stage 4 when assayed at 4, 5 and 6 days postemergence (Blomquist *et al.*, 1992). This demonstrated that JH was not directly required for pheromone production. Further work with the dipteran JH (6,7;10,11-bisepoxy JH III), a novel JH described in higher Diptera, including the housefly (Richard *et al.*, 1989), showed that it had no effect on sex pheromone production (Blomquist *et al.*, 1992).

In contrast to CA-CC removal, flies ovariectomized within 6 hr of emergence did not produce detectable amounts of any of the C23 sex

pheromone components. If a product from the developing ovary were involved directly or indirectly in sex pheromone synthesis, then implanting ovaries into ovariectomized insects should restore sex pheromone production. When insects which were ovariectomized within 6 hrs of emergence were implanted with ovaries on day 4 and then maintained for 3 days, they then produced the C23 sex pheromone components at levels similar to females with stage 5 ovaries. The implants reached the vitellogenic phases of development with the stage varying from 4-6 (Adams *et al.*, 1984b). Thus, it was concluded that a product from the ovary stimulated sex pheromone production.

3.4 *Ecdysteroids Induce Sex Pheromone Production in the Housefly*

Ecdysteroids play a role in regulating reproductive processes, including vitellogenin synthesis (Bownes, 1982; Adams *et al.*, 1985; Hagedorn, 1985; Adams *et al.*, 1989; Agui *et al.*, 1991) in a number of Diptera. To determine if ecdysteroids would restore sex pheromone production in ovariectomized flies, a single large dose (10 μg/insect) of 20-HE was injected into insects which had been ovariectomized within 6 hr of emergence. The incorporation of labeled acetate into the C23 sex pheromone components was determined at various intervals after treatment with 20-HE. At 18 and 24 hrs after 20-HE treatment, flies produced all of the C23 sex pheromone components (Adams *et al.*, 1984b). 20-HE has a very short half-life in houseflies. Thus, repeated treatments of ovariectomized flies with 20-HE at low dosages were performed. The results showed that 20-HE at doses as low as 50 ng repeated every 6 hrs induced sex pheromone production. Thus, it was concluded that ovarian-produced ecdysteroids induced sex pheromone production in the epidermal tissue of the housefly.

3.5 *Induction of Sex Pheromone Production in Male Houseflies*

Vitellogenin is normally produced only in females, but the injection of 20-HE into males of *Drosophila melanogaster* (Bownes, 1982) and *Sarcophaga bullata* (Huybrechts and De Loof, 1977) induced vitellogenin production. Thus, experiments were performed to determine whether male houseflies, which do not normally produce any of the C23 sex pheromone components, could be induced to produce the female-specific sex pheromone after ovary implants or injections of 20-HE.

Implanting ovaries or injecting 20-HE into male houseflies induced sex pheromone production, including all of the C23 sex pheromone components (Blomquist *et al.*, 1984b). The identity of the C23 pheromone components from induced males was verified by gas chromatography-mass

spectrometry. Control males did not produce any of the C23 pheromone components. These data demonstrate that males possess the biosynthetic capability for sex pheromone production, and that treatment with 20-HE alters the production of cuticular components such that Z9-23:Hy becomes a major product. Males at all ages readily metabolize Z9-23:Hy to the corresponding epoxide and ketone (Blomquist *et al.*, 1984b) via a cytochrome P-450 enzyme (Ahmad *et al.*, 1987).

Further evidence that ecdysteroids directly induce sex pheromone production came from studies in which isolated male abdomens were injected with 20-HE, and after a 24-hr period, they were assayed for the ability to produce pheromone. The results of this experiment showed that 20-HE induced sex pheromone production in isolated abdomens (Blomquist *et al.*, 1992). These data demonstrate that a head factor or other hormones are not required to induce sex pheromone production. Additional studies with combinations of 20-HE and JH further demonstrated that JH did not synergize the effect of 20-HE (Blomquist *et al.*, 1992). The data clearly demonstrate that sex pheromone induction is achieved solely by 20-HE.

3.6 *Biosynthesis of the Housefly Sex Pheromone*

Sex pheromone-producing tissue in insects varies in complexity from individual unicellular glands distributed throughout the integument to elaborate internal cellular glands (Percy-Cunningham and MacDonald, 1987). Schlein *et al.* (1980) reported that unicellular glands on the legs of female houseflies synthesized the sex pheromone components. Subsequent work (Dillwith and Blomquist, 1982; Dillwith *et al.*, 1981) using isolated tissue and radio-tracer techniques demonstrated that the sex pheromone components of the housefly were synthesized by epidermal tissue. Because the pheromone components are modified cuticular lipid components, it is not surprising that they are formed by epidermal tissue (Blomquist *et al.*, 1987a).

Z9-23:Hy formation in female houseflies occurs by the microsomal elongation of oleoyl-CoA to a 24 carbon fatty acyl moiety which is then converted to an alkene one carbon shorter (Fig. 2). The elongation of oleoyl-CoA requires malonyl-CoA and NADPH, and the highest elongation activity is in abdominal epidermal tissue (Vaz *et al.*, 1988).

3.7 *Chain Length Specificity: Elongation Reactions*

The regulation of Z9-23:Hy production was studied by examining the chain length specificity of the fatty acyl-CoA elongation reactions and the reductive conversion of 15-tetracosenoyl-CoA (24:1-CoA) to Z9-23:Hy in one- and four-day old male and female houseflies (Tillman-Wall *et al.*,

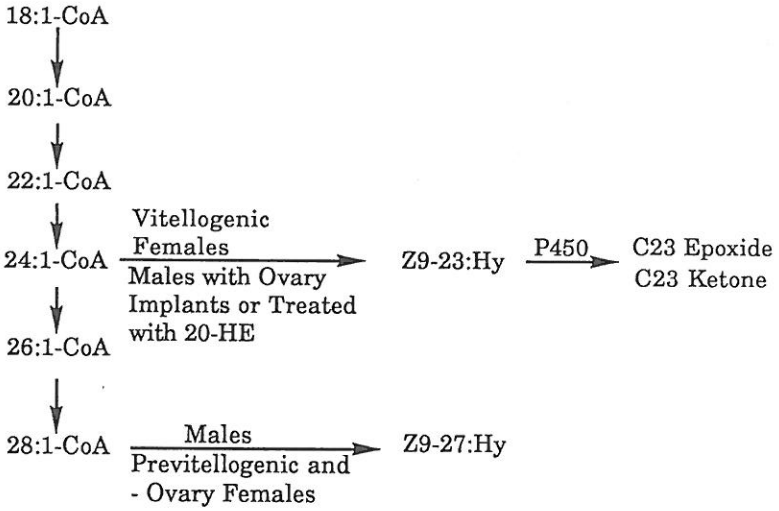

Fig. 2. Biosynthesis of the housefly sex pheromone components.

1992). Microsomal preparations from four-day old female insects produced Z9-23:Hy as the predominant alkene when incubated with malonyl-CoA, NADPH and [9,10-$^3H_2$]oleoyl-CoA (18:1-CoA), whereas microsomal preparations from four-day old male insects produced predominantly Z9-27:Hy. These are the major alkenes produced *in vivo* by day 4 females and males, respectively. Microsomes prepared from day 1 males and females produced Z9-27:Hy as the major alkene from labeled 18:1-CoA. This is the major alkene produced in vivo by both sexes at day 1. Hydrocarbon production is highly dependent upon 18:1-CoA concentration, with the highest rate occurring at 5 μM acyl-CoA and very low rates at concentrations above 20 μM.

The elongation of fatty acyl-CoA's occurs via condensation of the acyl-CoA with malonyl-CoA, with subsequent reduction and dehydration, to produce the final fatty acyl-CoA two carbons longer (Fig. 3). In rat microsomes, separate elongation systems have been proposed for the elongation of saturated and unsaturated fatty acids. The specificity is achieved at the condensation step (Bernert and Sprecher, 1977). Likewise, Goldberg *et al.* (1973) have provided evidence that there are at least two elongation systems in mouse brain microsomes: one involved in elongating 16:0 and another functioning with longer-chain fatty acids. Data from work on the elongase systems which produce very long-chain fatty acids in plants indicate that there are at least 3 elongation systems with different chain length specificities (Agrawal and Stumpf, 1985; Walker and Harwood, 1986). In order to determine if ecdysone induced changes in the

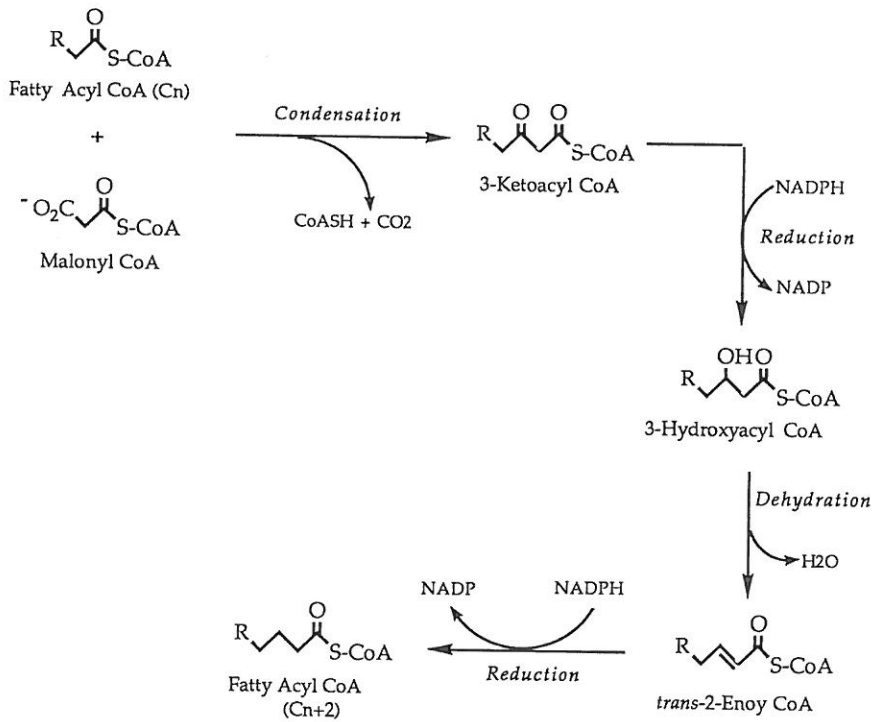

Fig. 3. Individual steps in the fatty acyl-CoA elongation reaction.

chain length specificity of the elongation reactions that could account for sex pheromone production, the elongation of labeled 18:1-CoA and 24:1-CoA was compared in day 1 and day 4 male and female houseflies. Day 1 male and female insects were similar in both total elongation activity and the chain length specificity of elongated products (Tillman-Wall *et al.*, 1992). In contrast, day 4 females did not efficiently elongate either 18:1-CoA or 24:1-CoA to fatty acyl groups longer than 24:1 (Fig. 4). However, day 4 males readily elongated both 18:1-CoA and 24:1-CoA to 28:1 (Fig. 4). These differences in the chain length specificity of the fatty acyl-CoA elongation reactions between day 4 males and females provide strong evidence that ecdysone exerts its regulatory effect at the elongation steps, perhaps via a repression of 24:1-CoA elongation.

The reductive conversion of 24:1-CoA to Z9-23:Hy was also examined in an attempt to recognize any ecdysone-mediated change in chain length specificity at this step(s). There is an abundance of evidence showing that hydrocarbon biosynthesis in insects occurs via the elongation of C16 and

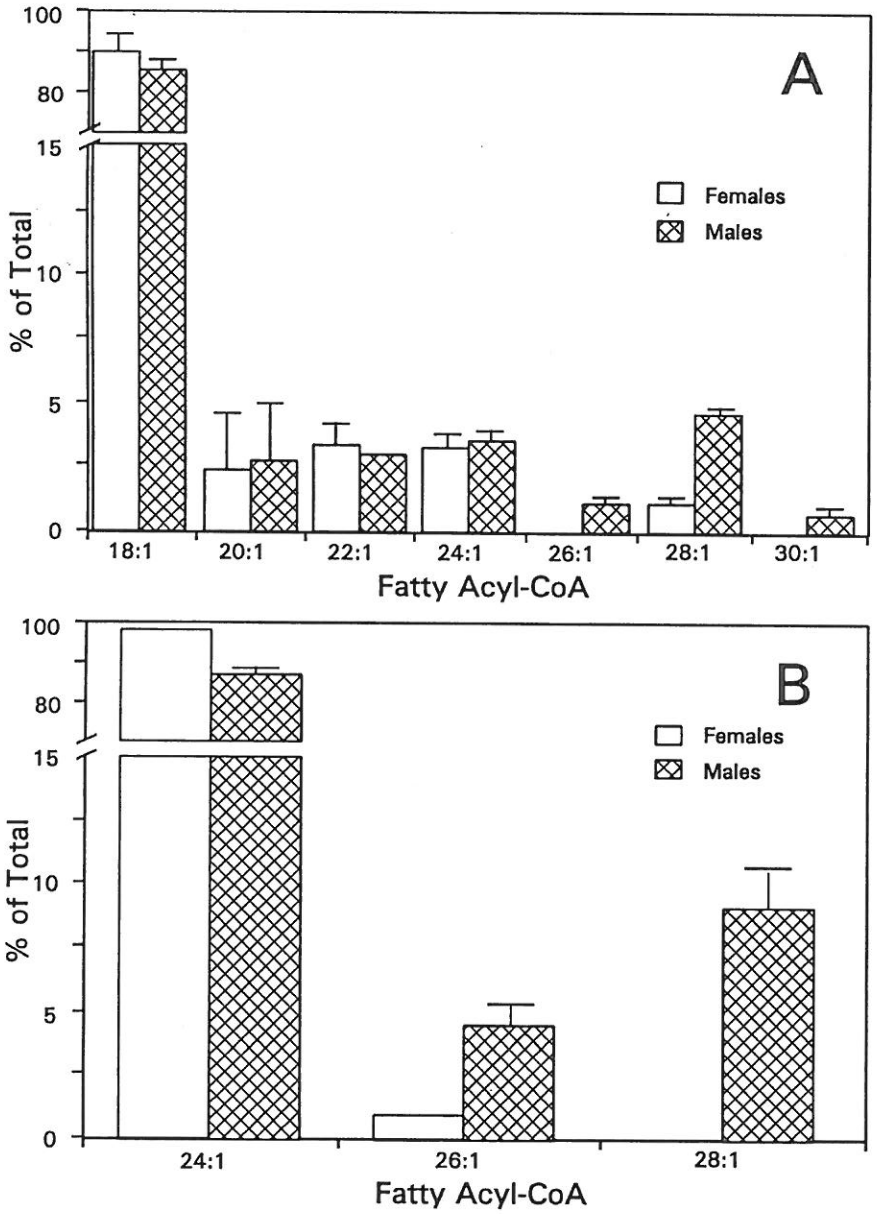

Fig. 4. Elongation of 18:1-CoA (A) and 24:1-CoA (B) by microsomes of four day old male and female houseflies. Data taken from Tillman-Wall *et al.* (1992).

C18 fatty acyl-CoAs to longer-chain acyl CoA's, with subsequent conversion to hydrocarbon one carbon shorter (Blomquist and Dillwith, 1985). Product versus time kinetic studies (Z9-23:Hy produced/time) at various 24:1-CoA concentrations were performed which showed that there was essentially no difference between microsomes prepared from day 4 male and female insects in Z9-23:Hy production.

Based on the results of these studies, we propose that ecdysone or an ecdysone-related factor suppresses the second of two elongation systems; the elongase that catalyzes elongation of 24:1-CoA to longer-chain moieties. This repression of 24:1-CoA elongation would allow an increased substrate pool for reductive conversion of 24:1-CoA to sex pheromone, Z9-23:Hy.

Recent studies have examined which specific step of the overall elongation reaction (Fig. 3) is endocrine-regulated during pheromone induction. Because the first step in many biochemical pathways is often the regulated or rate-determining step, initial studies have focused on acyl-CoA condensation with malonyl-CoA, and have involved comparison of the amount of 18:1- and 24:1-CoA condensation with malonyl-CoA in day 4 male and female. Preliminary data (Tillman-Wall, Kuenzli, Vanderwel and Blomquist, unpublished) have indicated that day 4 females have markedly decreased condensation activity with 24:1-CoA compared to day 4 males, thus lending support to the hypothesis that ecdysone represses the elongation activity that elongates 24:1-CoA.

3.8 *Mechanism of Alkene Formation in the Housefly*

The mechanism by which 24:1-CoA is reductively converted to Z9-23:Hy in the housefly has proven elusive. Kolattukudy and coworkers (Bognar *et al.*, 1984; Cheesbrough and Kolattukudy, 1984, 1988; Dennis and Kolattukudy, 1991) clearly showed that in plants and in an alga, hydrocarbons arise from the reduction of a fatty acyl-CoA to the aldehyde which is then decarbonylated to the hydrocarbon and carbon monoxide. In work on the housefly, we have obtained data demonstrating that 24:1-CoA is reduced to the aldehyde, and that the labeled aldehyde, at very low levels and under strict anaerobic conditions, is converted to the alkene. Furthermore, when [3,3-D_2, 3-^{13}C] and [2,2-D_2, 2-^{13}C]24:0-CoA are incubated with microsomes from male houseflies, chemical ionization mass spectral analysis showed that the deuteriums at positions 2 and 3 are retained intact when the fatty acyl-CoA is converted to an alkane one carbon shorter (Reed, Vanderwel, Pomonis, Choi, Reitz and Blomquist, unpublished). This observation is consistent with a decarbonylation reaction. However, when [1-^{14}C]24:1 is incubated with microsomal preparations under conditions where [^{3}H]24:1 is readily converted to Z9-

23:Hy, the label is recovered as $^{14}CO_2$ and not as ^{14}CO. When [1-^{14}C] and [15,16-^{3}H]24:1 are co-incubated, a 1/1 ratio of labeled alkene to CO_2 is obtained. Furthermore, when ^{14}CO is incubated with a microsomal preparation, it is not converted to $^{14}CO_2$. Thus, although the evidence favors a reductive decarbonylation mechanism for hydrocarbon production in the housefly, the data are not conclusive. It has been reported that 1-alkenes from both plants and insects are formed from a decarboxylation type reaction (Görgen *et al.*, 1989, 1990).

3.9 *Metabolism of Z9-23:Hy to Epoxide and Ketone*

Components of the housefly sex pheromone, (*Z*)-9,10-epoxytricosane (C23 epoxide) and (*Z*)-14-tricosen-10-one (C23 ketone) occur only in females and their appearance correlates closely with Z9-23:Hy synthesis (Blomquist *et al.*, 1984a). The application of [9,10-^{3}H]Z9-23:Hy to the surface of the female housefly resulted in the formation of radiolabeled C23 epoxide and C23 ketone (Blomquist *et al.*, 1984a). These findings showed that both the C23 epoxide and ketone are derived from Z9-23:Hy.

Both females and males of all ages metabolized Z9-23:Hy to C23 epoxide and ketone when it was applied to the surface of the insect (Blomquist *et al.*, 1984a). The transfer of Z9-23:Hy to males during the mating strike and subsequent copulation was studied by applying high specific activity [9,10-^{3}H]Z9-23:Hy to females and then determining the amount transferred to the males. The transferred Z9-23:Hy was metabolized by the males to C23 epoxide, C23 ketone and more polar metabolites (Ahmad *et al.*, 1989). Evidence was obtained that demonstrated the involvement of a cytochrome P-450 polysubstrate monooxygenase (PSMO) in the conversion of the alkene to the epoxide and ketone. Subcellular fractionation studies showed that the majority of the PSMO activity for the conversion of Z9-23:Hy to epoxide and ketone is associated with the microsomal fraction (Ahmad *et al.*, 1987). Well-known and commonly used inhibitors of the microsomal PSMO, a methylene dioxyphenyl compound, piperonyl butoxide and carbon monoxide inhibit both the *in vivo* and *in vitro* metabolism of Z9-23:Hy to epoxide and ketone (Ahmad *et al.*, 1987).

All major body parts of both females and males metabolized Z9-23:Hy when it was applied to the surface (Blomquist *et al.*, 1984a), which suggested that this PSMO is generally distributed. It appears that the metabolism of Z9-23:Hy to the epoxide and ketone is a pheromone biosynthetic step in females and a first step in pheromone catabolism in males.

It appears that the same enzyme catalyzes both the epoxidation at the double bond between carbons 9 and 10 of Z9-23:Hy and the hydroxylation

at carbon 14. The secondary alcohol thus formed is then converted to the unsaturated ketone (Guo *et al.*, 1991). Analogs of Z9-23:Hy bearing methyl substituents, cyclopropyl groups, fluorine substituents, and additional double bonds at the carbon 14 position (Latli and Prestwich, 1991) were used to probe the substrate specificity for the PSMO systems. Compounds with one or two fluoro groups or a methyl substituent at the 14 position of Z9-23:Hy were metabolized to the corresponding epoxide. However, compounds with two methyl groups, a cyclopropane group, a hydroxy group, or an additional double bond at the 14-position were not epoxidized at carbons 9 and 10 (Guo *et al.*, 1991). These results demonstrated that the PMSO which metabolizes Z9-23:Hy in the housefly has very strict structural requirements for the substrate. Analogs of Z9-23:Hy were also tested as potential inhibitors for Z9-23:Hy metabolism. The two compounds that were the most effective inhibitors in both female and male houseflies were (*Z*)-14-tricosen-10-one (a product of the reaction) and 1-nonyl-1-[(*Z*)-4-tetradecen-1-yl]-cyclopropane (Guo *et al.*, 1991).

3.10 *Methylalkane Biosynthesis in the Housefly*

The biosynthetic pathways for the methylalkane components of the housefly sex pheromone involve insertion of a methylmalonyl-CoA unit in place of a malonyl-CoA at specific points during the elongation process. Both radio-tracer work and studies monitoring the incorporation of stable isotopes by ^{13}C NMR and mass spectrometry (Blomquist *et al.*, 1987b; Dillwith *et al.*, 1981; 1982) demonstrated that a propionate unit derived from the amino acids valine, isoleucine and methionine is incorporated during the early steps of chain-elongation. The housefly does not have detectable levels of vitamin B_{12} (Wakayama *et al.*, 1984) and therefore cannot use succinate as a precursor to the methyl-branch unit. The lack of vitamin B_{12} has also apparently resulted in the housefly and other insect species (Halarnkar and Blomquist, 1989) evolving a different pathway from that of vertebrates for propionate catabolism. In the housefly, propionate, as the CoA derivative, is first dehydrogenated, then hydrated to 3-hydroxypropionate and finally oxidized to acetate with the loss of carbon 1 (Halarnkar *et al.*, 1986).

4 The German Cockroach Contact Sex Pheromone: Biology, Biochemistry and Endocrine Regulation

4.1 *German Cockroach Contact Sex Pheromone*

Nishida and Fukami (1983) summarized work on the isolation, identification and behavioral activity of components of the sex pheromone

of the German cockroach. The three components were identified as (S,S)-3,11-dimethylnonacosan-2-one and its derivatives which contain an alcohol or an aldehyde group at the 29 position (Fig 5). Each of the three components elicits the full range of behavioral responses in males, as do all combinations of stereochemical isomers at the 3,11 positions. Antennal contact with a female containing the sex pheromone elicits a "wing raising" behavior in males which is a required step in the courtship and mating of German cockroaches.

Recent work has identified another component of the sex pheromone from the German cockroach. Characterization of the cuticular hydrocarbons from this insect showed that major components were 3,11-, 3,9-, and 3,7-dimethylnonacosane (Augustynowicz *et al.*, 1987) and minor components were 3,11- and 3,9-dimethylheptacosane. One of the isomers of both homologs had the same methyl branching pattern as the reported sex pheromone. We re-examined the methyl ketone fraction from the German cockroach and showed that, in addition to 3,11-dimethylnonacosan-2-one, it contained 3,11-dimethylheptacosan-2-one (Jurenka *et al.*, 1989). Isolation and bioassay of the 3,11-dimethylheptacosan-2-one showed that it clearly elicited the wing-raising behavior in male cockroaches (Schal *et al.*, 1990), although higher levels of pheromone were needed to elicit the same response. The discovery of this fourth component provided insight into the biosynthetic pathways of the sex pheromone.

4.2 *Biosynthesis of the German Cockroach Sex Pheromone*

In order to examine the site of synthesis and transport of hydrocarbon in the German cockroach, labeled propionate, which labels only methyl-branched components, was injected into insects. The distribution of radioactivity was then analyzed in the cuticular lipids, epidermal cells, and internally (which includes hemolymph) after various incubation times. The data showed that the first site to contain labeled hydrocarbon was the epidermal tissue. This was followed by an increase in labeled hydrocarbon internally, and then after an interval, radiolabeled hydrocarbon increased on the surface of the insect (Quilici, Juárez, Schal and Blomquist, unpublished data). These data show that after synthesis by epidermal cells, hydrocarbon is then transported by hemolymph, presumably via lipophorin, and then is transported to the cuticle by an unknown route. It had been thought (Blomquist and Dillwith, 1985) that hydrocarbon is transported from the epidermal cells directly to the surface through pore and wax canals; the relative contribution of each route is still unknown. Recent data indicate that large amounts of hydrocarbons are incorporated into the developing oocytes and are found in the oviposited egg case (Schal

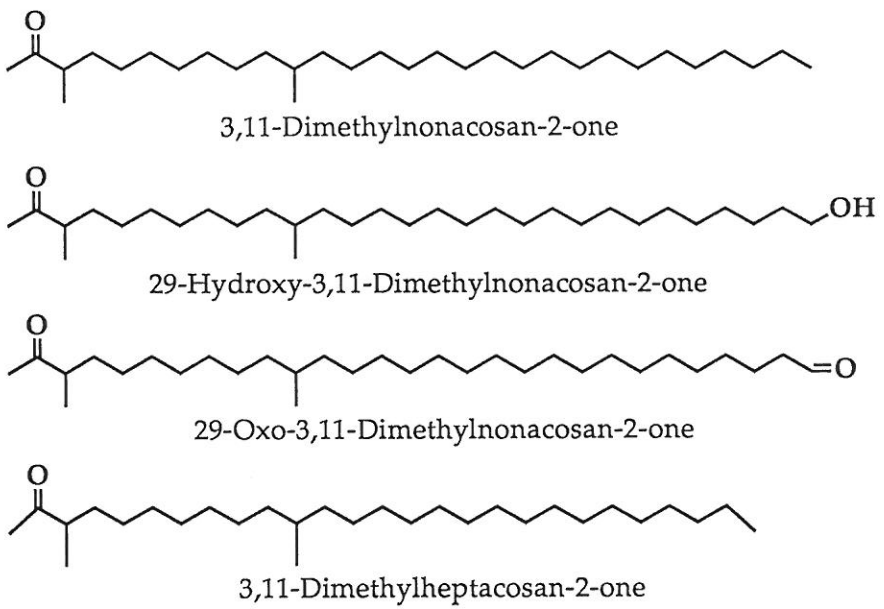

Fig. 5. Components of the contact sex pheromone of the female German cockroach.

et al., 1993) in the German cockroach. Ovarian hydrocarbons are qualitatively identical to integumentary hydrocarbons, suggesting that hemolymph transport of hydrocarbons may serve to both deliver hydrocarbons to developing oocytes as well as to redistribute newly synthesized hydrocarbons to the cuticular surface. *In vitro* studies using isolated tissues showed that both dorsal and ventral abdominal epidermal tissue from females metabolized labeled 3,11-dimethylnonacosane to the corresponding ketone, with no other tissue showing this activity. Thus, hydrocarbon and pheromone synthesis occur in epidermal tissue.

Work from our laboratory over the past several years using radiotracer and carbon-13 NMR techniques examined the precursors and directionality of biosynthesis of the methyl-branched cuticular hydrocarbons and the female contact sex pheromone (Chase *et al.*, 1990). The incorporation of labeled valine, isoleucine and methionine into hydrocarbon and sex pheromone demonstrated that these amino acids serve as precursors to the methylmalonyl-CoA which serves as the methyl-branch group donor (Fig. 6). Further evidence supporting this view came from carbon-13 NMR data showing that the labeled carbons from [3,4,5-$^{13}C_3$]valine labeled the tertiary carbon, the carbon adjacent to the tertiary

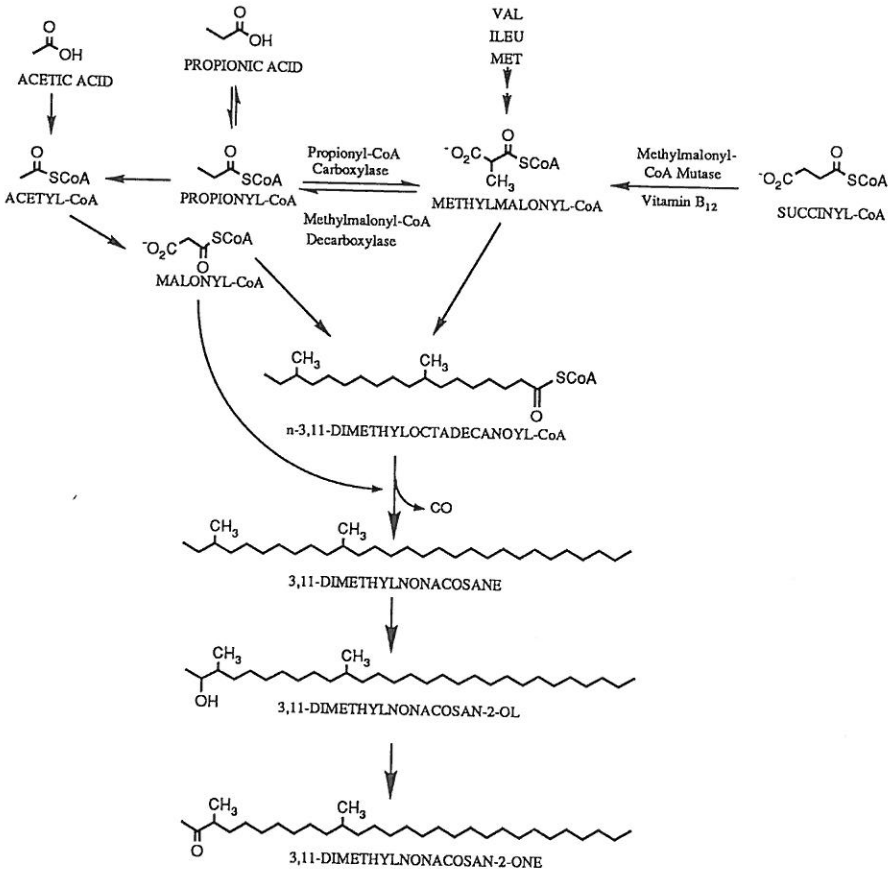

Fig. 6. Biosynthesis of the hydrocarbons and contact sex pheromone of the German cockroach.

carbon and the methyl-branch carbon of the pheromone. In addition, a comparison of the incorporation of [1,4-$^{14}C_2$]succinate and [2,3-$^{14}C_2$]succinate into the branched hydrocarbons and other lipids indicated that succinate also serves as a precursor to methylmalonyl-CoA (Fig. 6). Carbon-13 NMR studies showed that [1-^{13}C]propionate labeled the carbon adjacent to the tertiary carbon, and, for the 3,x-dimethylalkanes, that carbon-4 but not carbon-2 was enriched. This indicates that the methyl-branching groups of the 3,x-dimethylalkanes, as is the case in the housefly (Dillwith *et al.*, 1982), and for 3-methylalkanes in the American cockroach (Dwyer *et al.*, 1981), were inserted early in the chain-elongation process.

This observation suggested that methyl-branched fatty acids were precursors of the methyl-branched hydrocarbons, but until recently, such fatty acids had not been detected in insects. Therefore, it was of considerable interest that methyl-branched fatty acids with the same methyl branching positions as those observed for the major hydrocarbons, including n-3-, n-4-, n-5-, n-7-, n-8-, and n-9-monomethyl fatty acids and n-5,9-, n-3,9-, and n-3,11-dimethyl fatty acids with 16 to 20 carbons, were detected in the integument tissue of female German cockroaches (Juárez *et al.*, 1992). These fatty acids were present at very low levels and could be analyzed by GC-MS as their hydrocarbon derivatives only after removing the straight-chain components by molecular sieve (Juárez *et al.*, 1992).

Several lines of evidence have indicated that a microsomal fatty acid synthase might be involved in hydrocarbon production. In the cabbage looper, *Trichoplusia ni*, high rates of methyl-branched hydrocarbon synthesis were observed at times when soluble fatty acid synthase activity was very low (de Renobales *et al.*, 1989). Carbon-13 studies have shown that all the carbons in Z9-23:Hy in the housefly (Dillwith *et al.*, 1982), 3-methylpentacosane in the American cockroach (Dwyer *et al.*, 1981) and mono- and dimethylalkanes in the German cockroach (Chase *et al.*, 1990) were labeled. This suggested that the entire molecule was assembled in one place, presumably the endoplasmic reticulum.

The labelling studies provided direct evidence that a microsomal FAS is involved in hydrocarbon synthesis (Juárez *et al.*, 1992). A FAS in the microsomal fraction obtained from integument tissue of the German cockroach was able to incorporate [methyl-^{14}C]methylmalonyl-CoA into methyl-branched fatty acids. Moreover, the microsomal FAS has much higher ability to incorporate methylmalonyl-CoA into fatty acids than does the soluble FAS (Gu *et al.*, 1993). The microsomal FAS incorporated 0.26 mole of methylmalonyl-CoA by consuming one mole NADPH, whereas the soluble FAS only incorporated 0.015 mole of methylmalonyl-CoA under the same conditions.

Kinetic studies on the microsomal and soluble FAS revealed clear-cut differences between them in substrate preference (Gu *et al.*, 1993). The soluble FAS has almost no activity with methylmalonyl-CoA as the only elongating substrate whereas the microsomal FAS readily utilized methylmalonyl-CoA in the absence of malonyl-CoA. In contrast, the soluble FAS has a much higher activity with malonyl-CoA as substrate than does the microsomal FAS. The specific activity for soluble FAS is 144 ± 7 nmole NADPH oxidized/min/mg protein compared to only 20 ± 1 nmol/min/mg for the microsomal FAS. These comparisons between the two FASs strongly suggest that the microsomal FAS rather than soluble FAS plays a key role in methyl-branched fatty acid biosynthesis.

The mechanism of methyl-branched fatty acid synthesis in integument tissue of the German cockroach is quite different from the mechanism of uropygial gland FAS of goose (Buckner and Kolattukudy, 1976). Multimethyl-branched fatty acid synthesis results from the presence of a high methylmalonyl-CoA to malonyl-CoA ratio in the uropygial gland, and thus FAS uses only methylmalonyl-CoA to synthesize the multimethyl-branched fatty acids. In contrast, in the German cockroach, methyl-branched fatty acids with one or several methyl branches at specific points appear to be synthesized by a special FAS which is located in the microsomal fraction of integument tissue. The methyl-branched fatty acids are then presumably elongated and converted to hydrocarbon by microsomal enzymes in integument tissue.

4.3 *Conversion of 3,11-Dimethylnonacosane to Sex Pheromone*

The methyl ketone sex pheromone arises from the conversion of [11,12-$^3H_2$]3,11-dimethylnonacosane to 3,11-dimethylnonacosan-2-one via an alcohol intermediate (Chase *et al.*, 1992) (Fig. 6). The data indicated that a specific oxidase acts on the dimethylalkane at the 2 position to produce the alcohol and ketone and suggested that a similar oxidase might work on the 29 position to produce the 29-hydroxy-methyl ketone.

4.4 *Endocrine Regulation of Sex Pheromone Production in the German Cockroach*

In the German cockroach, the *in vivo* synthesis of sex pheromone and its accumulation on the cuticle are correlated to the assayed *in vitro* synthesis of JH by the CA and to oocyte development, suggesting common regulation by JH of sex pheromone production as well as other reproductive events (Schal *et al.*, 1991). The patterns of pheromone and hydrocarbon production in starved, allatectomized and head-ligated females, as well as in females rescued with hormone-replacement therapy, suggest two mechanisms of regulation of sex pheromone production: a JH-induced conversion of hydrocarbon to sex pheromone is related to the CA cycle and to oocyte development, while a JH-independent process, which is probably related to feeding, supplies precursors for hydrocarbon biosynthesis (Schal *et al.*, 1991).

Dependence of pheromone synthesis on JH levels is supported by the following findings: (a.) the pattern of accumulation of 3,11-dimethylnonacosan-2-one and 3,11-dimethylheptacosan-2-one on the cuticle is related to the pattern of JH synthesis through two gonotrophic cycles (Schal *et al.*, 1993), (b.) the rates of synthesis of methyl ketones, using labeled propionate, correspond to rates of JH synthesis (Schal *et al.*, 1991, 1993), (c.) pheromone production declines in allatectomized females and females with experimentally inhibited CA (e.g., starved, protein deprived, ootheca implanted, isolated), but JHA restores pheromone production in these females (Schal *et al.*, 1990).

However, whereas pheromone production is completely suppressed in other allatectomized cockroaches (Schal and Smith, 1990; Smith and Schal, 1990), allatectomized *B. germanica* females produce some pheromone (Schal *et al.*, 1990). Because JHAs are also less effective inducers of pheromone production in unfed females, we hypothesized that feeding might influence pheromone production by influencing the availability of substrate (hydrocarbon precursor) (Schal *et al.*, 1991). Our recent results lend support to this notion. The pattern of hydrocarbon synthesis generally corresponds to the pattern of feeding, with high rates in the first few days after the imaginal molt and low rates during maximal oocyte maturation and during pregnancy (Schal *et al.*, 1993). Since allatectomized females feed less, they synthesize less hydrocarbons and less methyl ketone pheromone. Without an ootheca, feeding continues in older allatectomized females, as does hydrocarbon synthesis. However, without an ovarian sink for internal hydrocarbons, deposition of hydrocarbons on the cuticle increases significantly, as does deposition of methyl ketone pheromone. Thus, accumulation of cuticular pheromone may result from a long-term mechanism involving feeding-induced hydrocarbon synthesis (precursor accumulation internally) and a stage-specific conversion of hydrocarbon to pheromone that is JH-mediated.

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