Structure and metabolism of cuticular lipids of the grasshopper melanoplus sanguinipes
by Gary James Blomquist

A thesis submitted to the Graduate Faculty in partial Fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Chemistry
Montana State University

Abstract:
The cuticular lipids of the grasshopper Melanoplus sanguinipes contain 60 percent hydrocarbons and 28 percent secondary alcohol wax esters, with lesser amounts of normal and sterol wax esters, triglycerides, alcohols, sterols, and free fatty acids. All the hydrocarbons are saturated and four types of alkanes are present: n-alkanes (61 percent), 3-methyl alkanes (trace amounts), internally branched monomethylallcanes (23 percent), and internally branched dimethylalkanes (16 percent). The principle n-alkanes are C29 and C27, with a range from C21 to C33. Trace amounts of 3-methyl alkanes of 28, 30, and 32 total carbons are present. The principle internally branched monomethylalkanes are C32 and C34, whereas the major dimethylaIkane contains 35 carbons. The total number of carbons in the secondary alcohol wax esters range from 37-54 with 41 predominating. The fatty acids of the wax esters are 18:0, 20:0, 16:0, 22:0, 14:0, 19:0, and 17:0 in decreasing quantity. The secondary alcohols from the. wax ester are C23, C25, C21, C27, C24, C22), and C26. Each secondary alcohol consists of two to four isomers with the hydroxyl group located near the center of the chain. The secondary alcohols do not correspond in chain length to the n-alkanes. The primary alcohols range from C22 to C32 with C24 and C26 predominating. The fatty acids in the triglyceride and free fatty acid fraction range from C12 to C24.

Dietary hydrocarbons are incorporated into cuticular lipids. Dietary secondary alcohols and ketones, however, are not incorporated into the cuticular lipids. In typical experiments, from 8 to 27 percent of the fed labelled n-alkanes are recovered in the cuticular lipids. Most of the radioactivity recovered from feeding n-tricosane-R-3H and a significant amount from n-pentacosane-R-3H is found as a secondary alcohol in the form of a wax ester. n-N onacosane-R-SH and n-hentria contane-R-3H were recovered primarily unchanged as the n-alkane.

Labelled acetate injected into the insect is incorporated into the hydrocarbon, secondary alcohol wax ester, normal plus sterol ester, triglyceride plus secondary alcohol, primary alcohol, and free fatty acid fraction in ca, the same distribution as the percent composition of these fractions. Labelled n-alkanes administered to the surface of the insect are hydroxylated at or near the middle of the carbon chain.

The secondary alcohols formed or exogenous labelled secondary alcohols are esterified. Chain length specificity is evident in both the hydroxylation of n-alkanes and the esterification of secondary alcohols, with the shorter chain C23, C21, C19, and C25 compounds converted to secondary alcohol wax esters more readily than the longer chain C27, C29, and C31 compounds. Secondary alcohols and ketones are not reduced to alkanes.

STRUCTURE AND METABOLISM OF CUTICULAR LIPIDS OF THE GRASSHOPPER MELANOPLUS SANGUINIPES

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ABSTRACT

The cuticular lipids of the grasshopper Melanoplus sanguinipes contain 60 percent hydrocarbons and 28 percent secondary alcohol wax esters, with lesser amounts of normal and sterol wax esters, triglycerides, alcohols, sterols, and free fatty acids. All the hydrocarbons are saturated and four types of alkanes are present: n-alkanes (61 percent), 3-methyl alkanes (trace amounts), internally branched monomethylalkanes (23 percent), and internally branched dimethylalkanes (16 percent). The principle n-alkanes are C_{29} and C_{27}, with a range from C_{21} to C_{33}. Trace amounts of 3-methyl alkanes of 28, 30, and 32 total carbons are present. The principle internally branched monomethylalkanes are C_{32} and C_{34}, whereas the major dimethylalkane contains 35 carbons. The total number of carbons in the secondary alcohol wax esters range from 37-54 with 41 predominating. The fatty acids of the wax esters are 18:0, 20:0, 16:0, 22:0, 14:0, 19:0, and 17:0 in decreasing quantity. The secondary alcohols from the wax ester are C_{23}, C_{25}, C_{21}, C_{27}, C_{24}, C_{22}, and C_{26}. Each secondary alcohol consists of two to four isomers with the hydroxyl group located near the center of the chain. The secondary alcohols do not correspond in chain length to the n-alkanes. The primary alcohols range from C_{22} to C_{32} with C_{24} and C_{26} predominating. The fatty acids in the triglyceride and free fatty acid fraction range from C_{12} to C_{24}.

Dietary hydrocarbons are incorporated into cuticular lipids. Dietary secondary alcohols and ketones, however, are not incorporated into the cuticular lipids. In typical experiments, from 8 to 27 percent of the fed labelled n-alkanes are recovered in the cuticular lipids. Most of the radioactivity recovered from feeding n-tricosane-R-^{3}H and a significant amount from n-pentacosane-R-^{3}H is found as a secondary alcohol in the form of a wax ester. n-Nonacosane-R-^{3}H and n-hentriacontane-R-^{3}H were recovered primarily unchanged as the n-alkane.

Labelled acetate injected into the insect is incorporated into the hydrocarbon, secondary alcohol wax ester, normal plus sterol ester, triglyceride plus secondary alcohol, primary alcohol, and free fatty acid fraction in ca. the same distribution as the percent composition of these fractions. Labelled n-alkanes administered to the surface of the insect are hydroxylated at or near the middle of the carbon chain. The secondary alcohols formed or exogenous labelled secondary alcohols are esterified. Chain length specificity is evident in both the hydroxylation of n-alkanes and the esterification of secondary alcohols, with the shorter chain C_{23}, C_{21}, C_{19}, and C_{25} compounds converted to secondary alcohol wax esters more readily than the longer chain C_{27}, C_{29}, and C_{31} compounds. Secondary alcohols and ketones are not reduced to alkanes.

INTRODUCTION

The physiology of insect cuticular lipids has been studied for many years, and general agreement has been reached concerning the main function of cuticular lipids. Until recently, however, little progress had been made in determining the structure and metabolism of cuticular lipids in insects and other organisms. With the advent of the powerful tools of gas-liquid-chromatography and mass spectrometry, it has now become possible to separate and determine the structure of these complex mixtures of lipids. Along with the increased interest and feasability of determining the structures of cuticular lipids, a concomitant rise of interest in their metabolism has occurred.

Physiology of Insect Cuticular Lipids

The physiology and function of insect cuticular lipids has been recently reviewed by Jackson and Baker (1). The epicuticle of insects is recognized as having distinct sublayers defined from inside to outside as inner epicuticle, cuticulum, lipid layer, and cement layer. The thickness is generally about 1μ, or only five percent of the thickness of the cuticle. The cuticular lipid consists of an innermost layer which orients the overlying crystals of paraffins. The degree of organization of the outermost lipid layer decreases the more removed it is from the organizing effect of the innermost layer (2). Locke (3) finds that part of the epicuticle is penetrated by pore canals containing lipid filaments. He proposes that the transport of lipid from the site of

synthesis across the endocuticle and epicuticle to the surface could be carried out in the hydrophobic center of the pore canal. He also proposes that the lipid soluble molecules to which the cuticle is permeable (insecticides, pheromones, hormones) could take up some of the space in the region occupied by the hydrocarbon ends of the pore lipids and eventually find their way into the insect.

Function of Insect Cuticular Lipids

Cuticular lipids are important to an insect's survival because (a) they prevent desiccation of the insect when humidity is low; (b) they protect the insect from toxic inorganic chemicals; (c) they serve as a barrier to the penetration of microorganisms; and (d) they affect the absorption of agricultural chemicals used in insect control (4). In addition, the role of chemical communication has been suggested for some insect cuticular lipids (5).

The large surface to volume ratio of insects makes it important that excessive evaporation be prevented. Many investigators have measured the transpiration of water from insects and these studies are reviewed by Barton-Browne (6), Beament (7, 8), Edney (9), Richards (10), Wigglesworth (11) and Locke (3). The evidence seems to agree that the cuticle is permeable to water vapors, and that the cuticular lipid layer plays a major role in the reduction of transpiration. A study of the grasshopper Melanoplus bivattatus (12) indicates that, "as for many

terrestrial insects so for the grasshopper, the most essential feature in its water economy is the lipid epicuticle. Damage to or removal of this fraction of the epicuticle resulted in a rapid loss of body moisture, leading ultimately to desiccation."

Along with their critical role in preventing desiccation, cuticular lipids also increase resistance to abrasive damage, which can facilitate the entry of pathogens and toxic chemicals into the organism and they play a role in controlling the entry of insecticides and other chemicals from the environment. Cuticular lipids may protect insects from microorganisms by making it difficult for the organisms to establish themselves due to the non-wetting nature of the surface. It is also possible that the cuticular lipid protects chemically by being toxic to microorganisms.

Composition and Structure of Insect Cuticular Lipids

In a review of the composition of waxes secreted by insects Tulloch (13) states, "clearly the study of the composition of insect waxes is an enormous field which has barely been scratched." A limited number of insects has been studied, however, and in general, the cuticular lipids consist of varying amounts of hydrocarbons, wax esters, aliphatic alcohols, free fatty acids, triglycerides, sterols, sterol esters (14), and other lipids which may be peculiar to certain insects, such as ceroplastol I and ceroplasteric acid, which are peculiar to Ceroplastes

destructor (15).

The hydrocarbons are an important component of the cuticular lipids of a number of insects and account for 68 percent of the cast skin lipids of the grasshopper Schistocerca vaga (16), 75 percent of the cuticular lipids from the American cockroach, Periplaneta americana (17), 90 percent of the surface lipids of the cockroaches Periplaneta australasiae, P. brunnea, and P. fuliginosa (18), 59-66 percent of the external lipids of the blowfly Lucilia cuprina (19), 49-67 percent of the unsaponifiable lipids of the housefly Musca domestica (20), and 12 percent of the external lipids of the adult and 3 percent of the external lipids of the naiad of a stonefly Pteronarcys californica (21).

The most commonly encountered hydrocarbons are n-alkanes, methyl branched alkanes, and alkenes, with odd chain lengths predominating. The major hydrocarbons in the cockroaches Leucophaea maderae and Blatta orientalis are n-heptacosane, 3-methylheptacosane, 11-methylheptacosane, and 13-methylheptacosane (22). The major hydrocarbons of the cockroaches P. australasiae, P. brunnea, and P. fuliginosa are n-tricosane, cis-9-tricosene, 3-methyltricosane, 11-methyltricosane, and 13-methylpentacosane (18). 2-Methyl alkanes have been reported in the house cricket Acheta domesticus (23) and 2-methylheptadecane has been reported as a sex pheromone in the tiger moth Holmelina nigricans (24). Another branched alkane, 13-methylhentriacontane has been identified in the larvae of the corn earworm, Heliothis zea (25), as the major constituent which

triggers the short range host-seeking response of the parasite Microplitis croceipes. Multiple branched alkanes with one of the methyl branches on the third or fourth carbon have been reported in the ants Atta columbica, A. sexdens, and A. cephalotes isthmicola (26). Dimethyl and trimethyl branched alkanes in which the methyl branches have isoprenoid spacing and are located internally in the carbon chain have been reported in the tobacco hornworm, Manduca sexta (27), in the grasshopper S. vaga (16), and in the Japanese beetle, Papillia japonica (28).

Wax esters are not as common in insect cuticular lipids, nor have they been as widely studied as hydrocarbons. Wax esters from different insects vary in composition, although agree in having as major constituents long chain normal primary alcohols and acids (29). Wax esters from C. destructor (15) consist of long chain normal fatty acids and alcohols with some of the alcohols unsaturated and having at least two double bonds per molecule. Wax esters of the adult and naiad P. californica (21) consist of total number of carbons C_{30} to C_{40}, both saturated and unsaturated, with the adult having more of the longer chain wax esters. No aliphatic wax esters of other than primary alcohols have been previously reported in insects.

The free fatty acids, triglyceride fatty acids, and primary alcohols usually found in insect cuticular lipids are principally of even carbon chain lengths, with fatty acids from C_{12} to C_{20} predominating

(19, 21, 29). In Ceroplastes rusci, however, the very long chain C_{32}, C_{30}, and C_{34} fatty acids predominate (30). The primary alcohols of C. rusci are n-tetracosanol (7.0 percent), n-hexacosanol (81.2 percent), and n-octacosanol (11.8 percent).

Aliphatic secondary alcohols are found in several insects. Tulloch (13) reviewed the literature referring to diols in bees wax. These diols are C_{24} to C_{30} and consist of one primary hydroxyl group and one at the penultimate position. Another secondary alcohol reported in the larva of Tenebrio molitar is pentacosan-8,9-diol, which makes up 55 percent of the cuticular lipids (31). Monohydroxyl aliphatic secondary alcohols with the hydroxyl group located near the center of the carbon chain have not been previously reported in insect cuticular lipids, although they are common constituents of plant cuticular lipids (32). The secondary alcohols in all plants studied correspond in chain length to the major n-alkanes and are predominately of odd chain lengths (33, 34, 35, 36). Wollrab (36) points out that in plants, secondary alcohols are not present in wax esters, in contrast to the situation found among the primary alcohols. Similarly, secondary alcohol wax esters have not been previously reported in insects.

Biosynthesis of Hydrocarbons and Secondary Alcohols

The biosynthetic pathways for insect cuticular lipids have not been worked out in detail. It is generally accepted that acetate units

are incorporated into the cuticular lipids, especially hydrocarbons (1, 37, 38, 39, 40, 41, 42), but the biosynthetic pathway is not known. Nelson (41) and others (1, 42), have reported that excised integuments, but not the fat body can synthesize hydrocarbons from acetate and a variety of fatty acids. The two most considered pathways for hydrocarbon biosynthesis are the condensation-reduction pathway, and the elongation-decarboxylation pathway. These two pathways are considered in depth in recent review articles by Kolattukudy (32, 33) dealing primarily with hydrocarbon biosynthesis in plants, and by Albro (43) dealing primarily with bacterial hydrocarbons. They will, therefore, be only briefly summarized here.

Condensation-Reduction Pathway

Occurrence of hydrocarbons, approximately twice as long as the usual fatty acids, together with the corresponding ketones and secondary alcohols with the functional group in the middle of the chain, prompted early workers to propose a head-to-head condensation pathway for the biosynthesis of hydrocarbons in plants (44, 45). According to this pathway (Fig. 1) two molecules of C_{15} acid condense head-to-head, with decarboxylation of one of them, to give nonacosan-15-one. This ketone is then converted to the corresponding secondary alcohol and hydrocarbon. This hypothesis was soon abandoned because pentadecanoic acid was not known to occur in plants (46). More recently, however, a modification of

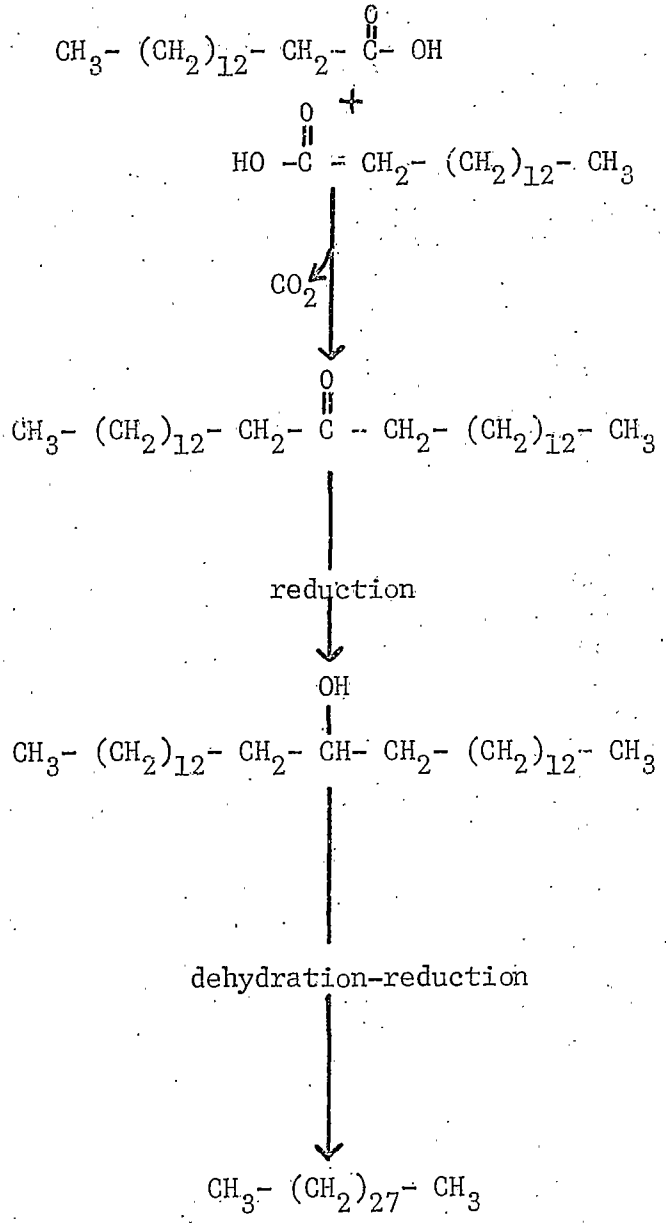

Fig. 1. Head-to-head condensation pathway for hydrocarbon biosynthesis.

the condensation pathway has been reported in microorganisms (43).

Elongation-Decarboxylation Pathway

In the elongation-decarboxylation pathway, a C_{16} fatty acid is elongated to a very long chain fatty acid and decarboxylated. The resulting n-alkane is then hydroxylated to a secondary alcohol and oxidized to the corresponding ketone (Fig. 2). Much indirect evidence favoring this pathway in plants has been presented (47, 48, 49, 50, 51, 52), and recently Kolattukudy has demonstrated the direct conversion of labelled triacontanoic acid to n-nonacosane (53) and the conversion of labelled n-nonacosane to the corresponding secondary alcohol and ketone (54) in Brassica oleracea.

As shown in Figures 1 and 2, secondary alcohols play a central role in each of the two commonly considered pathways for hydrocarbon biosynthesis. In plants, the demonstration of alkane $\rightarrow$ secondary alcohol and the lack of the reverse reaction implicated the elongation-decarboxylation pathway (54) for which direct evidence was later presented (53). No work has previously been reported in insects on the pathway of secondary alcohol biosynthesis, but such evidence could indicate which pathway of hydrocarbon biosynthesis occurs.

Incorporation of Dietary Lipids into the Cuticular Lipids

The effect of dietary fatty acids on the fatty acid composition of

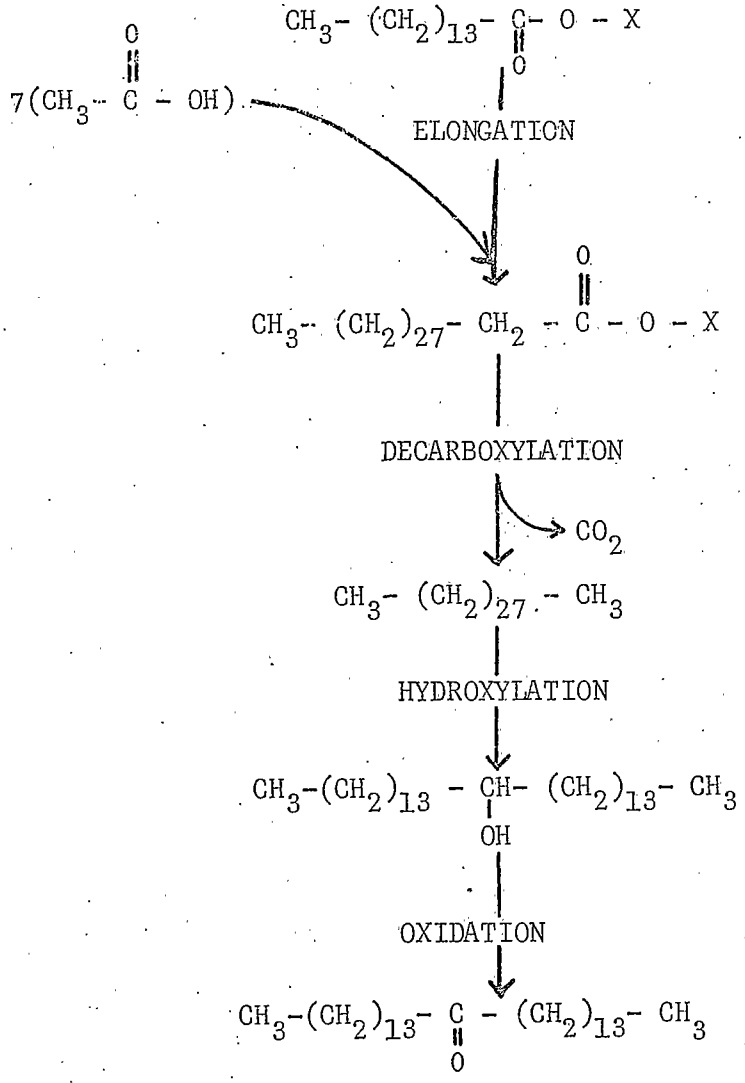

Fig. 2. Elongation-decarboxylation pathway for hydrocarbon biosynthesis.

insects has been clearly shown (55, 56, 57). The effect of dietary alkanes on the cuticular lipids is not as clear. However, in a comparative study of the integument, fat body, haemolymph, and dietary hydrocarbons, Nelson et al. (27) indicate a possible correlation between dietary n-alkanes and alkanes found in the cuticular lipids. The presence of branched chain alkanes in the tobacco hornworm and their absence in the diet suggest that this insect is capable of the biosynthesis of branched chain alkanes, whereas much of the n-alkanes may be dietary. No work on the possible incorporation of dietary secondary alcohols or ketones into cuticular lipids has been previously reported.

STATEMENT OF THE PROBLEM

Heuristic Data

A material with a unique R_f value on a silica gel thin layer plate was found in the outer surface lipids of Melanoplus sanguinipes defectus (Walker) (58). They found that the material chromatographed with an R_f value greater than that of a normal wax ester but less than the R_f value of paraffin hydrocarbons. Infrared spectrophotometry indicated that the material had ester linkages, and gas chromatography (GC) showed it to consist of four major compounds. Mild bromination of the mixture did not alter its gas chromatogram, indicating that the esters were not unsaturated. Saponification gave acid and alcohol moieties which were confirmed by IR spectrophotometry. A combination of thin layer chromatography (TLC) and GC indicated that the acid moiety was mainly octadecanoic acid, but small amounts of hexadecanoic, heptadecanoic and eicosanoic acids were also present. The IR spectra and TLC data indicated that the alcohol moieties were not primary alcohols and therefore might be secondary or tertiary alcohols. Likewise, the branching from the secondary or tertiary alcohols might interfere with the adsorption of the esters on the silica gel thin layer plate and thereby display greater R_f values than normal wax esters.

Avenues of Investigation

This unique material was characterized and found to consist of wax

esters of secondary alcohols. This was the first report of secondary alcohol wax esters in any organism, and the first report in insects of aliphatic secondary alcohols with the hydroxyl group located near the center of the carbon chain. The structure and metabolism of secondary alcohols in plants gave strong circumstantial evidence for the elongation-decarboxylation pathway for hydrocarbon biosynthesis (54), for which direct evidence was later presented (53). The occurrence of secondary alcohols in this insect prompted the following avenues of investigation.

1. Determine the structures of the secondary alcohols and relate them to possible hydrocarbon biosynthetic intermediates or metabolites. For the structures to be consistent with a condensation-reduction pathway, the hydroxyl group should be located primarily on even carbon numbers, having arisen from the condensation of two even chain fatty acids. Even chain fatty acids comprise the vast majority of fatty acids in most organisms. However, if an elongation-decarboxylation pathway followed by hydroxylation occurs, the hydroxyl group could be located on either even, odd, or both even and odd carbon numbers, depending upon enzyme specificity.

2. Determine the structural relationship between the secondary alcohols and the other cuticular lipids. In plants, the occurrence of secondary alcohols, ketones, and n-alkanes of the same carbon chain length suggested a common biosynthetic pathway. Likewise, the

occurrence of predominantly even carbon number fatty acids and primary alcohols and predominantly odd carbon number n-alkanes in both plants and insects implies the loss of a carboxyl group in alkane biosynthesis. The cuticular lipids of this insect were characterized to determine possible similar structural relationships.

3. Determine whether secondary alcohols and n-alkanes found in the cuticular lipids can arise via the diet. The indirect evidence suggesting that n-alkanes found in insect cuticular lipids can arise from the diet and the common occurrence of secondary alcohols and ketones in plant material, which comprises the majority of a grasshopper's diet, suggested that it would be necessary to determine if secondary alcohols can arise via the diet before attempting biosynthetic studies.

4. Determine the pathway of biosynthesis of the secondary alcohols and relate the pathway to hydrocarbon biosynthesis. An analysis of the structures of the secondary alcohols indicates that a condensation pathway does not take place and dietary studies indicate that dietary secondary alcohols are not incorporated into the cuticular lipids. This led to the hypothesis that secondary alcohols are formed from the hydroxylation of n-alkanes and not from the condensation of fatty acids to ketones followed by a reduction to secondary alcohols.

5. Determine the effects of age and sex on the composition and biosynthesis of some cuticular lipid components. Several insect pher-

omones have been discovered by comparing the composition of the cuticular lipids of male and female insects. Determining the effect of age on the incorporation of labelled compounds into cuticular lipids is necessary to obtain the optimum time for studying the biosynthesis of these compounds.

MATERIALS AND METHODS

Insects

Adult Melanoplus sanguinipes were collected from wild populations in southwestern Montana. The insects used for characterization of cuticular lipids were sacrificed at the time of collection by freezing with dry ice and stored at -29 C until needed. The grasshoppers used for metabolic studies with labelled substrates were either collected from wild populations or raised in wire cages at 27 C on a diet of rye shoots, lettuce leaves, and bran fed ad lib.

Insects from the same population and of equal age were selected at random and used one to four weeks after the larval-adult ecdysis unless otherwise indicated. Insects used in the dietary studies were fasted four hours prior to feeding labelled substrates and in all metabolic studies insects were fed dry bran after administration of substrates. Either three or five insects were used in each experiment and kept in a one liter beaker for the duration of the experiment.

Extraction and Separation of Lipids

Only intact specimens were used, thereby lessening possible contamination from internal lipids. Time studies indicated that slurrying the insects for up to 15 minutes in hexane provided a good yield of surface lipids without removing internal lipids.

The surface lipids were extracted by slurrying for 15 minutes in hexane. The extract was filtered through glass wool and then evaporated

to dryness and weighed. The surface lipids (200-700mg) were then chromatographed on a 2.5 x 18 cm column filled with silicic acid (Biosil A). An eight-fraction stepwise elution solvent system consisted of (a) 100 ml hexane; (b) 100 ml hexane; (c) hexane-chloroform 95:5 ml; (d) hexane-chloroform 90:10 ml; (e) hexane-chloroform 80:20 ml; (f) hexane-chloroform 80:40 ml; (g) 100 ml chloroform and (h) chloroform-methanol 67:33 ml. Further purification using preparative TLC on silica gel developed in hexane:chloroform (50:50) (Solvent I) yielded the following fractions: hydrocarbons, secondary alcohol wax esters, normal and sterol wax esters, triglycerides, aliphatic alcohols, sterols, and free fatty acids. Preparative TLC plates were visualized with rhodamine-6G under U.V. light. Preparative TLC was repeated until each fraction was shown to be pure by analytical TLC in Solvent I. Analytical TLC plates were visualized by spraying with concentrated sulfuric acid and charring. Quantitation of the fractions was obtained by weighing. Quantitation of the individual components in each fraction was obtained by disc integration of the gas chromatograms from each fraction.

Alkanes

Argenation chromatography using a TLC plate sprayed with a saturated silver nitrate in ethanol solution developed in hexane (Solvent II) was performed to check for unsaturation. Gas chromatography was carried out on a 5 ft. x 1/8 in. 2% SE-54 on Gas Chrom Q column programmed from

150-300 C in 10 min. Retention times were compared to standards. n-Alkanes were removed from the alkane mixture by inclusion in molecular sieve 5A suspended in a solution of 2,2,4-trimethylpentane. The branched alkanes were separated on a 6 ft. x 1/4 in. 3% OV-17 on Gas Chrom Q column operated isothermally at either 265 or 300 C, and were collected in glass capillary tubes. Mass spectra were obtained with a Varian CH5 instrument operating at ionizing voltage of 70 eV, collector current 100 uA. The samples were inserted into the ion source from gold crucibles.

Secondary Alcohol Wax Esters

Analytical GC of the secondary alcohol wax esters was carried out on a 2% SE-54 on Gas Chrom Q column 5 ft. x 1/8 in. programmed from 225-300 C in 15 min. Retention times were compared with those of a standard normal wax ester mixture and a secondary alcohol wax ester mixture. The secondary alcohol wax esters were reduced with lithium aluminum hydride (LAH) (59) or saponified with 5% KOH in an ethanol-hexane mixture (2:1). The products of both reactions were separated by preparative TLC in Solvent I. Analytical GC of the alcohol fractions was carried out on a 4 ft. x 1/8 in. 1% SE-30 on Gas Chrom Q column programmed from 150-250 C in 10 min. The alcohol fraction which migrated higher in the TLC system than normal alcohols was oxidized with Jones reagent (60). This alcohol fraction was separated and

collected by using preparative GC with a stream splitter on a 6 ft. x 1/8 in. 1% SE-30 on Gas Chrom Q column step programmed from 140-180 C. Mass spectra were obtained as described for alkanes. The fatty acids from the ethanolic KOH saponification were methylated by the method of Schlenk and Gellerman (61), and gas chromatographed on both a 6 ft. x 1/4 in. 15% EGS (ethylene glycol succinate) on Gas Chrom Q column at 165 C and a 9 ft. x 1/8 in. 3% OV-101 on Gas Chrom Q column programmed from 150-200 C in 10 min. Retention times were compared to those of standards.

Triglyceride Fatty Acids and Free Fatty Acids

The triglycerides were transesterified by the method of Morgan et al. (62). The free fatty acids were esterified by the method of Schlenk and Gellerman (61). The resulting fatty acid methyl esters were gas chromatographed as described above.

Aliphatic Alcohols and Sterols

The primary alcohols and sterols were acetylated by the method of Johnson et al. (63). The resulting primary alcohol acetates were gas chromatographed on a 4 ft. x 1/8 in. 1% SE-30 on Gas Chrom Q column programmed from 150-250 C in 10 min. The sterol acetates were gas chromatographed on the same column operated isothermally at 250 C. The retention times were compared with those of standards. Analytical GC

of the secondary alcohol fraction was carried out in the same manner as the primary alcohols and compared with standards.

Synthesis of Tricosan-12-one and Secondary Alcohol Wax Esters

Synthesis of tricosan-12-one used for a mass spectrometry standard was carried out by pyrolysis of the barium salts of dodecanoic acid (64). Synthesis of secondary alcohol wax ester standards and a labelled secondary alcohol wax ester containing tricosan-12-ol-R-^{3}H was carried out by mixing 10 mg myristryl chloride with ca. 1 mg of the appropriate secondary alcohol and adding 0.5 ml pyridine. The reaction was allowed to proceed at room temperature for 15 minutes and the wax ester extracted from the reaction mixture with hexane and purified by TLC in Solvent I.

Labelled Substrates

Tricosan-12-one, heptacosan-14-one, hentriacontan-16-one, nonadecan-10-ol, heneicosan-11-ol, n-nonadecane, n-heneicosane, n-pentacosane, n-nonacosane and octacosanoic acid were purchased from Pfaltz and Bauer. The ketones were reduced to their corresponding secondary alcohols with LAH (59) and to alkanes by the Huang-Minlon modification to the Wolff-Kishner reduction (65). The alkanes, ketones, and secondary alcohols were purified by double development TLC first in Solvent I and then in Solvent II, and their purity checked by GC. The C_{23}

compounds were tritiated by New England Nuclear and the C_{19}, C_{21}, C_{25}, C_{27}, C_{28}, C_{29}, and C_{31} compounds by ICN using the Wilzbach method (66). Some of the labelled octacosanoic acid-R-^{3}H was reduced to the corresponding primary alcohol using LAH (59). The labelled C_{28} acid and alcohol were purified using TLC in diethylether - conc. ammonia hydroxide (100:0.25) (Solvent III). The fatty acid, which remained at the origin, was clearly separated from the fatty alcohol. The labelled alkanes, secondary alcohols, and ketones were separated and purified by repeated TLC double developed in Solvents I and II prior to use. The purity of each compound was checked by TLC and the n-alkanes and secondary alcohols by radio-GC. The specific activities of labelled compounds used in dietary, excised integuments, and the cell free preparation were the C_{23} compounds 4.5 mCi/mg, the C_{27} and C_{31} compounds 0.8 mCi/mg, the C_{29} n-alkane 0.2 mCi/mg, and the C_{25} compounds 0.1 mCi/mg. The specific activities of the n-alkanes and secondary alcohols used in all other studies were adjusted to 0.1 mCi/mg by the addition of unlabelled compounds. Each substrate was repurified by TLC just prior to use. Sodium acetate-1-^{14}C (sp. ac. 0.85 mCi/mg), palmitic acid-9,10-^{3}H (sp. ac. 1.95 mCi/mg), stearic acid 1-^{14}C (sp. ac. 0.2 mCi/mg), and linoleic acid 1-^{14}C (sp. ac. 0.2 mCi/mg) were purchased from Amersham Searle.

Administration of Labelled Substrates

Between 0.5×10^{6} and 1.5×10^{6} counts/min. were used in each feeding

experiment. The labelled substates in diethylether were placed on fresh lettuce leaves. The insects were fasted for 4 hours prior to feeding them the labelled substrates.

Between 10^4 and 10^5 counts/min. were used in each experiment when n-alkanes, secondary alcohols, ketones, or the C_{28} acid and alcohol were administered to the insect's surface. Similar amounts of each labelled substrate were used when comparisons were being made. A comparison of the hydroxylation and esterification of n-tricosane-R-^{3}H and tricosan-12-ol-R-^{3}H showed that a similar metabolism occurred whether the substrate was injected beneath the cuticle at the third or fourth abdominal segment or layered on the surface of the abdomen. Because of the ease of administering the substrate on the surface of the abdomen, this method was used for the alkanes, secondary alcohols, and ketones. These substrates were administered in diethylether at a concentration so that one ul per insect was used. Sodium acetate in a water solution and palmitic, stearic, and linoleic acid in diethylether were injected between the third and fourth segment of the abdomen. Two ul were injected into each insect.

Excised Integument Studies

The grasshoppers were sacrificed by decapitation and the external organs were removed by making a cut in the ventral side and removing

the loose tissues. The excised integument was then cut in half longitudinally and placed in chambers saturated with water. The fat body was placed in a similar chamber. The labelled substrates were then administered to the fat body and on either the inside or the outside of the excised integument in diethylether. The incubation was stopped after two hours by extraction with hexane. A similar procedure was used in comparing the hydroxylation and esterification in the abdomen, thorax, and head, except that whole body parts were used.

Cell Free System

Appropriate amounts of whole insects or insect abdomens were homogenized with 5 or 9 vol. of 0.15M KCl with a mortar and pestle, virtis homogenizer, or teflon pestle in a glass mortar. The cell free preparation from both the virtis homogenizer and the teflon pestle in a glass mortar were subjected to centrifugation and the 10,000 g x 10 min. pellet, the 100,000 g x 90 min. pellet and the supernatant fraction obtained. The pellets were resuspended in either 0.15M KCl or Tris buffer. All the above operations were carried out at 2-4 C. For the hydroxylation enzyme assay, 100,000 cpm of n-tricosane-R-^{3}H was added in 1 ul diethylether or a tween 80 and water suspension to a test tube containing 50 umoles Tris buffer, pH 8.0 or 7.0, 20 umoles G-6-P dehydrogenase and 0.5 ml of enzyme preparation. The final volume was 1.0 ml. The incubation mixture for the esterification studies contained

100,000 cpm tricosan-12-ol-R-^{3}H, 10 umoles $MgCl_2$, 0.5 umoles CoA, 10 umoles ATP, 1 to 10 umoles palmitic acid and 0.5 ml of enzyme preparation. Incubation for both reactions was done at either 30 or 33 C for 2 hrs under either air or O_2:CO_2 (95-5). The reaction was stopped by addition of 3 ml hexane.

Extraction and Separation of Labelled Compounds

The insects were sacrificed or the reaction stopped at the time indicated and the cuticular lipid extracted as described earlier. The frass was collected and placed in a small vial. Two ml of hexane was added and the frass ground with a glass rod. The frass was extracted for 24 hours with shaking to insure complete extraction. In the studies using labelled n-alkanes and secondary alcohols, the extracts were evaporated to dryness under nitrogen, spotted on a TLC plate, and developed in hexane-toluene (80:20) (Solvent IV). The TLC plate was visualized with rhodamine-6G under U.V. light. Three fractions were obtained: (1) hydrocarbons, (2) secondary alcohol wax esters, and (3) the more polar lipids. In all studies using ketones-R-^{3}H and in several of the studies using n-alkanes-R-^{3}H and secondary alcohols-R-^{3}H as substrates, the TLC plate was double developed in Solvent IV and four fractions obtained: (1) hydrocarbons, (2) secondary alcohol wax esters, (3) ketones, and (4) the more polar lipids.

In the studies using labelled acetate, the extract was evaporated to dryness under nitrogen and the residue transferred to a Pasteur pipette column containing ca. 50 mg of column silica gel (Biosil A 100-200 mesh), and eluted with 6 ml hexane. The column retained the polar lipids and allowed the hydrocarbons to pass through in the hexane. The polar lipids were then eluted with 10 ml chloroform-methanol (2:1). The hydrocarbon in hexane was divided into two equal fractions and evaporated to dryness. One fraction was counted to obtain the total radioactivity in the hydrocarbon fraction and the branched alkanes were isolated from the other fraction by inclusion of the n-alkanes in molecular sieve 5A suspended in a solution of 2,2,4-trimethylpentane. The efficiency of separating the branched alkanes from n-alkanes was checked with tritiated C_{27} n-alkane. Greater than 99.5 percent of the normal C_{27} hydrocarbon is removed. The polar lipid fraction was evaporated to dryness and separated on a TLC plate in Solvent I. The origin plus free fatty acids, primary alcohols plus sterols, triglyceride, and normal wax ester plus sterol ester fractions were scraped into counting vials. The secondary alcohol wax ester fraction was scraped into a Buchner funnel and eluted with toluene. This was evaporated to dryness and the wax ester reduced with LAH. The resulting secondary and primary alcohols were separated by TLC in Solvent I and scraped into counting vials. In one set of experiments, total hydrocarbon and wax ester fractions were also counted. In the studies

with labelled octacosanoic acid and octacosanol, four fractions were obtained by TLC first in Solvent II, then in Solvent III: hydrocarbon, secondary alcohol wax ester, primary alcohol plus triglyceride, and polar lipids.

Liquid Scintillation Counting

Each TLC fraction was scraped into a counting vial and 15 ml of a counting solution (0.4% PPO and 4% Cabosil in toluene) was added. In samples obtained from other than a TLC plate, the Cabosil was omitted. Counting efficiency was between 37 and 39 percent for all tritium samples counted.

Radio-GC

After the total radioactivity of the lipid sample had been determined, the lipid was recovered from the counting solution by adding 5 ml of water to the cocktail and repeatedly extracting with hexane. The hydrocarbon was placed on a small column of Biosil A and eluted with 5 ml hexane. The secondary alcohol wax ester was separated by TLC developed in hexane-diethylether-acetic acid (80:20:1) (Solvent V) and isolated as described above. The secondary alcohol wax ester was saponified by refluxing in 5% ethanolic KOH and hexane (2:1) for 3 hr. The reaction mixture was acidified with HCl and extracted with hexane. The resulting secondary alcohols and acids were separated by TLC developed

in Solvent I. The acid fraction was scraped into a counting vial. The secondary alcohol fraction was scraped into a Buchner funnel, eluted with toluene, and evaporated to dryness. A radio-GC of the recovered hydrocarbons and secondary alcohols was run on a 6 ft. x 1/4 in. 1% OV-17 on Gas Chrom Q column. A thermal conductivity detector was used. The C_{21} and C_{23} compounds were run at 190 C, the C_{25} compounds at 210 C, and the C_{27}, C_{29}, and C_{31} compounds at 230 C. The samples were collected on 1 cm cigarette filters.

All data points are the average of 3 to 6 experiments and 3 or 5 insects were used in each experiment.

RESULTS

STRUCTURE AND COMPOSITION OF THE CUTICULAR LIPIDS

The hexane-extracted cuticular lipids from M. sanguinipes amounted to ca. 1.6 mg lipid/gram insect. Hydrocarbons account for 60 percent of the cuticular lipids, secondary alcohol wax esters 28 percent, free fatty acids 6 percent, aliphatic alcohols 2 percent, and sterols, triglycerides, and other wax esters one percent each.

Alkanes

The hydrocarbon fraction was analyzed by GC (Fig. 3) and found to be composed of over 30 components ranging in chain length from 21 to 33 carbons (based on their retention times). The hydrocarbons eluting after n-tritriacontane are all branched chain hydrocarbons and account for 33 percent of the hydrocarbon fraction. All of the hydrocarbons are saturated as indicated by argenation chromatography and GC.

The branched chain alkanes were isolated by treating the total hydrocarbon fraction with 5A molecular sieve. The branched chain alkanes account for 39 percent of the alkane fraction and elute from the gas-chromatograph between the elution points of the n-alkanes. The n-alkanes were identified by their disappearance after inclusion in 5A molecular sieve and by GC comparison with standards. They account for 61 percent of the alkane fraction. The four types of alkanes present as homologous series are n-alkanes (61 percent), 3-methylalkanes (trace amounts), internally branched monomethylalkanes (23 percent), and internally branched

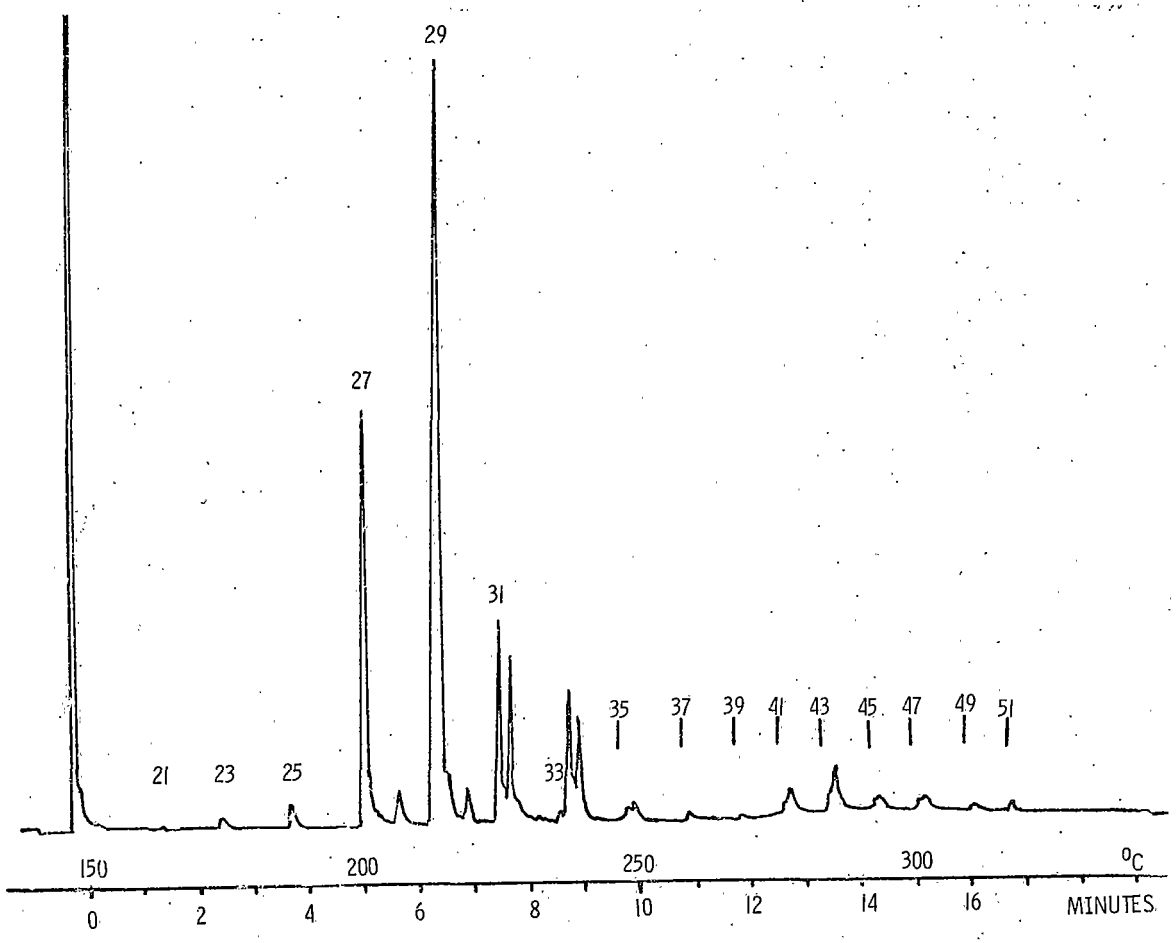

Fig. 3. Gas chromatogram of the cuticular hydrocarbons of M. sanguinipes. The numbers by the peaks 21 to 33 indicate n-alkanes. The numbers with lines beneath them from 35 to 51 indicate where an n-alkane of that chain length would appear.

dimethylalkanes (16 percent). The quantitative results of the GC and mass spectral analysis are shown in Table I.

The n-alkanes are primarily of odd carbon chain length and range from 21 to 33 carbons. n-Nonacosane and n-heptacosane are the principle n-alkanes, comprising 38 and 16 percent of the alkane fraction respectively. The only even carbon-number n-alkanes are of 26, 28, and 30 carbons and are present in trace amounts.

The 3-methyl alkanes are of 28, 30, and 32 total carbons and are present in trace amounts. The 3-methylalkanes elute 0.3 carbon units in front of n-alkanes of the same total carbons and were identified by mass spectral data. The 3-methylalkanes gave the characteristic M-29 and smaller M-57 peaks (Fig. 4A) when subjected to mass spectrometry. They also chromatographed identical with 3-methylalkanes in a mixture of alkanes from Kusner lemon given us by H. E. Nordby (67).

The internally branched monomethylalkanes elute 0.6 to 0.7 carbon units in front of n-alkanes of the same total carbons. These monomethylalkanes fragment by cleavage on either side of the branch point with partial loss of a hydrogen atom to give two major odd mass peaks (C_nH_{2n} + 1) and two major even mass peaks (C_nH_{2n}) when subjected to mass spectrometry. The mass spectrum of the C_{32} internally branched monomethylalkanes is shown in Figure 4B. The mass spectra peaks at 309 (308) and 169 (168) arise from cleavage on either side of the 11-methyl isomer. Peaks at 337 (336) and 141 (140) arise from cleavage on either side of

TABLE I

COMPOSITION OF THE CUTICULAR HYDROCARBONS OF *M. SANGUINIPES*

--- indicates not detectable at the 0.1% level
Tr equals less than 1% but greater than 0.1%

NUMBER OF CARBONS	PERCENT COMPOSITION			
	NORMAL	3-METHYL	INTERNALLY MONOMETHYL	DIMETHYL
21	Tr	---	---	---
23	Tr	---	---	---
25	1	---	---	---
26	Tr	---	---	---
27	16	---	---	---
28	Tr	Tr	1	---
29	38	---	---	---
30	Tr	Tr	2	---
31	6	---	1	---
32	---	Tr	9	---
33	Tr	---	1	---
34	---	---	7	---
35	---	---	---	4
36	---	---	1	---
37	---	---	---	2
38	---	---	Tr	---
39	---	---	---	1
40	---	---	Tr	---
41	---	---	---	Tr
42	---	---	Tr	---
43	---	---	---	2
44	---	---	1	---
45	---	---	---	2
46	---	---	Tr	---
47	---	---	---	1
48	---	---	Tr	---
49	---	---	---	1
51	---	---	---	1
53	---	---	---	1

the 9-methyl isomer. These are the two major isomers present. Peaks at 365 (364) and 111 (110), 281 (280) and 197 (196) and 253 (252) and 226 (225) arise from cleavage on either side of the 7, 13, and 15-methyl isomers. The 5-methyl isomer may also be present, as indicated by the peaks at 393 (392). The mass spectra from the C_{28}, C_{30}, C_{31}, C_{33}, and C_{34} internally branched monomethylalkanes were analyzed in a similar manner and the isomers present summarized in Table II. The C_{32} and C_{34} internally branched monomethylalkanes account for 9 and 7 percent of the alkane fraction. Internally branched monomethylalkanes are also present at one percent levels or less at 36, 38, 40, 42, 44, 46, and 48 carbons as shown by GC data. Due to insufficient quantities, only limited spectral data was obtained for the C_{36} monomethylalkanes and spectra were not obtained for the monomethylalkanes longer than C_{36}.

The dimethyl alkanes are of odd number total carbons and elute 1.3 to 1.4 carbon units in front of n-alkanes of the same total carbons. The difference of 1.3 to 1.4 carbon units is consistent with the presence of two methyl branches towards the center of the molecule with each methyl branch decreasing the GC retention time the equivalent of 0.7 carbon atoms (4, 16, 68). The shortest dimethylalkane present is the C_{35} homologue, which comprised 4 percent of the alkane fraction. Dimethylalkanes of odd carbon number 35 through 53 appear to be present

as indicated by GC and mass spectral data. Due to the insufficient quantities, mass spectra were obtained only for the C_{35}, C_{37}, C_{39}, and C_{43} homologues.

Internally branched dimethylalkanes fragment on both sides of each methyl group when subjected to mass spectrometry. Cleavage internal to a methyl group results in a secondary fragment ion with even mass predominating. Cleavage external to a methyl group results in a secondary fragment ion containing a second methyl group with odd mass predominating (16). In each case the positive ion remains on the secondary fragment.

The mass spectra of the C_{35} dimethylalkanes is shown in Figure 4C. The major even mass and odd mass peaks show that the C_{35} dimethylalkane is a mixture of 11,15-, 13,17-, 9,13- and 15,19-dimethyltritriacontane. The peaks at 168, 351, 280, and 239 arise from cleavage on each side of the 2 methyl branches of 11,15-dimethyltritriacontane with loss or retention of the hydrogen atom as indicated above. The peaks at 196, 323, 252, and 267 arise from cleavage on each side of the two methyl branches of 13,17-dimethyltritriacontane. The peaks at 140,379, 211, and 308 arise from cleavage on each side of the two methyl branches of 9,13-dimethyltritriacontane. The peaks at 225 (224) and 295 arise from cleavage on either side of the symmetrical 15,19-dimethyltritriacontane. The mass spectra of the C_{37}, C_{39}, and C_{43} homologues were interpreted

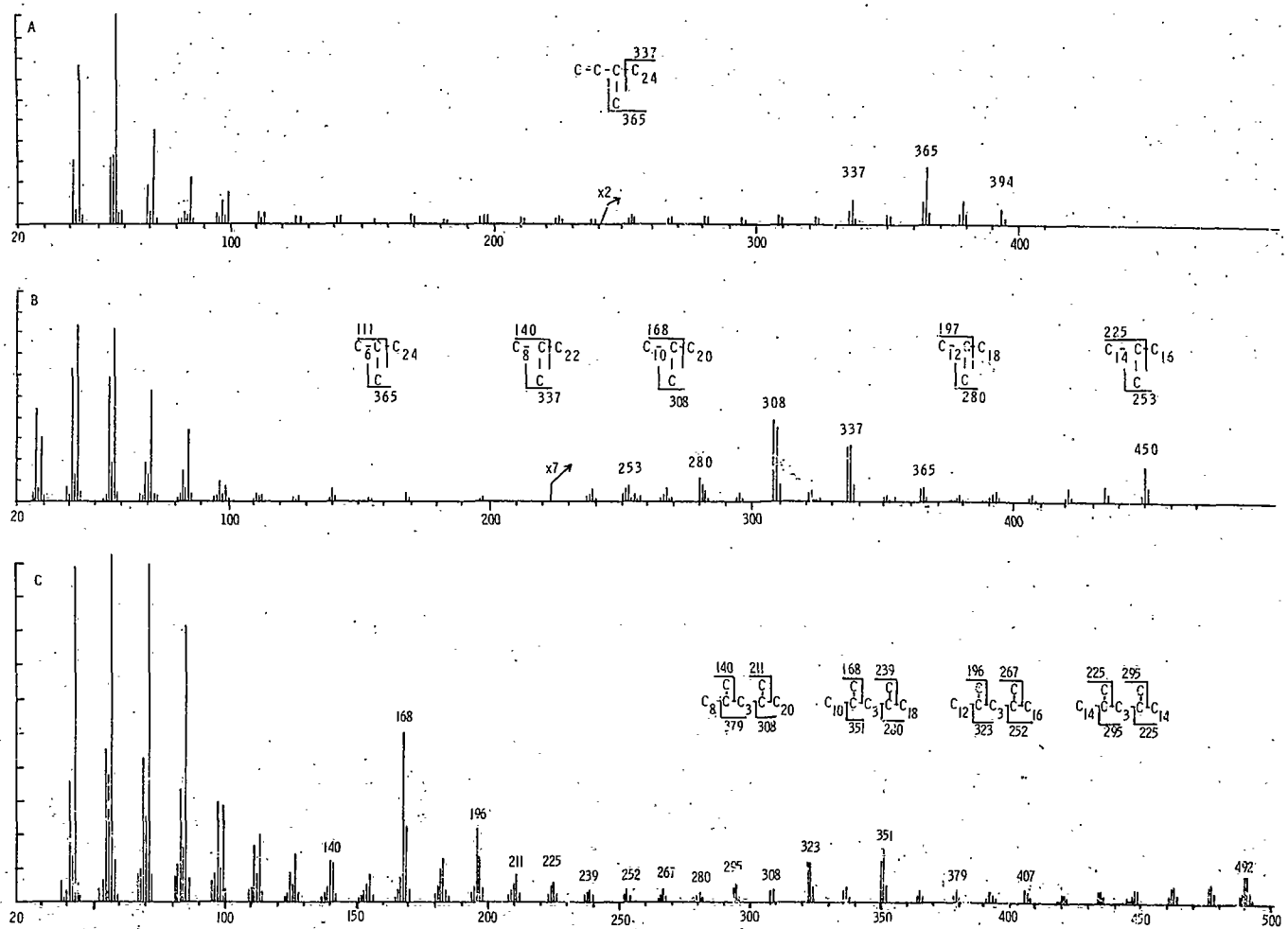

Fig. 4. Mass spectra of some of the branched alkanes of M. sanguinipes. A is a mass spectrum of 3-methylheptacosane, B is a mass spectrum of the C_{32} internally branched monomethylalkanes, and C is a mass spectrum of the C_{35} internally branched dimethylalkanes.

TABLE II

POSITION OF THE METHYL GROUPS OF THE INTERNALLY BRANCHED MONO- AND DIMETHYLALKANES OF *M. SANGUINIPES*

Position of the methyl group(s) indicated in order of decreasing quantities

NUMBER OF CARBONS	POSITION OF THE METHYL GROUP(S)
	MONOMETHYLALKANES
28	11-, 13-, 9-, 5-, 7-
30	11-, 9-, 7-, 5-, 13-
31	9-, 8-, 10-, 11-
32	11-, 9-, 13-, 7-, 15-
33	11-, 10-, 9-, 12-
34	11-, 13-
	DIMETHYLALKANES
35	11,15-, 13,17-, 9,13-, 15,19-
37	11,15-, 13,17-, 15,19-, 9,13-
39	13,17-, 15,19-, 11,15-, 17,21-
43	11,15-, 13,17-, 15,19-, 17,21-, 19,23-

in a similar manner and the isomers summarized in Table II. The C_{37} compounds are a mixture of 9,13-, 11,15-, 13,17-, and 15,19-dimethylpentatriacontane, with the 11,15-, 13,17-, and 15,19- isomers predominating. The C_{39} dimethyl compounds are a mixture of 11,15-, 13,17-, 15,19-, and 17,21-dimethylheptatriacontane. The C_{43} dimethyl compounds are a mixture of 11,15-, 13,17-, 15,19-, 17,21-, and 19,23-dimethylhentetracontane, with the 11,15- and 13,17- isomers predominating.

Wax Esters

The secondary alcohol wax esters from M. sanguinipes range from 37-54 carbons (Table III) with the major peak at 41 carbons, lesser peaks at 39, 43, and 45 carbons and trace quantities at 37, 40, 42, 44, and 47-54 carbons. The wax esters in the 47-54 carbon range are very likely esters of aliphatic secondary alcohols and long chain fatty acids, as well as esters of β-amyrin and in this region the GC peaks are not well defined and were therefore not accurately measurable.

Saponification of the secondary alcohol wax ester samples yielded only saturated fatty acids (Table 3). The major fatty acid is stearic acid 18:0 with 83 percent of the fatty acids being stearic acid or longer. The results from the GC of the methylated fatty acids from saponification and from the GC of normal alcohols from LAH reduction gave similar quantitation for the fatty acid composition.

TABLE III

COMPOSITION OF CUTICULAR WAX ESTERS OF SECONDARY ALCOHOLS OF M. SANGUINIPES

NUMBER OF CARBONS	PERCENT COMPOSITION
WAX ESTERS	
37	Tr
39	18
40	Tr
41	38
42	Tr
43	27
44	Tr
45	11
47-54	Tr
RESULTING FATTY ACID	
14:0	4
16:0	11
17:0	1
18:0	48
19:0	1
20:0	28
21:0	1
22:0	5
RESULTING SECONDARY ALCOHOL	
21	12
22	2
23	59
24	2
25	20
26	1
27	3
β-Amyrin	2

The alcohol portion of the wax esters under consideration was found to be a series of aliphatic secondary alcohols, most of which have an odd number of carbons (Table 3). Jones oxidation of this fraction gave aliphatic ketones, some of which were subjected to mass spectrometry to substantiate the structures of the secondary alcohols. The GC retention times of these secondary alcohols are quite different from normal alcohols, with the secondary alcohol retention time being almost one carbon unit shorter than a normal alcohol under the same conditions.

The mass spectrum of each secondary alcohol peak shows that the sample is a mixture of two to four isomers with the hydroxyl group located near the middle of the chain. In all cases the parent peak is very small, while the usual M-18 peak observed in alcohols is predominant.

The C_{21} alcohol consists of two isomers with about equal amounts of heneicosan-10-ol and heneicosan-11-ol. The mass spectrum (Fig. 5A) shows major peaks of interest at 294, 185, 171 and 157. The 294 peak is the M-18 peak. Peaks at 185 and 157 arise from cleavage at either side of carbon 10 giving ions $(C_{12}H_{25}O)^+$ and $(C_{10}H_{21}O)^+$. The 157 peak is slightly larger than the 185 peak arising from preferential cleavage of the larger group as a neutral fragment. The peak at 171 arises from the heneicosan-11-ol, with cleavage at either side of the symmetrical molecule resulting in an ion of $(C_{11}H_{23}O)^+$.

The C_{22} alcohol consists of two isomers (Fig. 5B) with about twice as much docosan-11-ol as docosan-10-ol. The peaks at 171 and 185 result from cleavage at either side of carbon eleven, giving ion fragments of $(C_{11}H_{23}O)^+$ and $(C_{12}H_{25}O)^+$. These peaks are about twice as large as the 157 and 199 peaks, which arise due to cleavage at either side of the carbon 10. Again, as noted earlier, the cleavage of the larger alkyl group as a neutral fragment is preferred, as the 171 and 157 peaks are larger than the 189 and 199 respectively. The peak at 308 is the M-18 peak.

The C_{23} peak consists primarily of tricosan-11-ol (Fig. 5C) with smaller amounts of tricosan-12-ol and tricosan-10-ol. The peaks at 171 and 199 arise from cleavage at either side of carbon eleven, giving ions of $(C_{11}H_{23}O)^+$ and $(C_{13}H_{27}O)^+$. The peaks at 157 and 213 arise from cleavage at either side of carbon ten. The cleavage at either side of the center carbon of the symmetrical tricosan-12-ol gives rise to the peak at 185. The 322 peak is the M-18 peak. A mass spectrum of the C_{23} alcohol oxidized to ketone is shown in Fig. 5D. The peaks at 169 and 197 arise from cleavage at either side of the carbon eleven of the tricosan-11-one. β-Cleavage (33) of the same isomer gives rise to peaks at 184, 185, and part of 183, 212, 213 and part of 211. The cleavage of symmetric tricosan-12-one gives rise to peaks at 183 and through β-cleavage at 198, 199 and part of the 197 peak. The mass spectrum of the synthesized tricosan-12-one gives similar results.

The C_{24} peak consists primarily of tetracosan-11-ol and tetracosan-12-ol(Fig. 5E). The peaks at 171 and 213 arise from cleavage at either side of carbon 11, and the peaks at 185 and 199 arise from cleavage at either side of carbon 12. The M-18 peak is at 336.

The C_{25} secondary alcohol is comprised of three isomers (Fig. 5F), with a hydroxyl group at the 11, 12, or 13 position. The peaks at 171 and 227 arise from cleavage at either side of carbon eleven, peaks at 185 and 213 from cleavage at either side of carbon twelve, and a peak at 199 from cleavage at either side of the central carbon of the symmetrical pentacosan-12-ol. The M-18 peak is at 350.

The C_{26} peak consists of three isomers with a hydroxyl group at the 11, 12, or 13 position (Fig. 5G). The peaks at 171 and 241 arise from cleavage at either side of carbon 11, peaks at 185 and 227 from cleavage at either side of carbon 12, and peaks at 199 and 213 from cleavage at either side of carbon 13. The M-18 peak is at 364.

The C_{27} peak contains four isomers (Fig. 5H), with a hydroxyl group at the 11, 12, 13, or 14 position. Peaks at 171 and 255 result from cleavage at either side of carbon 11, peaks at 185 and 241 from cleavage at either side of carbon 12, peaks at 199 and 227 from cleavage at either side of carbon 13, and the peak at 213 from cleavage at either side of the central carbon of the symmetrical heptacosan-14-ol. The peak at 378 is the M-18 peak. The peaks at 169, 183, 197, 211, 225, 239, and 253 are thought to arise from dehydrogenation at high

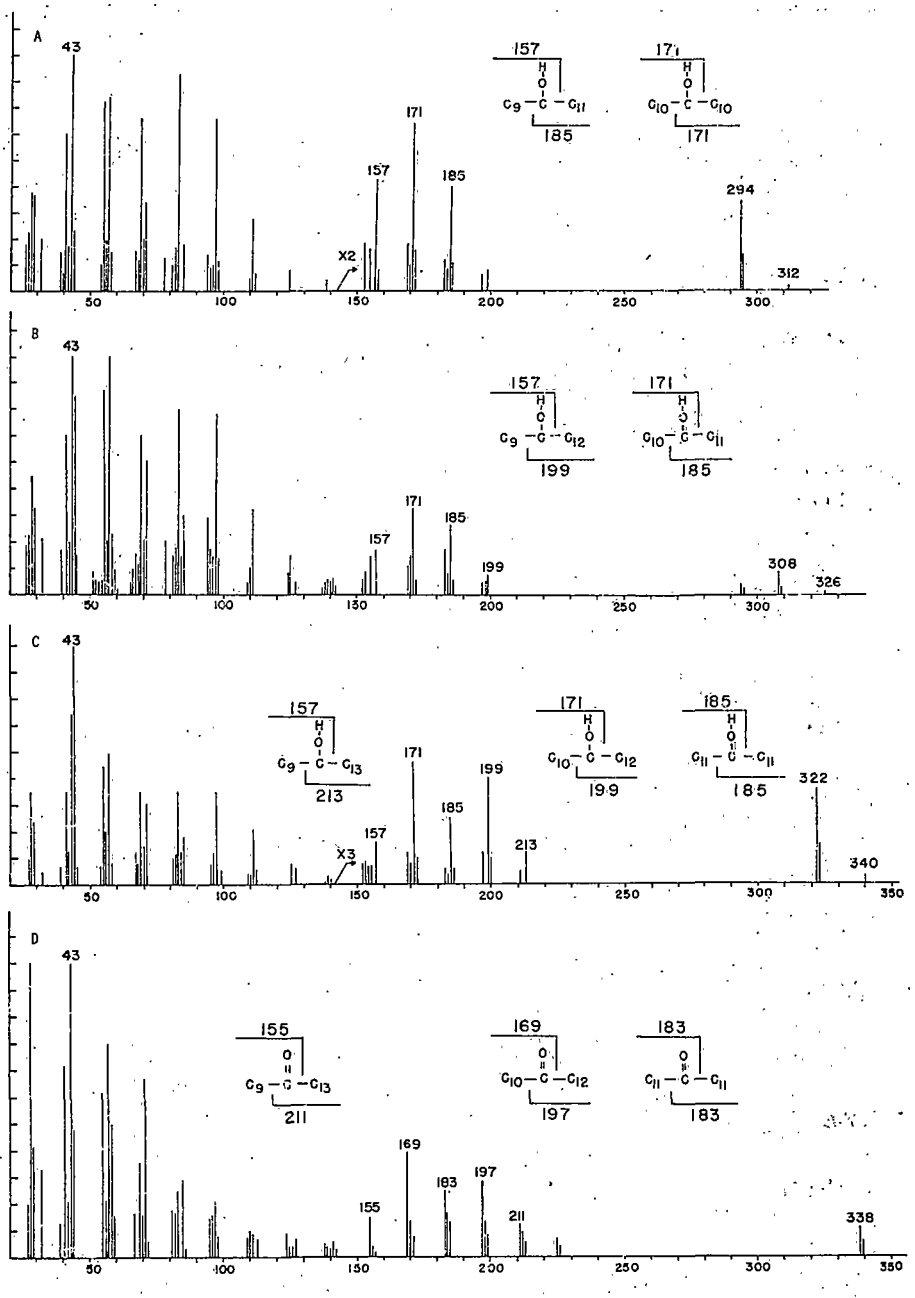

Fig. 5. Mass spectra of secondary alconols from the secondary alcohol wax esters of M. sanguinipes. Spectra D is of ketones resulting from oxidation of the secondary alcohols in Spectra C. Sequence continues on following page.

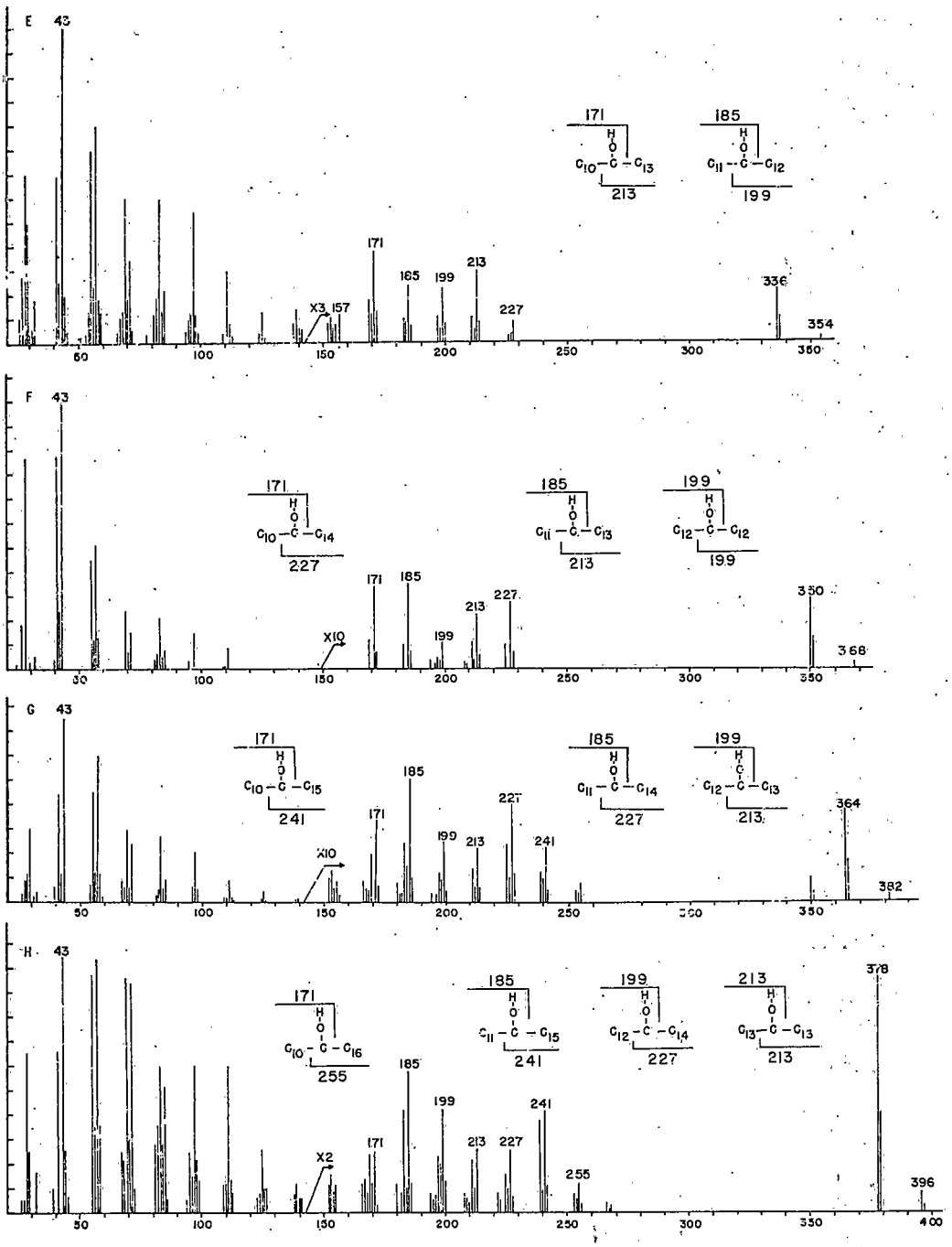

Fig. 5. Sequence continued from previous page.

temperatures (67).

In addition to the aliphatic secondary alcohols, the wax ester after either LAH reduction or saponification yields an alcohol migrating near the normal alcohols on TLC which is indicated by GC to have ca. 30 carbons as compared to a normal alcohol standard. After subsequent isolation of the compound, it was found to cochromatograph with authentic β-amyrin and the mass spectrum was similar to that of authentic β-amyrin. β-amyrin constitutes ca. 2 percent of the alcohol fraction of the secondary alcohols from M. sanguinipes.

Both sterol and other wax esters are observed, but due to the insufficient quantity of these fractions and the complexity of the mixture, they were not characterized.

Triglyceride Fatty Acids and Free Fatty Acids

The major triglyceride fatty acids are 16:0, 18:1, 18:3, and 18:2 in decreasing quantity. The triglyceride fatty acids range from C_{12} to C_{24} (Table IV). The free fatty acids range from C_{12} to C_{22}. The major free fatty acids are 18:3, 18:2, and 18:1 (Table IV).

Aliphatic Alcohols and Sterols

The primary alcohols range from C_{22} to C_{32}. They are predominantly even chain lengths with the major primary alcohols being C_{26} and C_{24} (Table IV). Free secondary alcohols are observed in trace amounts in the

cuticular lipids and are found in the same composition as the secondary alcohols found in the secondary alcohol wax ester fraction.

The major sterol is cholesterol, comprising 96 percent of the sterol fraction. Three percent stigmosterol and one percent unidentified sterol are also present.

TABLE IV

TRIGLYCERIDE FATTY ACIDS, FREE FATTY ACIDS, AND PRIMARY ALCOHOLS OF M. SANGUINIPES

--- indicates not detectable at the 0.1% level
Tr equals less than 1% but greater than 0.1%

	TRIGLYCERIDE FATTY ACIDS	FREE FATTY ACIDS	PRIMARY ALCOHOLS
12:0	3	2	---
14:0	2	2	---
15:0	Tr	---	---
16:0	27	13	---
16:1	5	---	---
17:0	Tr	---	---
18:0	6	6	---
18:1	23	21	---
18:2	14	24	---
18:3	19	28	---
20:0	Tr	Tr	---
22:0	Tr	Tr	3
24:0	Tr	---	34
25:0	---	---	Tr
26:0	---	---	36
27:0	---	---	Tr
28:0	---	---	9
29:0	---	---	5
30:0	---	---	12
32:0	---	---	Tr

INCORPORATION OF DIETARY LABELLED SUBSTRATES INTO CUTICULAR LIPIDS

Table V shows the data from a typical feeding experiment using randomly tritiated hydrocarbons, ketones, and secondary alcohols. It shows that fed hydrocarbons are transported to the surface and incorporated into cuticular lipids. Because of unlabelled dietary hydrocarbons, the difference in the specific activities of the n-alkanes used and the physiological state of the insects, the differences in the percentage incorporated do not necessarily indicate preferential incorporation of one n-alkane over another. Secondary alcohols and ketones are not incorporated into cuticular lipids when fed. The relatively small amount of radioactivity recovered in the zero time experiments probably arises from contamination of the insect surface as the grasshoppers eat the labelled substrate. It appears that the small amount of radioactivity recovered in 42 hr experiments with secondary alcohols and ketones also arises from similar contamination.

Most of the radioactivity recovered from feeding the C_{23} n-alkane and a significant amount from the C_{25} n-alkane was found in the secondary alcohol wax ester fraction. The radioactivity from the fed C_{27}, C_{29}, and C_{31} hydrocarbons was recovered in the cuticular lipids primarily as the unchanged hydrocarbon. In control zero time experiments, the radioactivity recovered is found in the same fraction as that fed. The small amount of radioactivity recovered from the dietary secondary

TABLE V

INCORPORATION OF FED TRITIATED HYDROCARBONS, SECONDARY ALCOHOLS AND KETONES INTO CUTICULAR LIPIDS OF M. SANGUINIPES

	ZERO TIME		42 HR	
CARBON NUMBER	COUNTS/MIN	% FED	COUNTS/MIN	% FED
	HYDROCARBONS			
C_{23}	5,360	1	323,140	27
C_{25}	20,113	2	113,854	8
C_{27}	6,676	2	88,777	20
C_{29}	20,028	2	149,313	10
C_{31}	4,378	1	97,833	11
	SECONDARY ALCOHOLS			
C_{23}	20,619	3	32,197	4
C_{27}	3,714	2	5,547	3
C_{31}	13,207	3	10,938	3
	KETONES			
C_{23}	34,434	3	23,432	2
C_{27}	23,115	3	31,932	3
C_{31}	26,920	3	5,973	1

alcohol was present as the secondary alcohol wax ester after 42 hr., while the radioactivity recovered from the fed ketones was in the ketone fraction. About 6 percent of the fed C_{23} hydrocarbon was recovered in the more polar lipid fraction. Only one percent or less of the C_{25}, C_{27}, and C_{31} fed hydrocarbons was recovered in this fraction.

Frass

The percent of radioactivity recovered in the frass varied considerably. From 7 to 66 percent of the fed labelled n-alkanes, secondary alcohols, and ketones were recovered in the frass. However, in each case, the radioactivity recovered from the frass was in the same chemical class of compounds as that fed.

BIOSYNTHESIS OF CUTICULAR LIPIDS

Incorporation of Acetate-1-^{14}C into the Cuticular Lipids

The percent of radioactivity from acetate recovered in the hydrocarbon, secondary alcohol wax ester, normal and sterol wax ester, triglyceride and secondary alcohol, primary alcohol, and free fatty acid fractions compared to the percent composition of the cuticular lipids is shown in Table VI. Sixty-nine percent of the recovered radioactivity from injected acetate is found in the hydrocarbon fraction, 16 percent in the secondary alcohol wax ester fraction, 10 percent in the triglyceride

TABLE VI

INCORPORATION OF RADIOACTIVITY FROM ACETATE-1-^{14}C INTO THE CUTICULAR LIPIDS OF M. SANGUINIPES COMPARED TO THE COMPOSITION OF THE CUTICULAR LIPID

FRACTION	PERCENT RECOVERED RADIOACTIVITY	PERCENT COMPOSITION
HYDROCARBON	69 ± 9	60
SECONDARY ALCOHOL WAX ESTER	16 ± 5	28
NORMAL AND STEROL ESTER	1 ± 1	1
TRIGLYCERIDE AND SECONDARY ALCOHOL	10 ± 4	1
PRIMARY ALCOHOL	2 ± 1	2
FREE FATTY ACID	2 ± 1	6

INCORPORATION INTO HYDROCARBONS

SUBSTRATE	COUNTS/MIN INJECTED	PERCENT INCORPORATED	COUNTS/MIN INCORPORATED	PERCENT IN BRANCHED	PERCENT IN NORMAL
Na acetate-1-^{14}C a	900,000	7	62,100	85±5	14±5
Na acetate-1-^{14}C b	2,000,000	1	22,200	62±8	38±8
Palmitate 9,10^{3} H	6,000,000	0.2	9,200	39±9	61±9

and secondary alcohol fraction, and 2 percent or less in the other fractions. The incorporation of acetate into the various fractions compares well with the composition of the cuticular lipids, except in the triglyceride plus secondary alcohol fraction which makes up only 1 percent of the cuticular lipids.

Labelled acetate is incorporated into both the branched and normal alkane fractions. The percent incorporated into the branched fraction varies from 50 to 80 percent in different experiments. It appears that the age, sex, and dietary preconditioning may play a part in the distribution of label from acetate into these fractions. Labelled palmitate is also incorporated into both the branched and normal alkane fractions. The radioactivity in the secondary alcohol wax ester fraction is found in both the secondary alcohol and acid moieties, with 82 $\pm$ 5 percent of recovered radioactivity in the secondary alcohol portion, and 18 $\pm$ 5 percent in the acid part.

Incorporation of Fatty Acids into the Cuticular Lipids

Labelled palmitate, stearate, and linoleate are incorporated into both the hydrocarbon and secondary alcohol wax ester fractions when injected into the insect. Between 0.2 and one percent of each substrate is incorporated into both the hydrocarbon and wax ester fraction with approximately equal amounts and distribution of label from both stearate and linoleate into the hydrocarbon and wax ester fraction. Labelled

octacosanoic acid and octacosanol are not incorporated into either hydrocarbons or secondary alcohol wax esters to any appreciable extent.

Hydroxylation and Esterification of n-Alkanes and Esterification of Secondary Alcohols

Both labelled n-alkanes and secondary alcohols applied to the insect's surface are incorporated into the secondary alcohol wax ester fraction. Figure 6 shows the hydroxylation and esterification of n-alkanes-R-^{3}H in M. sanguinipes. n-Heneicosane-R-^{3}H (C_{21}) is hydroxylated and esterified most readily, with 41 ± 8 percent of the recovered radioactivity found in the secondary alcohol wax ester fraction after 18 hours. Twenty-five ± 7 percent, 24 ± 5 percent, and 13 ± 5 percent of the recovered radioactivity from n-tricosane-R-^{3}H (C_{23}), n-nonadecane-R-^{3}H (C_{29}) and n-pentacosane-R-^{3}H (C_{25}) is recovered as secondary alcohol wax esters after 18 hours. Saponification and LAH reduction of the secondary alcohol wax ester shows that the radioactivity is in the secondary alcohol portion. Radio-GC of the recovered secondary alcohols from experiments using C_{21}, C_{23}, and C_{25} n-alkanes shows that the radioactivity is in the secondary alcohol of the same carbon chain length as that of the n-alkane-R-^{3}H administered. Figure 7 shows a radio-GC of n-tricosane -R-^{3}H applied to the insect and Figure 8 a radio-GC of the recovered secondary alcohols. The radioactivity recovered is in the C_{23} secondary alcohols.

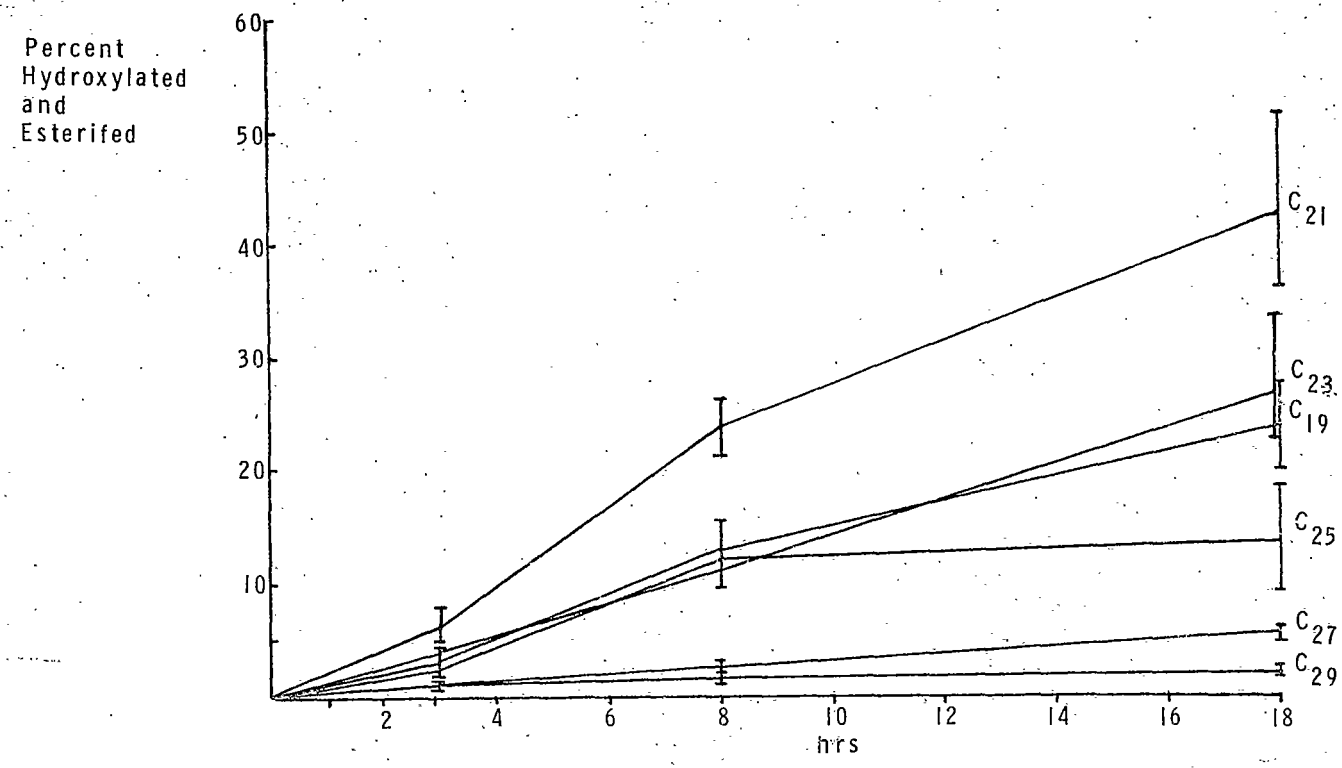

Fig. 6. Hydroxylation and esterification of n-alkanes in M. sanguinipes.

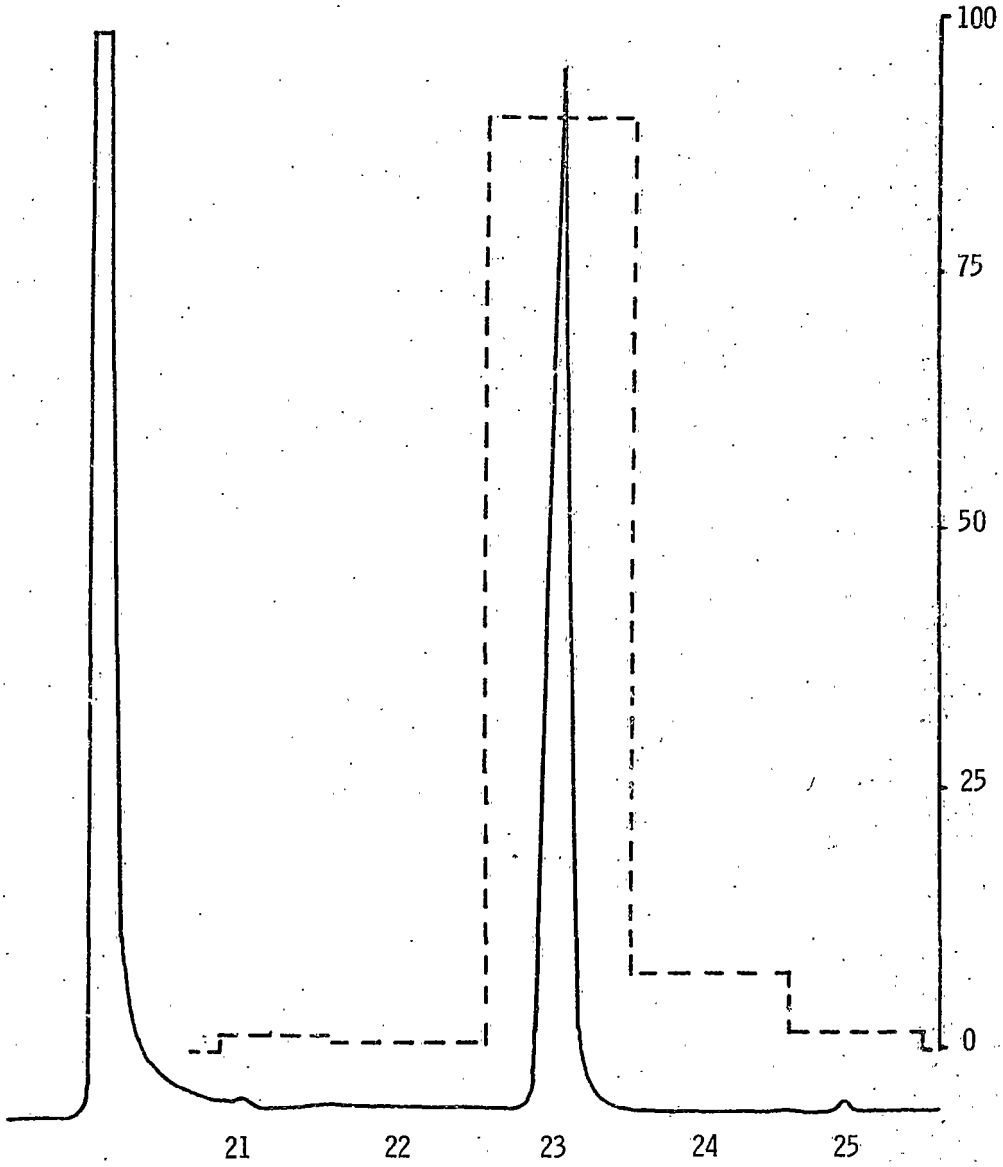

Fig. 7. Radio-GC of n-tricosane-R-^{3}H. The solid line is the GC trace. The dashed line represents percent recovered radioactivity.

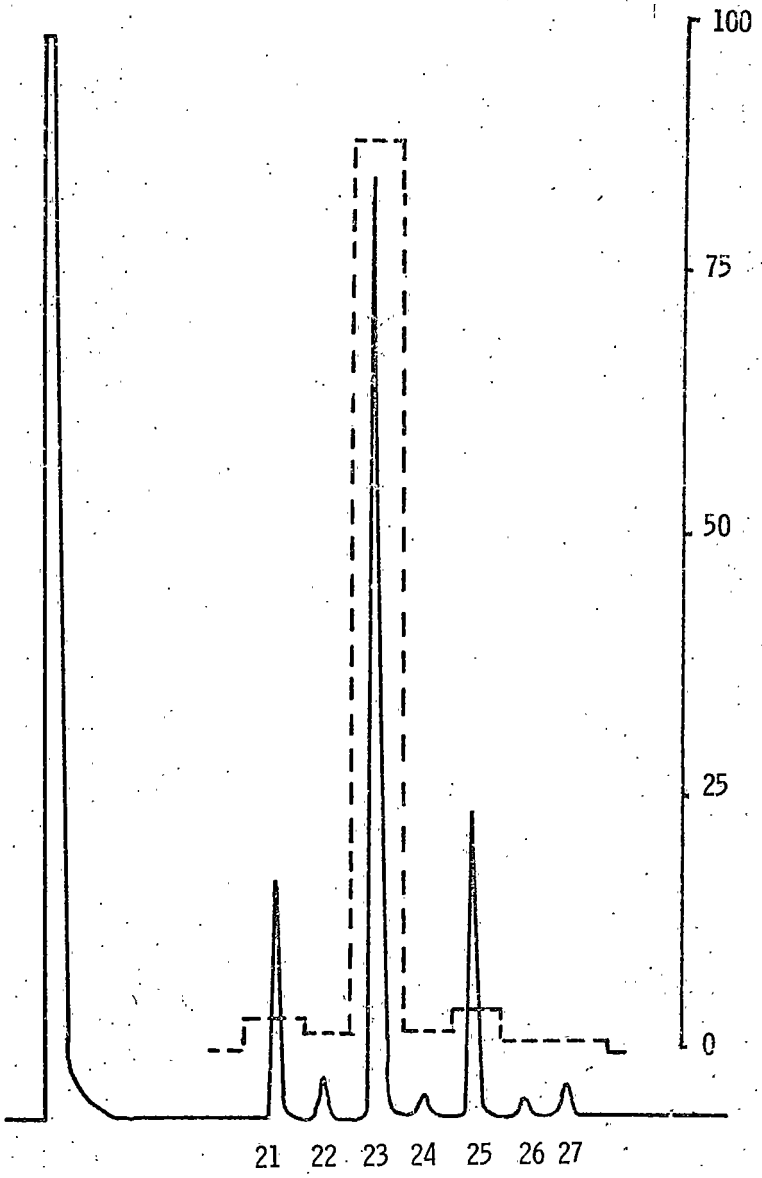

Fig. 8. Radio-GC of the recovered secondary alcohols from n-tricosane-R-^{3}H administered to M. sanguinipes. The solid line is the GC trace. The dashed line represents percent recovered radioactivity.

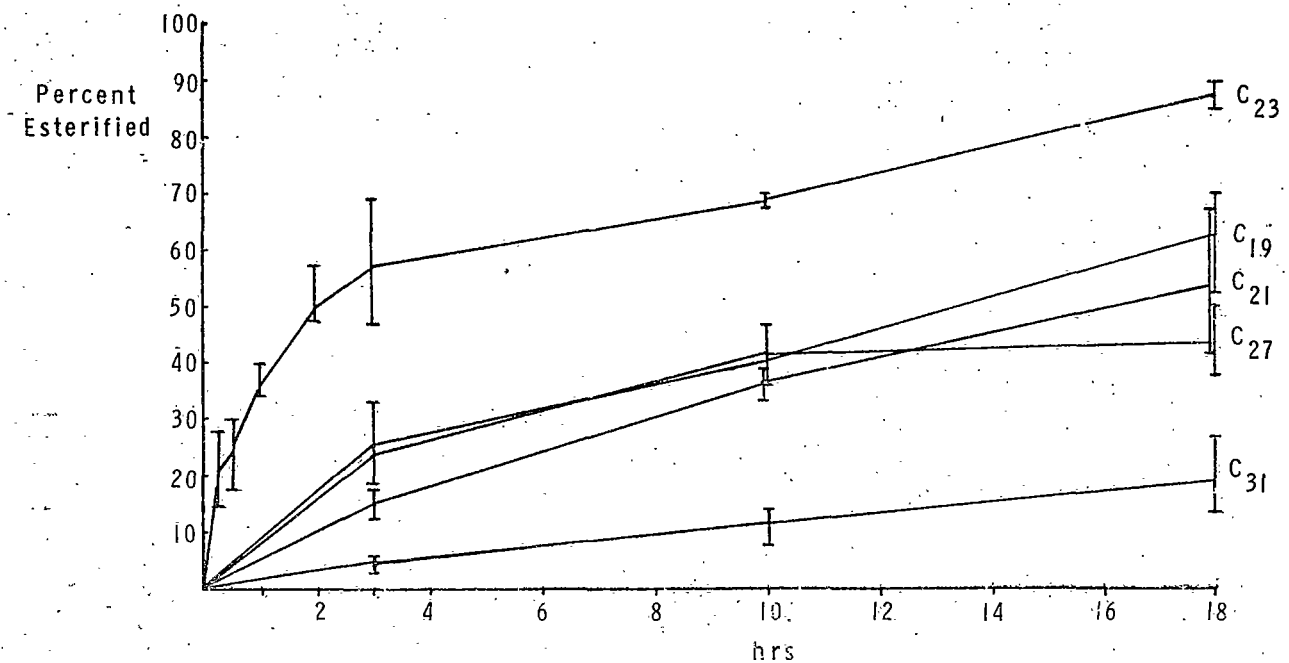

Fig. 9. Esterification of labelled secondary alcohols by M. sanguinipes.

Most of the hydroxylated n-alkanes appear as the secondary alcohol wax esters, however, a small portion remains as the free secondary alcohol. Only 2.2 ± 0.3 percent of n-nonadecane-R-^{3}H, 4.9 ± 0.5 percent of n-heneicosan-R-^{3}H, 6.8 ± 1.2 percent of n-tricosane-R-^{3}H, 1.7 ± 0.5 percent of n-pentacosane-R-^{3}H, 1.1 ± 0.1 percent n-heptacosane-R-^{3}H, and 1.0 ± 0.3 percent of n-nonacosane-R-^{3}H appears in the polar lipid fraction. The polar lipid fraction contains all lipids more polar than ketones including primary and secondary alcohols, triglycerides, sterols and free fatty acids.

It is shown in Figure 9 that the esterification of secondary alcohols occurs readily with chain length specificity evident. After three hours, 57 ± 13 percent of tricosan-12-ol-R-^{3}H is esterified. After 18 hours, 87 ± 3 percent, 62 ± 8 percent, 53 ± 18 percent, and 43 ± 11 percent of tricosan-12-ol-R-^{3}H, nonadecan-10-ol-R-^{3}H, heneicosan-11-ol-R-^{3}H, and heptacosan-14-ol-R-^{3}H are esterified. Only 18 ± 8 percent of hentriacontan-16-ol-R-^{3}H is esterified in 18 hours.

Metabolism of Ketones

The metabolism of ketones applied to the surface of *M. sanguinipes* and allowed to metabolize 18 hours is shown in Table VII.

Hentriacontan-16-one-R-^{3}H and heptacosan-14-one-R-^{3}H are not incorporated into either the hydrocarbon or wax ester fraction as less than 1 percent of the radioactivity recovered is found in these fractions. Tricosan-12-one-R-^{3}H is incorporated into both hydrocarbons and wax esters in small amounts. Twenty-eight ± 4 percent of the recovered radioactivity from tricosan-12-one-R-^{3}H is found in the polar fraction whereas only 9 ± 1 percent and 5 ± 1 percent of the radioactivity from heptacosan-14-one-R-^{3}H and hentriacontane-16-one-R-^{3}H are found in the polar fraction.

TABLE VII

METABOLISM OF LABELLED KETONES OF M. SANGUINIPES

FRACTION	TRICOSAN-12-ONE-R-^{3}H	HEPTACOSAN-14-ONE-R-^{3}H	HENTRIACONTAN-16-ONE-R-^{3}H
ALKANE	1 ± 1	<1	<1
SECONDARY ALCOHOL WAX ESTER	2 ± 1	<1	<1
KETONE	69 ± 4	90 ± 1	94 ± 3
POLAR LIPIDS	28 ± 4	9 ± 1	5 ± 1

Metabolism of Secondary Alcohol Wax Esters

A wax ester containing tricosan-12-ol-R-^{3}H and myristic acid was applied to live insects, both on the inside and outside of excised integuments, and to the fat body. The cuticular lipids were extracted after four hours. In each set of experiments, all of the radioactivity recovered was found in the secondary alcohol wax ester fraction.

Excised Integument Studies

Studies using excised integument and fat body tissue indicate that secondary alcohols are esterified when applied to the outside of the insect's cuticle. It is shown in Table VIII that no esterification occurs when tricosan-12-ol-R-^{3}H was applied to the fat body and only 2 $\pm$ 1 percent of tricosan-12-ol-R-^{3}H was converted to wax ester when applied to the inside of the cuticle. However, 26 $\pm$ 3 percent of the recovered radioactivity from n-tricosan-12-ol-R-^{3}H is found as wax ester after 2 hours when applied to the outside of the excised integument. This is in comparison to 47 $\pm$ 5 percent of tricosan-12-ol-R-^{3}H converted to wax ester in live insects after 2 hours.

Hydroxylation of n-alkanes did not occur in excised integument experiments. No hydroxylation of n-tricosane-R-^{3}H could be demonstrated in the fat body or if applied to either the inside or outside of the excised integument. Likewise, no hydroxylation was observed when the insect was frozen for 4 hours, brought to room temperature and labelled

TABLE VIII

PERCENT INCORPORATION OF TRICOSANE-R-^{3}H AND TRICOSAN-12-OL-R-^{3}H INTO WAX ESTERS BY THE INTEGUMENT AND FAT BODY OF M. SANGUINIPES

TRICOSAN-12-OL-R-^{3}H

FRACTION	LIVE INSECT	FAT BODY	EXCISED INTEGUMENT INSIDE	EXCISED INTEGUMENT OUTSIDE
ALKANE	<1	<1	<1	<1
SECONDARY ALCOHOL WAX ESTER	47 ± 5	1 ± 1	2 ± 1	26 ± 3
POLAR LIPIDS	53 ± 6	98 ± 1	97 ± 1	74 ± 3

TRICOSANE-R-^{3}H

FRACTION	EXCISED INTEGUMENT FAT BODY	EXCISED INTEGUMENT IN-SIDE	EXCISED INTEGUMENT OUT-SIDE	PRE FROZEN RM. TEMP.	LIVE INSECT	WHOLE BODY PARTS HEAD	WHOLE BODY PARTS THO-RAX	WHOLE BODY PARTS ABDO-MEN
ALKANE	99±1	99±1	99±1	99±1	92	96±2	92±2	91±2
SECONDARY ALCOHOL WAX ESTER	<1	<1	<1	<1	6	2±1	6±2	6±1
POLAR LIPIDS	<1	<1	<1	<1	2	1±1	2±1	2±1

substrate administered. Hydroxylation was observed when whole body parts were used, with the abdomen and thorax containing more enzyme activity than the head. The abdomen and thorax both hydroxylated and esterified 6 ± 2 percent of the recovered radioactivity from n-tricosane-R-^{3}H after 4 hours, whereas the head hydroxylated and esterified only 2 ± 1 percent.

Attempt to Obtain a Cell Free System Which Would Hydroxylate n-Alkanes or Esterify Secondary Alcohols

An attempt was made to achieve a cell free preparation which would hydroxylate n-alkanes or esterify secondary alcohols. Strips of cuticle, cell free preparations obtained from insect abdomens by mortar and pestle, a teflon pestle and glass mortar, a virtis homogenizer and the 10,000 g x 90 min. pellet and the supernatant fraction all failed to either hydroxylate n-tricosan-R-^{3}H or esterify tricosan-12-ol-R-^{3}H when added to appropriate reaction mixtures. Thus, the attempt to obtain a cell free system failed.

Attempt at Induction of the Hydroxylating System by Phenobarbitol, DDT, and 3,4,3',4'-Tetrachlorobiphenyl

Attempts were made to induce the hydroxylation of n-tricosane-R-^{3}H by pretreating the insects with phenobarbitol, DDT, and a PCB (3,4, 3',4'-tetrachlorobiphenyl), all known inducers of mixed function oxidases

(70, 71, 72). Including phenobarbitol, DDT, or 3,4,3',4'-tetrachlorobiphenyl in the insect's diet for 2 to 6 days resulting in no significant increased hydroxylating activity (Table IX). In the control insects, 11 percent of the n-tricosane-R-^{3}H is converted to either secondary alcohol wax ester or free secondary alcohols in 4 hours, whereas the pretreated insects metabolized 10 to 13 percent of the same substrate. Likewise, the topical application of phenobarbitol at a dose of 25 ug per insect per day resulted in no increased hydroxylating activity.

TABLE IX

HYDROXYLATION OF n-TRICOSANE-R-^{3}H AFTER PRETREATMENT OF THE INSECT WITH PHENOBARBITOL, DDT, OR A PCB

Phenobarbitol given orally at 0.7 mg per gr insect per day for 2 days
DDT given orally at 30 ppm for 6 days
The PCB (3,4,3',4'-tetrachlorobiphenyl) given orally at 100 ppm for 6 days

FRACTION	CONTROL	PHENOBARBITOL	DDT	PCB
HYDROCARBON	89 ± 2	90 ± 2	87 ± 3	89 ± 1
SECONDARY ALCOHOL WAX ESTER	8 ± 2	7 ± 1	9 ± 3	7 ± 1
POLAR LIPIDS	3 ± 1	3 ± 1	4 ± 2	4 ± 1

AGE AND SEX - EFFECT ON THE CUTICULAR LIPIDS

Effect of Age and Sex on the Composition of the Major Cuticular Hydrocarbons

The percent composition of the major hydrocarbons of each instar, one week old adults, and one week old adults which had been infected with Nosema locustae (73) in the 4th instar are shown in Table X. The percentages of n-nonacosane and n-heptacosane are reversed between 1st, 2nd, and 3rd instar insects compared to 5th instar and adult insects. In the 1st instar insects, n-nonacosane comprises 15 percent of the alkanes compared to 22 percent n-heptacosane. In the one week old adult insects, n-nonacosane comprises 29 percent of the alkane fraction compared to 17 percent n-heptacosane. No other significant differences are observed among the hydrocarbons of 1st through the 5th instars. No significant differences are observed between the alkanes of one week old insects and insects of the same age and group infected with N. locustae.

The comparison between male and female mixed age adults (2-4 weeks old) and 6-7 week old insects is shown in Table XI. n-Nonacosane comprises 41 and 36 percent of the alkanes in male and female mixed age insects compared to 15 and 20 percent n-heptacosane. The relative amounts of these n-alkanes are reversed in 6-7 week old insects. n-Nonacosane comprises 18 and 13 percent of the alkanes in male and female 6-7 week old insects compared to 38 and 26 percent n-heptacosane. In the male mixed age insects, n-tricosane and n-pentacosane comprise one percent

TABLE X

COMPOSITION OF THE MAJOR CUTICULAR HYDROCARBONS FROM FIRST THROUGH FIFTH INSTARS, ADULT, AND N. LOCUSTAE INFECTED ADULT M. SANGUINIPES

---- indicates not detectable at the 0.1% level
Tr equals less than 1% but greater than 0.1%
a series indicates internally monomethylalkanes
b series indicates dimethyl alkanes

NUMBER OF CARBONS	1st	2nd	3rd	4th	5th	ADULT	INFECTED
23	Tr	2	3	3	1	1	1
25	4	4	6	4	3	3	3
26	Tr	1	1	--	--	Tr	Tr
27	22	19	22	22	20	17	18
28a	6	5	4	2	2	1	Tr
28	Tr	1	1	1	1	2	1
29	15	14	15	20	26	29	22
30a	6	6	5	4	4	4	8
30	Tr	1	--	--	--	Tr	Tr
31	3	3	3	3	5	4	3
32a	19	17	16	14	13	13	13
32	--	1	--	--	--	1	1
34a	7	8	5	9	8	11	11
35b	8	9	8	7	7	6	8
>35	9	9	10	11	10	8	10

TABLE XI

COMPOSITION OF THE CUTICULAR HYDROCARBONS OF MALE AND FEMALE MIXED AGE ADULT AND 6-7 WEEK OLD ADULT M. SANGUINIPES

--- indicates not detectable at the 0.1% level
Tr equals less than 1% but greater than 0.1%
a series indicates internally monomethylalkanes
b series indicates dimethyl alkanes

	MIXED AGE		6-7 WEEK OLD	
NUMBER OF CARBONS	MALE	FEMALE	MALE	FEMALE
23	1	Tr	Tr	1
25	1	1	Tr	2
26	Tr	Tr	Tr	1
27	15	20	38	36
28a	1	1	1	1
29	41	36	18	13
30a	2	2	2	3
30	2	1	--	--
31	8	5	1	1
32a	6	8	6	9
32	Tr	Tr	Tr	Tr
34a	5	7	7	8
35b	4	4	7	7
36a	Tr	Tr	Tr	Tr
37b	2	3	1	1
38a	Tr	Tr	--	Tr
39b	1	3	--	Tr
40a	Tr	Tr	Tr	Tr
41b	Tr	Tr	1	1
42a	Tr	Tr	1	1
43b	2	2	4	4
44a	1	Tr	1	1
45b	2	2	5	5
46a	Tr	Tr	Tr	Tr
47b	1	1	1	1
48a	Tr	Tr	Tr	Tr
49b	2	1	2	2
51b	Tr	1	2	1
53b	Tr	1	1	Tr

each of the alkanes and in the 6-7 week old insects they comprise only trace amounts. In the female, n-tricosane and n-pentacosane comprise trace and one percent of the alkanes in mixed age adults and one and two percent in 6-7 week old adults.

Effect of Age and Sex on the Composition of Secondary Alcohol Wax Esters

A comparison of the major secondary alcohol wax esters from each of the 5 instars, mixed age adults, 6-7 week old adults and adult insects infected with a fat body protazoan Nosema locustae (73) is shown in Table XII. There is a small increase in the longer chain wax esters with age. The C_{39} secondary alcohol wax ester comprises 16, 22, 20, 17, and 15 percent of the secondary alcohol wax ester fraction in instars 1 through 5, but only 10 percent of mixed age adults and 7 and 5 percent of 6-7 wk old adult females and males. In contrast, the C_{45} secondary alcohol wax ester comprises between 25 and 30 percent of this fraction in instars 1 through 5, goes up to 33 percent in mixed age adults and to 45 percent in both male and female insects 6-7 weeks old. No significant differences were observed between males and females in the wax esters or the secondary alcohol fractions. The only difference in the fatty acids from the wax esters is in 14:0 and 16:0 with the male having 2 and 35 percent 14:0 and 16:0 respectively, and the female having 7 and 28 percent. No significant differences were observed between mixed age adults and similar

TABLE XII

EFFECT OF AGE AND SEX ON THE COMPOSITION OF THE MAJOR SECONDARY ALCOHOL WAX ESTERS OF M. SANGUINIPES

	INSTARS					ADULTS			
						1 WEEK OLD		6-7 WEEKS OLD	
CARBON NUMBER	1st	2nd	3rd	4th	5th	CONTROL	INFECTED	MALE	FEMALE
37	1	2	2	1	1	--	--	--	--
39	16	22	20	17	14	10	9	6	7
41	51	49	50	47	47	40	41	30	28
43	25	23	25	28	30	33	37	46	46
45	6	4	3	6	7	13	11	15	15
47-54	--	--	--	Tr	Tr	2	2	2	3

SIX-SEVEN WEEK OLD INSECTS

RESULTING SECONDARY ALCOHOLS			RESULTING FATTY ACIDS		
CARBON NUMBER	MALE	FEMALE	FATTY ACID	MALES	FEMALES
21	8	8	12:0	1	1
22	1	1	14:0	2	7
23	74	74	16:0	35	28
24	1	1	18:0	59	60
25	15	16	20:0	4	4
26	1	1			
27	1	1			

aged insects which had been infected with N. locustae during the fourth instar (73).

Effect of Age and Sex on the Hydroxylation and Esterification of n-Tricosane-R-^{3}H

The effect of age and sex on the hydroxylation and esterification of n-tricosane-R-^{3}H in M. sanguinipes is shown in Figure 10. Males and females exhibit a marked difference in the ability to metabolize n-tricosane-R-^{3}H to secondary alcohol wax esters. Age also plays a role in the ability to metabolize this substrate. Males and females present a similar metabolism for newly-emerged adults and for one week old and 5 and 6 week old adult insects. However, the female insects demonstrate a lesser ability to metabolize n-tricosane-R-^{3}H at weeks 2 through 4 than do the males. In the females, the hydroxylation activity peaks at one week and rapidly declines until three weeks, at which time it levels off. In the males, the hydroxylation activity peaks at two weeks, slowly declines until four weeks, and then drops off rapidly.

The difference in the percent of recovered radioactivity found in the secondary alcohol wax esters from males and females appears to reflect differences in the hydroxylation step and not the esterification step, as similar amounts of free secondary alcohol are observed in both males and females at each age period looked at.

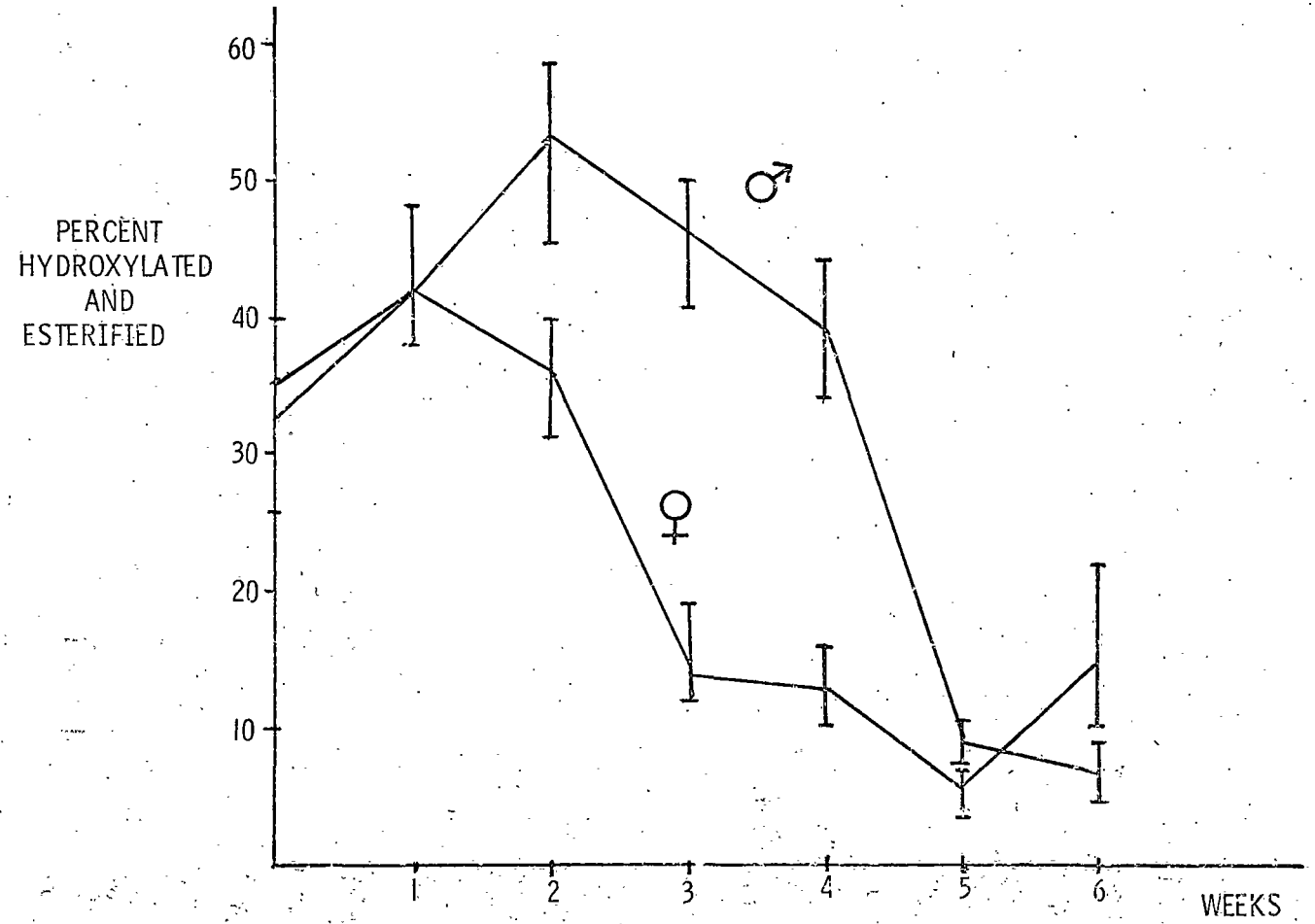

Fig. 10. Effect of age on the hydroxylation and esterification of n-tricosane-R-^{3}H in male and female M. sanguinipes

DISCUSSION

STRUCTURE AND COMPOSITION OF THE CUTICULAR LIPIDS

Branched Alkanes

Methyl branched alkanes are commonly found in insect cuticular lipids. Nelson *et al.* (16) reported that the branched alkanes of the grasshopper *S. vaga* consist of internally branched monomethylalkanes, dimethylalkanes, and trimethylalkanes. The branched alkanes of *M. sanguinipes* consist of 3-methylalkanes, internally branched monomethylalkanes, and internally branched dimethylalkanes. No trimethylalkanes were observed.

3-Methylalkanes have been identified in plants, Mollusca, and Arthropoda. Insects from which 3-methylalkanes have been isolated include the stonefly *P. californica* (21), five cockroaches of the *Periplaneta* genus (17, 18), and the cockroaches *L. maderae* and *B. orientalis* (22). The majority of the 3-methylalkanes isolated from all sources have an even number of carbon atoms (16), as do the 3-methylalkanes found in *M. sanguinipes*.

Monomethylalkanes in which the methyl branch is located towards the center of the molecule have been identified mainly in algae and insects. This type of branched alkane has been identified from a large number of insects, including the classes Coleoptera, Hymenoptera, Lepidoptera, Orthoptera, and Tricoptera (16). The majority of the internally branched monomethylalkanes have the methyl branch located on odd-numbered carbon atoms, and in plants and insects, this is usually on carbons 11, 13 or

15 (16). The majority of the internally branched monomethylalkanes from M. sanguinipes have the methyl branch on odd-numbered carbons ranging from 5 to 15, with the 11-methyl isomer predominating in most cases. The C_{31} and C_{33} internally branched monomethylalkanes have methyl branches located on both odd and even carbons.

Recently, two new homologous series of methylalkanes were reported in the insects M. sexta (4, 68) and S. vaga (16), in which two or three methyl branches with isoprenoid spacing are located towards the center of the molecule. The Japanese beetle, P. japonica has present in its cuticular lipids the dimethylalkanes 9,13-dimethyltricosane and 11,15-dimethylpentacosane, (28) and the grasshopper Melanoplus packardii has dimethylalkanes ranging from C_{35} to C_{51} with isoprenoid spacing (74). Thus, to date, the five insects M. sexta, S. vaga, P. japonica, M. packardii, and M. sanguinipes are the only sources of the long chain internally branched dimethylalkanes. Bennet et al. (28) also reported the presence of dimethylalkanes in which the methyl branches did not have isoprenoid spacing; these were 9,11-dimethyldocosane and 9,11-dimethyltetracosane. No trimethylalkanes were reported in P. japonica, M. packardii or M. sanguinipes.

Secondary Alcohols and Secondary Alcohol Wax Esters

Wax esters of aliphatic secondary alcohols have not previously

been reported in insect or plant cuticular lipids. Likewise, monohydroxyl secondary alcohols with the hydroxyl group located at or near the center of the carbon chain have not previously been reported in insects. They are, however, common constituents of plant cuticular lipids (33, 34, 35, 36). Wollrab (36) points out that in plants, secondary alcohols are not esterified, in contrast to the situation found among the primary alcohols. In both plants and insects, wax esters generally have one or more of the long chain, n-series primary alcohols and acids (32, 29).

The location of the hydroxyl group of secondary alcohols has usually been determined by first oxidizing the secondary alcohol to the corresponding ketone (35, 36). Less ambiguous results are secured by obtaining the mass spectrum of the secondary alcohol directly. This is especially helpful when more than one isomer exists, as it eliminates the β-cleavages found in mass spectra of ketones (34). β-cleavage results in a mass spectrum peak 14 amu higher than the cleavage on either side of the carboxyl group.

In Figure 5D, β-cleavage of tricosan-10-one accounts for the mass spec peak at 225. In mass spectrum of the corresponding C_{23} secondary alcohols shown in Figure 5C, peaks resulting from a similar cleavage are absent. Quantitation of the isomers is possible when mass spectra of the secondary alcohols are obtained directly. Quantitation of the isomers using a spectra from the ketones would be more difficult

because some peaks are enriched via the β-cleavage of isomers having the carboxyl group on adjacent carbons. Similar comparisons illustrating β-cleavages were shown in lower molecular weight ketones and secondary alcohols by Friedel et al. (75) and Sharkey et al. (76).

Relationship Between the Secondary Alcohols and n-Alkanes

Figure 11 illustrates the relationship between the n-alkanes and secondary alcohols of M. sanguinipes. It shows that the major secondary alcohols are C_{25} and shorter, whereas the n-alkanes are predominantly C_{27} and longer. This is in contrast to the situation found in plant cuticular lipids, where secondary alcohols and/or ketones correspond in chain length to the major alkanes (32, 33, 34, 36). In most plants studied, much of the alkane and secondary alcohol fraction consists of a single isomer, and the alkanes are usually either n-nonacosane or n-hentriacontane, with secondary alcohols and/or ketones corresponding in chain length (32). In B. oleracea, most of the hydrocarbon fraction is n-nonacosane. The secondary alcohols, however, consist of two isomers with nonacosan-15-ol and nonacosan-14-ol present at 3:2 ratio (32). The alkanes of M. sanguinipes, as for most insects, are much more complex. Over 30 different alkanes are present in the cuticular lipids of M. sanguinipes. Likewise, the secondary alcohols of this insect are more complex than that found in most plants. The secondary alcohols contain

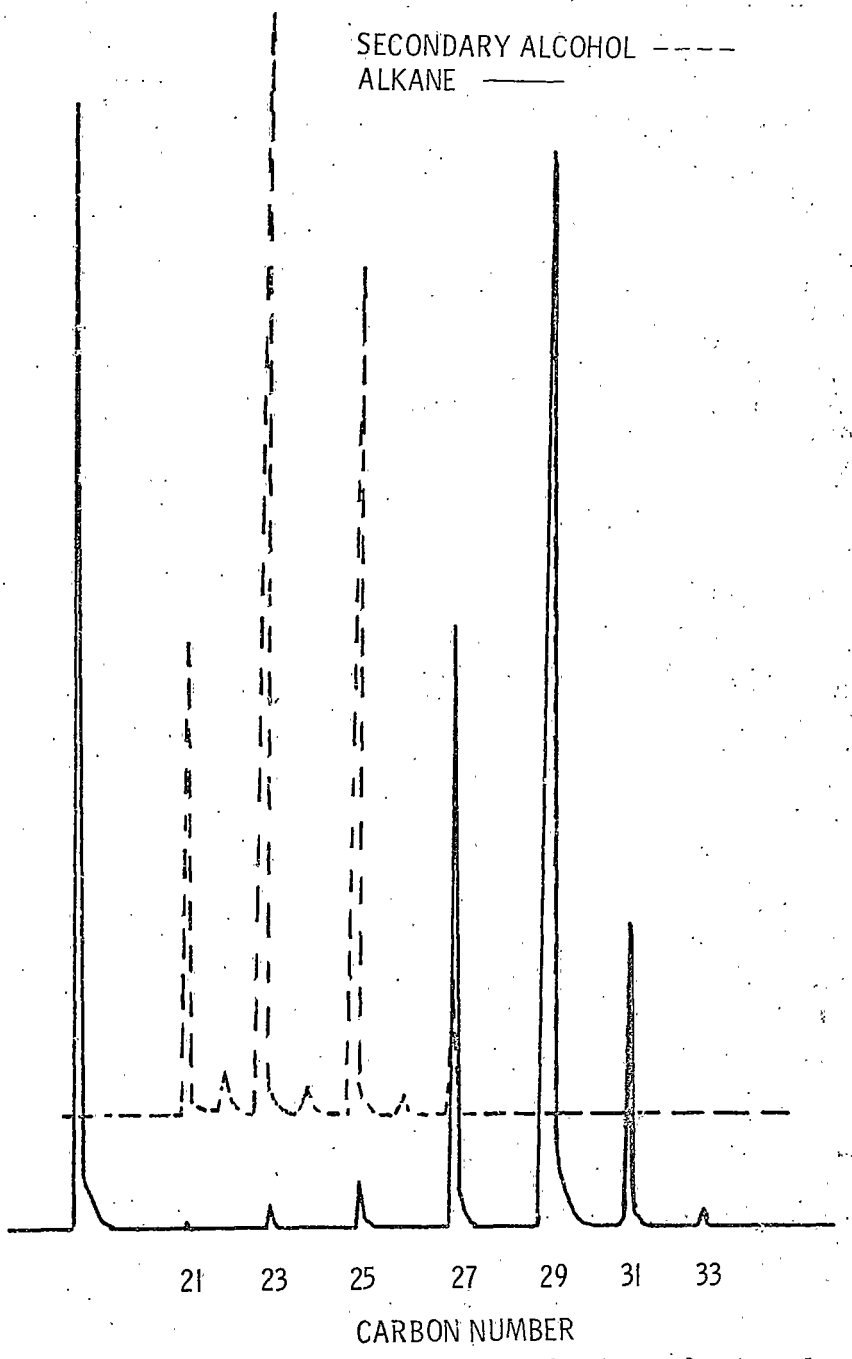

Fig. 11. Relationship between the secondary alcohols and n-alkanes of M. sanguinipes.

7 homologues differing in chain length with 2 to 4 isomers of each homologue.

Biosynthetic Implications from the Structures of the Secondary Alcohols

In plants, the occurrence of alkanes and secondary alcohols of the same chain lengths suggested that both arose from a common metabolic pathway. The requirement for a large amount of 15:0 acid for a condensation pathway of biosynthesis to occur led early workers (46) to reject this pathway for the synthesis of nonacosan-15-one and the corresponding secondary alcohol and alkane in B. oleracea. The percentage of each secondary alcohol in M. sanguinipes and the fatty acids required for a condensation mechanism to have occurred in their biosynthesis is shown in Table XV. Over 50 percent of the secondary alcohols would have to arise from odd chain fatty acids if a condensation mechanism were operative. Tricosan-11-ol is the major isomer comprising 37 percent of the secondary alcohols. The fatty acids 11:0 and 13:0 would be required for a condensation pathway of biosynthesis to occur. Odd chain fatty acids have not been extensively studied in insects, but have been demonstrated to occur in small amounts only, both by de nova synthesis and α-oxidation in plants and animals (32, 77). Unless insects possess an unusually active odd chain fatty acid synthesizing system, the structures of the secondary alcohols make it appear unlikely that they arise from a condensation pathway.

TABLE XIII

COMPOSITION OF THE SECONDARY ALCOHOLS IN THE SECONDARY ALCOHOL WAX ESTERS OF M. SANGUINIPES

Percent determined by GC (Table III) and by measuring the mass spectral peak heights adding together the peaks arising from cleavage on each side of the hydroxyl groups.

SECONDARY ALCOHOL	PERCENT	FATTY ACID REQUIRED FOR CONDENSATION PATHWAY TO HAVE OCCURRED
henicosan-10-ol	7	10:0 + 12:0
henicosan-11-ol	5	11:0 + 11:0
docosan-10-ol	1	10:0 + 13:0
docosan-11-ol	1	11:0 + 12:0
tricosan-10-ol	12	10:0 + 14:0
tricosan-11-ol	37	11:0 + 13:0
tricosan-12-ol	10	12:0 + 12:0
tetracosan-10-ol	<1	10:0 + 15:0
tetracosan-11-ol	1	11:0 + 14:0
tetracosan-12-ol	1	12:0 + 13:0
pentacosan-11-ol	10	11:0 + 15:0
pentacosan-12-ol	9	12:0 + 14:0
pentacosan-13-ol	2	13:0 + 13:0
hexacosan-10-ol	<1	10:0 + 17:0
hexacosan-11-ol	<1	11:0 + 16:0
hexacosan-12-ol	<1	12:0 + 15:0
hexacosan-13-ol	<1	13:0 + 14:0
heptacosan-11-ol	<1	11:0 + 17:0
heptacosan-12-ol	1	12:0 + 16:0
heptacosan-13-ol	1	13:0 + 15:0
heptacosan-14-ol	<1	14:0 + 14:0

The difficulty in quantitating isomers from mass spectral data is recognized. However, calculating percentages by measuring peak heights of these closely related isomers, each with hydroxyl groups on adjacent carbons and near the center of the chain, does give an indication of the relative amount of each isomer in a GC peak.

Relationship Between the Primary Alcohols and n-Alkanes

Kolattukudy (53) has demonstrated in broccoli the direct decarboxylation of very long chain fatty acids to alkanes one carbon shorter. Lambremont (78) has demonstrated the reduction of fatty acids to fatty alcohols and the oxidation of fatty alcohols to fatty acids in the tobacco budworm. The primary alcohols in M. sanguinipes have a similar carbon chain length range as do the n-alkanes, ranging from C_{22} to C_{32}. This suggests that n-alkanes may arise from the decarboxylation of very long chain (C_{24}-C_{34}) fatty acids and that primary alcohols are formed from reduction of very long chain fatty acids. It appears unlikely that the primary alcohols arise from oxidation of n-alkanes, since the primary alcohols are predominantly even carbon chain length and the alkanes predominantly odd carbon chain lengths. If a decarboxylation and reduction system is operating in M. sanguinipes, the very long chain fatty acids must be either efficiently decarboxylated to alkanes or reduced to the primary alcohols since only trace amounts of fatty acids 20:0, 22:0, and 24:0 are present and no fatty acid longer than

24:0 is observed. It appears that the very long chain fatty acids may not be released from the enzyme system until it is metabolized. Attempts to demonstrate the direct decarboxylation or reduction of labelled 28:0 in M. sanguinipes failed.

INCORPORATION OF DIETARY SUBSTRATES INTO CUTICULAR LIPIDS

In a comparative study of the integument, fat body, haemolymph, and dietary hydrocarbons of the tobacco hornworm, Nelson et al. (27) indicate a possible correlation between dietary n-alkanes and integument n-alkanes. On the other hand, the presence of branched long-chain hydrocarbons in the insect and their absence in the diet suggests that the tobacco hornworm is capable of the biosynthesis of a number of branched hydrocarbons. The results of the dietary studies and incorporation of labelled acetate indicate that the grasshopper M. sanguinipes is similar in that labelled dietary n-alkanes can be incorporated unchanged into cuticular hydrocarbons, and the insect also has the capability of biosynthesis of branched long-chain hydrocarbons from acetate.

Dietary preconditioning and feeding during labelled hydrocarbon experiments effects the quantity of the labelled n-alkanes reaching the cuticular lipids. If the grasshopper is preconditioned on generous quantities of lettuce and moist grass, less of the dietary labelled n-

alkane reaches the cuticular lipids. Likewise, if the insect is allowed to eat generous quantities of lettuce and moist grass following the labelled n-alkane, less of the label reaches the cuticular lipids. The efficiency of the n-alkane absorption appears to reflect the mass as well as possibly the moisture content of the diet. This also is reflected in the amount of frass collected over a comparable time period. Where there is considerable mass and moisture to the diet there is more frass and more labelled n-alkane recovered in the frass.

The recovery of the fed labelled n-alkane unchanged from the frass indicates that the n-alkane is not metabolized in the gut or that if metabolized is converted to efficiently absorbed metabolites. The lack of incorporation of dietary secondary alcohols or ketones into cuticular lipids suggests that these compounds are either not efficiently absorbed, or are not transported to the cuticular lipids. This also suggests that dietary plant secondary alcohols and ketones are not incorporated into the cuticular lipids of M. sanguinipes.

BIOSYNTHESIS OF CUTICULAR LIPIDS

Incorporation of Acetate Into the Cuticular Lipid

The results of the incorporation of labelled acetate into the cuticular lipids indicates that the insect is capable of the biosynthesis of both branched and normal alkanes, the acid and alcohol moiety of the secondary alcohol wax ester, the primary alcohol wax ester and sterol

ester, triglycerides, primary alcohols and free fatty acids. Insects are not capable of the biosynthesis of sterols (14, 79). The similarities in the distribution of labelled acetate and the percent composition of the cuticular lipids is noteworthy. The only major discrepency is the large amount of radioactivity in the triglyceride plus secondary alcohol fraction, which may suggest an active synthesis of secondary alcohols.

Biosynthesis of Secondary Alcohols

The results of the dietary studies indicate that secondary alcohols as such do not arise via the diet but that dietary n-alkanes may be incorporated into the secondary alcohol wax ester. The incorporation of labelled acetate into both the secondary alcohol and acid part indicates that the insect is capable of the biosynthesis of both components of the wax ester. An analysis of the structures of the secondary alcohols indicates that a condensation pathway of biosynthesis does not occur. Results of studies using labelled n-alkanes, secondary alcohols, and ketones show that n-alkanes are hydroxylated to secondary alcohols and esterified, and that secondary alcohols and ketones are not reduced to hydrocarbons. It appears that the biosynthesis of secondary alcohols in the insect is similar to that shown in plants: alkanes ⟶ secondary alcohols ⟶ ketones. However, no ketones were observed in M. sanguinipes' cuticular lipids suggesting that the enzyme system responsible for oxidizing secondary alcohols to ketones is absent in this

insect.

The very small incorporation of n-heptacosane-R-^{3}H and n-nonacosane-R-^{3}H into secondary alcohol wax esters or free secondary alcohols demonstrates that chain length specificity is evident in the hydroxylation system and explains the distribution of secondary alcohols and n-alkanes in the cuticular lipids of M. sanguinipes. In contrast, as mentioned earlier, the secondary alcohols correspond in chain length to the major n-alkanes in all plants studied. Chain length specificity in n-alkane hydroxylation has not been studied in plants.

The direct demonstration of the metabolism of alkanes to secondary alcohols and the lack of conversion of the proposed intermediates in the condensation pathway (secondary alcohols and/or ketones) to alkanes indicate that a condensation pathway for the biosynthesis of n-alkanes does not occur in M. sanguinipes. A similar nonconversion of ketones to secondary alcohols or hydrocarbons was observed in B. oleracea (80). It is possible that lackoff accessibility of ketones and/or secondary alcohols to the site of hydrocarbon biosynthesis or their failure to equilibrate with enzyme bound intermediates could account for their lack of incorporation into hydrocarbons. However, a more likely pathway for hydrocarbon biosynthesis in insects is the elongation-decarboxylation pathway, as was suggested for replacement hydrocarbon biosynthesis in P. americana (42).

There is no detectable incorporation of either heptacosan-14-one-R-^{3}H or hentriacontan-16-one-R-^{3}H into the hydrocarbon or wax ester fraction. Tricosan-12-one-R-^{3}H is converted to both hydrocarbon and wax ester in small but significant amounts. This does not appear to be a direct conversion of ketones to hydrocarbon or wax ester, but rather appears to occur via a metabolism of the ketone to more polar products, probably fatty acids and then the synthesis of wax esters and hydrocarbons from the fatty acids. Over one fourth of the recovered radioactivity from the labelled C_{23} ketone is found in the polar fraction. It is clear that the ketone is not simply reduced to the corresponding secondary alcohol as then most of the radioactivity would appear in the wax ester fraction.

Biosynthesis of the Secondary Alcohol Wax Ester

Both the acid and alcohol moiety of the secondary alcohol wax ester are saturated. Yet, stearate-1-^{14}C and linoleate-1-^{14}C are incorporated equally into both the secondary alcohol wax ester and hydrocarbon fraction. This suggests that β-oxidation of the injected substrates occurs, and the resulting labelled acetate is incorporated into these fractions. It appears that whereas labelled secondary alcohols and n-alkanes are incorporated directly into the wax ester as secondary alcohols with the carbon skeleton unchanged, labelled long chain acids are metabolized before incorporation. Only a very low percentage of label is incorporated

from fatty acids. Large metabolic pools of unlabelled fatty acids may be responsible for the low incorporation of fatty acids compared to secondary alcohols. Pools of free, unlabelled secondary alcohols are present in trace amounts only.

Location of the Hydroxylation and Esterification Enzymes

It appears that both the hydroxylation and esterification enzymes are located very near the surface of the insect, as n-alkanes and secondary alcohols applied to the surface are readily incorporated into wax esters. Likewise, the excised integument studies show that secondary alcohols are much more readily esterified when applied to the outside of the integument. Esterification does not take place on the inside of the integument or the fat body. The possibility that esterification did occur on the inside of the integument and fat body but that strong esterase activity saponified the wax ester was considered. However, the lack of metabolism of exogenous labelled secondary alcohol wax ester indicated that this was not the case.

Use of the whole body parts demonstrated that hydroxylation activity was present in the abdomen, thorax, and head. It appears that the abdomen and thorax contain the most enzyme activity, but it is possible that cutting the insect inactivates the hydroxylating enzyme located on the head more readily.

Cuticular hydrocarbons have usually been considered metabolic end products and oriented in crystalline form (1). The rapid metabolism of the shorter chain n-alkanes to secondary alcohol wax esters is thus very surprising, especially if one considers the hypothesis by Locke (3) that lipid soluble molecules travel into insects via pores. If that is the case, the molecule must be hydroxylated and then esterified before traveling back to the surface. The ease with which n-alkanes are hydroxylated in this insect is also surprising when compared to data Kolattukudy presents for the hydroxylation of n-alkanes in B. oleracea (54), where only a very small percentage of the recovered radioactivity is present as secondary alcohols or ketones.

Lability of the Hydroxylating System

It appears that the hydroxylation system is very labile. All attempts to obtain a hydroxylating system from other than live insects or whole body parts failed. Even freezing the insect and then bringing it to room temperature resulted in a complete inactivation of the hydroxylating system. Mixed function oxidase inhibitors have been isolated from eye pigment of houseflies (81, 82) and the abdomen of the house cricket (83). However, due to the complete lack of metabolism of n-alkanes in all systems in which tissue disruption occurs, it is difficult to visualize that a specific inhibitor is responsible. It is possible that freezing or cutting the tissue causes a physical disruption

of the enzyme system or that phenol oxidases may be released from cells and cause the inactivation.

Attempt at Induction of the Hydroxylating System

The nature of the reaction which introduces the oxygen into a long chain alkane is not well understood. Two pathways have been suggested. One is a dehydrogenation of the type observed with lower homologues in bacteria (84) followed by hydration to give secondary alcohols. Alternatively, a mixed function oxidase-type enzyme could hydroxylate a carbon at or near the center of the alkane chain (32). Evidence favoring this second pathway has been presented for the hydroxylating system in plants and includes (a) the incorporation of low levels of ^{18}O from molecular oxygen; (b) the inhibition of the reaction by chelating agents such as phenanthroline, and (c) the partial reversal of the phenanthroline inhibition by exogenous Fe (32). Failure to obtain a cell free or tissue slice system capable of hydroxylating n-alkanes prevented the use of phenanthroline or similar chelating agents in this insect to determine if a mixed function oxidase system was operative in the hydroxylation of n-alkanes.

Induction of mixed function oxidase activity in insects has been demonstrated with DDT and phenobarbitol (71). PCBs have been shown to be potent inducers of mixed function oxidase activity in mammals (72). It was hoped that use of these mixed function oxidase inducers would

indicate if such a system was operative here. However, lack of induction by these compounds suggests that either a mixed function oxidase system is not responsible for the hydroxylation of n-alkanes in M. sanguinipes, or that, if present, the mixed function oxidase system is not affected by the inducing agents used.

Age and Sex - Effect on the Composition and Metabolism of Cuticular Lipids

Very little differences are observed between the hydrocarbons and wax esters of male and female insects. However, the small increase in the amount of n-tricosane and n-pentacosane in 6-7 week old females versus males appears to be of interest. This may be due to the marked advantage observed from weeks two through four in the ability of the males to metabolize n-alkanes compared to the females. This difference in the hydroxylation and esterification of n-alkanes between males and females may be due to the fact that at weeks two through five much of the metabolic energy of the females may be diverted to reproductive processes (85). This difference in the hydroxylation ability of males and females suggests experiments comparing male and female insect's ability to metabolize various insecticides. It is recognized that many of the common insecticides are detoxified by mixed function oxidase reactions (71). If females exhibit a lesser ability to metabolize and hence detoxify insecticides, this may prove of economic

importance relating to the time and dosage of insecticide application.

SUMMARY

The cuticular lipids of the grasshopper M. sanguinipes consist of hydrocarbons, secondary alcohol wax esters, normal and sterol esters, triglycerides, alcohols, sterols, and free fatty acids. The structures and composition were determined by TLC, GC, and mass spectrometry. An analysis of the structures indicated certain possible biosynthetic relationships between some of the cuticular lipid components. Studies with labelled acetate, n-alkanes, secondary alcohols, ketones, fatty acids, and primary alcohols demonstrated some of these biosynthetic relationships. Figure 12 illustrates the possible biosynthetic relationships between the cuticular lipid components. The dashed lines are steps which other workers have shown in different organisms and are what structural relationships of the cuticular lipids of this insect suggest. The solid lines are steps directly demonstrated with appropriate labelled substrates. Acetate is incorporated into all the cuticular lipid components except sterols. Long chain fatty acids are incorporated into the alkanes and secondary alcohol wax esters. No very long chain fatty acids are present in the cuticular lipids, but the structural relationship between the primary alcohols and n-alkanes, and reports in the literature demonstrating the interconversion of fatty acids and alcohols in insects and the decarboxylation of very long chain fatty acids to n-alkanes in plants support the proposed pathways. Labelled n-alkanes from the diet or administered to the insect's surface are incorporated into secondary alcohol wax esters.

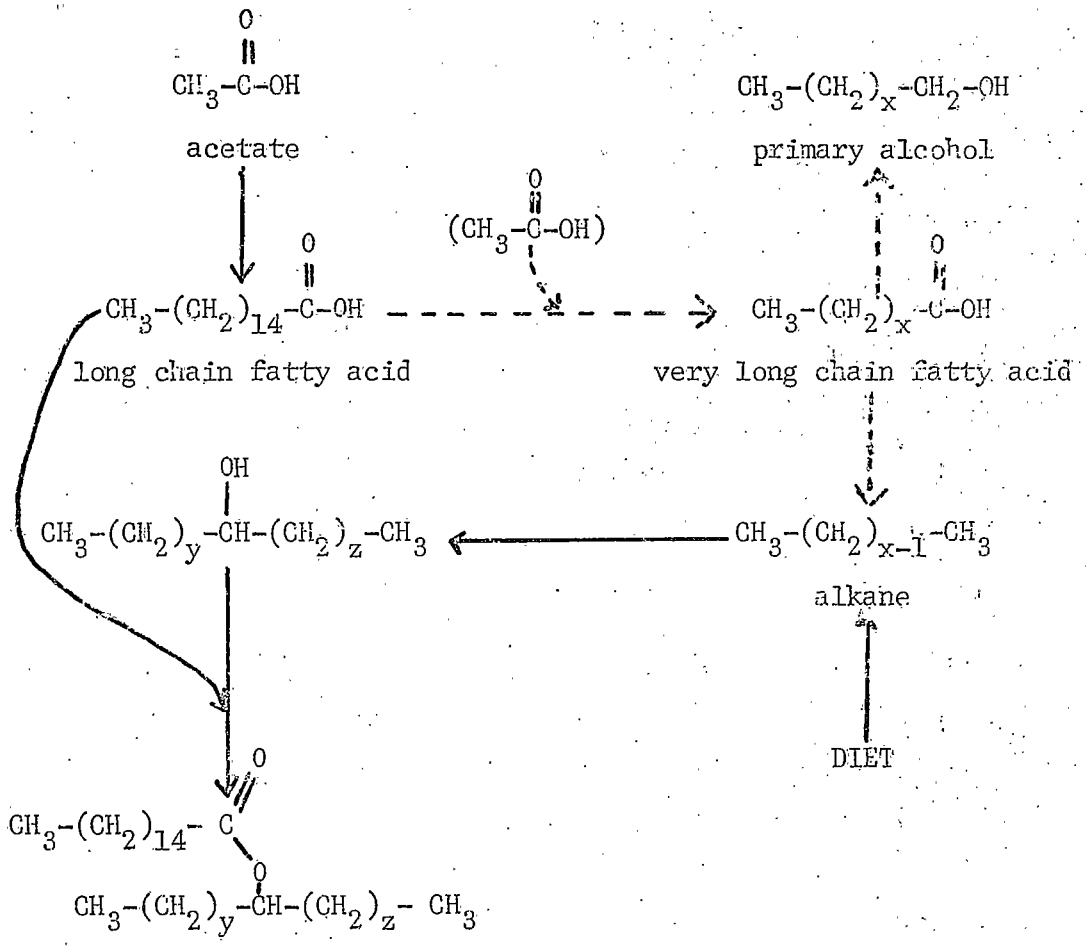

FIG. 12. Proposed Biosynthetic Relationships Between Some of the Cuticular Lipids of M. sanguinipes.

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