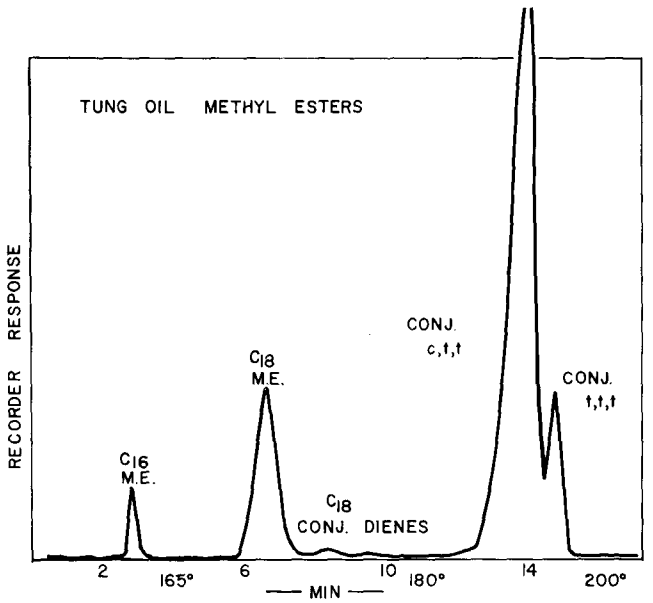

FIG. 8. Chromatogram of tung oil methyl esters, 4 ft column, 5% Versamid 900 on 100–120 mesh Gas Chrom P, isothermal at 165C for 5 min, temp programmed at 2.9°/min to 200C, inlet 300C, detector 275C, flow rate 100 ml/min.

column allows the unreacted hydroxy acid to be determined simultaneously while the polyester column does not. In all cases the GLC values are higher than the UV data and reflect more closely the predicted composition of the dehydrated oils. The reasons for the discrepancy are not yet known, but it is suspected that the absorption coefficient used for the UV analyses is not correct. The coefficient is a composite of the absorption coefficients of the conjugated dienes found in dehydrated castor oil.

The separation of α-eleostearic and β-eleostearic acid has been reported for both Apiezon (1,7) and polyester (7,8) columns with the α-eleostearic (c,t,t) eluting first. The order of elution is the same on a polyamide column, as shown in Figure 8. With temp programming the conjugated trienes are eluted in less than 15 min. The conjugated dienes precede the conjugated trienes, and the unconjugated components travel with the saturated components.

The retardation of the conjugated trienes, with respect to the unconjugated trienes, is such that a direct measurement of the total triene conjugation may be made, without recourse to UV spectrophotometric techniques which are at best only comparative. The situation is quite comparable to that encountered in the analysis of dehydrated castor oil.

The polyamide substrate has proved to be useful for the analysis of polar derivatives not readily separable on other columns by GLC. The versatility and stability of the polyamide when used as a column substrate at high temp will undoubtedly lead to other applications in the analysis of fatty derivatives.

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Composition of Insect Waxes. I. Waxes of Exotic Coccidae: *Gascardia madagascariensis, Coccus ceriferus* and *Tachardia lacca*

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Abstract

Composition of several coccid waxes has been determined by means of alumina and gas-liquid chromatography. *Coccus ceriferus* wax is a mixture of the esters of C_{26} and C_{28} alcohols with C_{24},C_{26} and C_{28} acids. *Tachardia lacca* wax has a high percentage of free alcohols (essentially C_{28} alcohol); *Gascardia madagascariensis* wax contains a large proportion of free acids. In addition to C_{26},C_{32} and C_{34} normal chain acids, there are several C_{30},C_{32} and C_{34} hydroxy acids, in which the hydroxyl function is situated in the middle of the hydrocarbon chain. Small proportions of odd and even hydrocarbons are present in all of the waxes investigated.

Introduction

NATURAL WAXES have been the subject of considerable study, and the results of these investigations were reviewed some years ago by Warth (1). The methods previously available, such as crystallization and distillation, were inadequate for the fine separation of distinct homologous higher fatty acids, fatty alcohols, or hydrocarbons. For this reason, it was desirable to apply the new and precise chromatographic techniques now available for more accurate determination of the nature and proportions of these compounds. Gas-liquid chromatography (GLC) is particularly useful for this purpose, although its application to compounds with boiling points in the higher ranges present some experimental difficulties. In recent years, these methods have been applied by several workers to plant waxes (2,3,4); mineral waxes (5,6); and human waxes (9). It was our purpose to investigate the composition of some insect waxes, more precisely those of the Coccidae, or scale insects.

Materials and Methods

Commercial samples of China wax from the insect *Coccus ceriferus,* and of shellac wax from the Indian lac insect, *Tachardia* (or *Carteria,* or *Laccifer*) *lacca,* were available from several firms. Samples of waxy material from *Gascardia madagascariensis,* a coccid of Madagascar, were extracted from the cocoons of this insect.

Extraction of Waxes. Cocoons (1 kg) were pounded,

then refluxed with 1.5 liters petroleum ether (bp 50–70C) for several hr. The warm mixture was filtered and the residue refluxed with three 1-liter portions petroleum ether. The solvent was removed by distillation, leaving the crude wax (30% cocoon wt), a mixture of resinous and waxy substances.

Purification of Wax. The crude wax (300 g) was refluxed 3 times with acetone (3 liters) to dissolve the nonwaxy substances, chiefly resins and pigments. Then the wax was filtered out as a white powder.

Saponification. 10 g purified wax, or of a chromatographic fraction of waxy substance, was dissolved in 100 ml benzene and refluxed 4 days with 100 ml 10% KOH methanolic solution. The solvents were removed by distillation and 100 ml water added to the residue. The mixture was acidified with H_2SO_4 and warmed in a water bath until the waxy material melted. The crust that formed during cooling was removed and washed several times with water. The mixture of saponified products was dissolved in warm chloroform or warm benzene and the solution dried on Na_2SO_4.

Separation of Acidic and Neutral Substances. The usual method of separating acids by use of an aqueous alkaline solution could not be used in this case, because sodium and potassium salts of higher fatty acids are not soluble in water. Another procedure, that proposed by Savidan (10), was used successfully. Saponification products were dissolved in 100 ml warm chloroform or warm benzene and the solution passed through a column packed with 50 g Na_2CO_3 for 8 g of product. Temp was maintained at about 56C by refluxing acetone vapors in a jacketed chromatographic tube. Neutral substances were eluted by the solvent, and acidic substances retained on the column. The sodium carbonate and sodium salts of fatty acids were extracted from the tube and dissolved in boiling water. Acidification with HCl gave a precipitate of fatty acids which could be separated by filtration. After several washings and dryings, the mixture of fatty acids was recovered.

Methylation. The small quantities of acids required for GLC were dissolved in benzene at 35C, an ethereal solution of diazomethane added in small portions, and the solution held over night at 35C.

Esters for separation by column chromatography were prepared by esterification with anhydrous methanol, 10 g acids being dissolved in 500 ml benzene-methanol (1:1) and 5 ml concn H_2SO_4 added. The solution was refluxed 20 hr in a Soxhlet apparatus containing anhydrous $MgSO_4$, mixed with 400 ml water and 200 ml benzene and decanted in a warm separatory funnel. The upper phase was washed, and dried on Na_2SO_4.

Adsorption Chromatography. Alumina II was used for the separation of esters and for fractionation of the unsaponifiable mixture. Because of the difficulty in dissolving the materials, the process was carried out in chromatographic tubes with heating mantles. Acetone vapors maintained the temp at ca. 56C. Solvents used in most cases were hexane, hexane-benzene (1:1), benzene, benzene-methanol (9:1), and benzene-methanol (1:1).

Gas-Liquid Chromatography. Apparatus was an Aerograph A-90 P unit with thermal conductivity cells for detection. A stainless steel column, 5 ft x ¼ in. OD, packed with 60/80 mesh fire brick coated with 20% silicone rubber was used to separate homologous methyl esters, hydrocarbons, and alcohols. Temp was programmed from 180–320C. Pure standards and natural products were dissolved in warm dioxane. Relative proportions of constituents were calculated by triangulation (11).

TABLE I
Constituents of Coccid Waxes in % of Total Wax

Coccidae	Hydrocarbons	Alcohols	*n* Acids	Hydroxy acids
Gascardia madagascarienis	0.6	28.0	38.0	33.4
Coccus ceriferus	2.6	47.4	50.0	.....
Tachardia lacca	1.8	77.2	21.0	.....

Reductions. Fatty alcohols or fatty esters (800 mg) were refluxed with 24 ml 65% HI and 40 mg red phosphorus for 18 hr. The alkyl iodide was extracted with benzene, the solution washed first with Na_2SO_3 solution and then with water. The solution was dried on Na_2SO_4 and the benzene evaporated, leaving the iodide. This (800 mg) was dissolved in 50 ml ethanol-methanol-water (40:10:50) solvent and refluxed with 250 mg zinc powder and 4 ml concn HCl, the solvents evaporated and remaining compounds dissolved in benzene.

Reduction of carboxylic esters with $LiAlH_4$ was carried out as follows: to 500 mg of substance dissolved in 40 ml tetrahydrofuran warmed at 40C were added 500 mg $LiAlH_4$ suspended in 10 ml of the same solvent. The mixture was held for 30 min at 40C then 5 ml water and 15 ml 3N H_2SO_4 added. The tetrahydrofuran phase was washed and dried on Na_2SO_4.

Oxidations. Hydroxylated esters were oxidized to keto esters by CrO_3; 300 mg of the substance suspended in 75 ml acetone was added to a solution of 300 mg CrO_3 in 10 ml H_2O and 1.5 ml H_2SO_4. The mixture was stirred 30 min at 40C, poured into 100 ml warm water and the keto ester extracted with 100 ml warm benzene. The keto ester was purified by alumina chromatography.

A more drastic chromic oxidation was performed to split the hydrocarbon chain of the hydroxy acid. To 500 mg hydroxy acid dissolved in 35 ml acetic acid and maintained at 78C, 300 mg CrO_3 was added. After several hr, another 300 mg and later an additional 150 mg CrO_3 was added. The reaction was stopped by addition of methanol and oxidation products were extracted with warm benzene.

Permanganate oxidation of hydroxy acids was carried out in acetone solution. To a solution of 430 mg hydroxy acid in 300 ml acetone, 600 mg $KMnO_4$ was added in small portions. The mixture was refluxed 9 hr, acetone distilled off and the residue extracted 3 times with 100 ml hot absolute ethanol. The alcoholic solution was acidified with 6N HCl and concentrated to 30 ml. The precipitate was filtered, alcohol-washed and dried in vacuum.

Results

Tables I and II summarize the composition of coccid waxes.

A. *Gascardia madagascariensis.* 5 Kg cocoons furnished 1,390 g petroleum ether-soluble substances from which 430 g wax and 960 g resinous material was separated. The saponification of 25 g wax followed by treatment with Na_2CO_3 as described above yielded 12.5 g unsaponifiables. This fraction was submitted to further saponification and separation on Na_2CO_3. A total of 9.5 g unsaponifiables and 15.5 g acids was thus obtained.

Unsaponifiables. This fraction was extracted repeatedly with hexane. The soluble part, representing 2% of the total unsaponifiables, was eluted with hexane by alumina chromatography. The IR spectrum

TABLE II

Constituents of Coccid Waxes in % of Each Group

No. of C atoms	*Gascardia madagascariensis*			*Coccus ceriferus*			*Tachardia lacca*		
	Hydrocarbons	Alcohols	Non-hydroxy Acids	Hydrocarbons	Alcohols	Acids	Hydrocarbons	Alcohols	Acids
10	....	....	....	....	....	....	....	....	0.1
11	....	....	....	....	....	....	....	....	0.2
12	....	....	....	....	....	....	....	....	0.1
13	....	....	....	....	....	....	....	....	0.3
14	....	....	....	....	....	6.9	....	....	1.0
15	....	....	....	....	....	....	....	....	....
16	....	....	0.4	....	....	2.4	....	....	3.4
17	....	....	....	....	....	....	....	....	....
18	....	....	0.1	....	....	3.4	....	....	0.4
19	....	....	....	....	....	....	....	....	....
20	....	....	0.1	....	....	traces	....	....	0.3
21	....	....	....	....	....	....	....	....	....
22	....	....	traces	....	....	1.2	....	....	0.2
23	....	....	....	....	....	....	....	....	....
24	....	4.6	0.5	....	7.0	14.4	....	....	0.2
25	3.4	....	....	3.9	....	....	....	....	....
26	1.4	72.0	26.0	0.6	63.0	49.0	....	0.6	0.3
27	72.0	....	....	5.2	....	....	42.0	....	1.2
28	2.2	11.6	4.4	1.0	28.0	15.5	4.1	66.6	18.9
29	20.0	....	....	7.6	....	....	35.1	....	1.7
30	....	5.9	5.8	2.7	2.0	5.6	2.8	21.0	25.1
31	1.0	....	traces	42.1	....	....	13.4	....	1.1
32	....	5.9	19.8	1.0	....	1.5	traces	9.0	27.2
33	....	....	0.1	29.2	....	....	2.6	....	0.2
34	....	....	39.5	0.5	....	....	....	2.8	17.6
35	....	....	....	6.2	....	....	....	....	....
36	....	....	3.2	....	....	....	....	....	0.5

showed only hydrocarbon bands and the analysis was in good agreement with that of heptacosane. Calculated for $C_{27}H_{56}$: C 85.17, H 14.83. Found: C 85.17, H 14.86. GLC demonstrated that there are two principal components: heptacosane 72%, and nonacosane 20% of total hydrocarbons, with smaller quantities of the even hydrocarbons, hexacosane and octacosane (Table II). It is noticeable that even hydrocarbons are found in very low proportions while Mazliak reported similar amounts both of odd and even homologous paraffins in Carnauba wax (3).

Alumina chromatography of the hexane-insoluble fraction of unsaponifiables yielded above 50% of a white compound eluted by benzene. The IR spectrum showed −OH bands at 3300,1060 and 1110 cm^{-1}. The last chromatographic fraction eluted by benzene-ethanol (1:1) was a yellowish resinous compound not yet studied. The benzene eluates were pooled and analysed after crystallization from ethyl acetate. Calculated for $C_{26}H_{54}O$: C 81.60, H 14.22, (C)−CH_3 3.93. Found: C 81.82, H 14.26, (C)−CH_3 3.98. GLC showed hexacosanol to be the major constituent, with smaller proportions of homologous C_{24},C_{28},C_{30} and C_{32} fatty alcohols (See Table II).

Acids. Crude acids retained on the Na_2CO_3 column were methylated with sulfuric acid-methanol as indicated above. Methyl esters had the characteristic hydroxyl band in their IR spectra. Chromatography on alumina II at 56C yielded only two fractions: one of them (58%) was eluted by hexane-benzene (1:1), the other (47%) was eluted by benzene-methanol (9:1). The IR spectra showed only methyl ester bands at 1741,1250,1190,1170 and 870 cm^{-1} in the first fraction and additional −OH bands in the last one.

The major constituents identified by GLC are the normal esters of tetracontanoic, dotriacontanoic, and hexacosanoic acids. Odd homologs are of minor importance.

Hydroxylated methyl esters were studied. Recrystallization from ethyl acetate yielded a microcrystal line powder, having mp 90C. Analysis, calculated for $C_{34}H_{68}O_3$: C 77.80, H 13.06, (O)−CH_3 5.90, (C)−CH_3 2,86. Found: C 77.86, H 13.04, (O)−CH_3 5.94, (C)−CH_3 2.70. Analysis agreed with a non-branched chain monohydroxy acid $C_{33}H_{66}O_3$. Trials to identify individual acids by GLC of methyl esters, even at high temperature (350C), were unsuccessful. No peak was detected on the chromatogram. Determination of the length of the carbon chain was attempted by another method as described by Downing, et al. (7). The hydroxylated methyl esters were reduced with $LiAlH_4$ as described above. After saponification and treatment of the saponified mixture by Na_2CO_3 a diol (mp 112C) was isolated and purified by recrystallization. Analysis, calculated for $C_{33}H_{68}O_2$: C 79.77, H 13.80. Found: C 79.24, H 13.47. Acetylation of this compound by acetic anhydride-pyridine yielded an ester with the mp 76.5C. Analysis, calculated for $C_{37}H_{72}O_4$: C 76.49, H 12.49, (CO)−CH_3 (for 2) 5.16. Found: C 76.15, H 12.46, (CO)−CH_3 (for 2) 5.19. This compound is a diacetate which agrees with an initial monohydroxy acid. The diol treated with HI and red phosphorus followed by reduction with $LiAlH_4$ yielded products that were purified by chromatography on alumina. Hexane eluted a white compound recrystallized from absolute ethanol, mp 70C. Its IR spectrum was that of a saturated hydrocarbon. Two major compounds were separated by GLC: tetratriacontane (57%), dotriacontane (38%) with a smaller yield of triacontane (5%).

Other determinations of the chain length were performed by reducing hydroxylated methyl esters with HI and P followed by Zn−HCl treatment and purification by chromatography on alumina. Normal methyl esters obtained were identified by GLC as methyl triacontanoate, dotriacontanoate and tetratriacontanoate. In the original wax, hydroxy triacontanoic, hydroxy dotriacontanoic and hydroxy tetratriacontanoic acids are present.

Oxidation of hydroxylated ester by CrO_3 gave a keto compound which was purified by alumina chromatography. No diacid or ketone was detected after saponification. Permanganate oxidation yielded a keto acid (12). The −OH is not at the alpha, beta or omega position. More drastic chromic oxidation of the keto acid was performed, as described above, to determine the position of the hydroxyl. Scission products were methylated and analysed by GLC for ester of mono- and diacids. Accordingly, the original hydroxy acids are mixtures of C_{30},C_{32},C_{34} acids with a −OH function in the approximate middle of the hydrocarbon chain.

B. *Coccus ceriferus.* 5 g commercial wax was saponified by methanolic KOH for 4 days. After Na_2CO_3 treatment, the non-acidic fraction showed ester bands by IR spectra and was saponified again for 4 days. The final yield was about 50% of the acidic fraction and 50% unsaponifiables (Table I). Column chromatography of unsaponifiables furnished only 2.6% hydrocarbons eluted by hexane. GLC showed a major fraction of hentriacontane and a smaller one of tritriacontane. Fatty alcohols eluted by benzene were essentially hexacosanol (63%) and octacosanol (28%), as indicated in Table II.

Fatty acids were methylated by diazomethane. The IR spectrum showed no hydroxyl band. Column chromatography of methyl esters furnished one fraction eluted by hexane. Its composition as determined by GLC shows in Table II. Hexacosanoic acid is the major constituent with smaller amounts of homologous tetracosanoic and octacosanoic acids.

C. *Tachardia lacca.* Crude yellowish commercial wax was purified by extraction of resinous compounds with acetone, 300 g raw material furnishing 200 g white purified wax. After two successive saponifications, 21% acidic material and 79% unsaponifiables were obtained. Column chromatography of the unsaponifiable fraction yielded about 2% hydrocarbons and 98% fatty alcohols. The hydrocarbons were essentially a mixture of heptacosane, nonacosane and hentriacontane, with minute amounts of even homologs as shown by GLC. Octacosanol is the major constituent of the fatty alcohols (Table II).

Fatty esters were chromatographed on alumina columns after methylation of acids by diazomethane. No hydroxy acid was detected. The whole fraction eluted by hexane had no hydroxyl band in the IR spectrum. GLC showed a mixture of C_{28},C_{30},C_{32}, and C_{34} acids with traces of other homologs (Table II).

Discussion

The latest prior papers on the chemical composition of waxes from the coccidae are rather old. Henriques investigated the composition of China wax from *Coccus ceriferus* in 1897 (13), and found hexacosanol and hexacosanoic acid. In 1920, Gascard (14) assigned other formulas to these constituents; $C_{27}H_{56}O$ to the alcohol and $C_{27}H_{54}O_2$ to the acid. These conclusions were corroborated by Huminski (15) in 1935. In the same year, Collins (16) found C_{24},C_{26},C_{28} and C_{30} acids by fractional distillation, and more recently Warth (1) notes the occurrence of slight amounts of heptacosane (1%).

Obviously, these earlier investigators were hampered by lack of equipment and techniques sufficiently sensitive to separate homologous constituents with high bp, and for this reason the results they obtained were approximate. It is interesting to compare our own results with theirs. We find a much greater variety of components in China wax (Table II). Heptacosane represents a low percentage of the total hydrocarbons, while hentriacontane and tritriacontane are in major proportion. It must be specified, however, that all odd and even hydrocarbons from C_{25} to C_{35} are present. Of alcohols and acids, only the even homologs were found. The major constituents for which formulas were discussed earlier are in fact C_{26} alcohol and C_{26} acid, and are probably combined as ceryl cerotate in the original wax. The shorter chain fatty acids, C_{14},C_{16} and C_{18}, were also recovered in appreciable yields. They were not mentioned in reports of previous work.

Shellac wax from *Tachardia lacca* was investigated by Gascard (17), who found C_{32} alcohol and C_{32} acid. Warth (1) gives a more extensive list of constituents, including pentacosane, hentriacontane, free alcohols, neoceryl (C_{25}) alcohol or tachardiacerol, lacceryl (C_{32}) alcohol, and ceryl aleuritate (aleuritic acid is the 9,10,16 trihydroxy palmitic acid). Our own conclusions do not agree entirely with those of Warth. Although a notable proportion of free alcohols was found, 77.2% alcohols against 21% acids, no alcohol having an odd number of C atoms was detected. Acids have an even number of C atoms from C_{28} to C_{34}, and only traces of odd-numbered carbon homologs are present. No hydroxy acid was detected. The aleuritic acid mentioned by Warth must be attributed to resinous impurities in the samples of crude wax. The hydrocarbons are different from those previously noted. No pentacosane was found. Heptacosane and nonacosane are the essential constituents, with smaller proportions of hentriacontane and the even hydrocarbons. It must be mentioned that several terpenic acids have been found in the stick-lac from *Tachardia lacca* (18). The structure of shellolic acid, established by Yates and Field (19), was recently confirmed by Carruthers, et al. (20).

The wax from *Gascardia madagascariensis* has not been investigated previously, although Brochere and Polonsky (21) isolated gascardic acid ($C_{25}H_{34}O_2$) from the resinous fraction. Wax from this insect differs widely from the composition of other coccid waxes, consisting of 28% alcohols and 71.4% acids. These acids have a higher carbon number than other coccid waxes investigated, 40% tetratriacontanoic and 3% hexatriacontanoic acid. No acid of higher carbon number than these was detected. One other peculiarity of *Gascardia* wax is the high yield of hydroxy acids, 47% of the total acid fraction. Alpha or omega hydroxy acids are commonly found in natural waxes, but the −OH function is never found in the approximate middle of the hydrocarbon chain. *Gascardia* hydroxy acids are a complex mixture of C_{30},C_{32} and C_{34} compounds of the general formula:

$$CH_3-(CH_2)_n-CHOH-(CH_2)_m-COOH$$

$n = 9$ to 24

$m = 15$ to 24

and $n + m = 27,29$, or 31.

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