ON NATURAL WAXES. XIX.*

COMPLEX ESTERS OF THE WAX OF THE HONEYBEE (*Apis mellifera* L.)

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The ester fraction 2 isolated by gradient elution chromatography of unsaponified beeswax on silica gel was divided into a saturated and an unsaturated fraction. The individual groups of compounds obtained by saponification of both fractions of esters 2 were further separated by column chromatography on silica gel and analyzed in detail by TLC, GLC, and by IR, MS and NMR spectrometry. Homologous series of saturated monocarboxylic acids (mostly palmitic acid, C_{16}), unsaturated monocarboxylic acids (mostly oleic acid, C_{18}), hydroxyalkanes (C_{24} to C_{34}), monocarboxylic monohydroxy acids (mostly 15-hydroxypalmitic acid, C_{16}) and 1,(ω−1)-dihydroxyalkanes ($C_{20}-C_{32}$) were thus identified. The chemical composition of some minor components is also discussed. On the basis of the results obtained, views are proposed on the possibilities of mutual arrangement of the individual components in the original saturated and unsaturated esters 2.

While in a previous communication[1] we took up the chemical composition of the group of simple saturated and unsaturated alkyl esters of beeswax (esters 1), the present paper is devoted to a detailed analysis of another ester group (esters 2) which were also isolated by gradient elution chromatography on a column of silica gel.

This group of compounds (designated as esters II) was isolated from unsaponified beeswax by chromatography on a column of silica gel by Fuchs and de Jong[2]. Carlier and coworkers[3] separated unsaponified beeswax by preparative chromatography on a thin layer of silica gel. These authors did not separate the above group of esters from the following group of esters. Both groups of compounds were then saponified together and homologous series of acids and alcohols were analyzed directly in a mixture by mass spectrometry, using molecular or other characteristic ions. They found a homologous series of saturated monocarboxylic acids (C_6-C_{37}) with maxima at C_{14} and C_{16}, considerable amounts of singly unsaturated acids (C_{13}, C_{14}, C_{16}, C_{17}, C_{18}) and traces of hydroxy acids (C_{15}, C_{16}, C_{18}, C_{24}). Alcohols were present from C_8 to C_{34} with C_{22}, C_{24} and C_{26} predominating.

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Recently, Tulloch[4] published a review on the composition of beeswax where he mentions (without experimental evidence) some of his analyses. His views are generally in good agreement with our results.

Esters 2 form the third larger group of compounds (12·8%) which was isolated as a single fraction from column chromatography[1] of unsaponified beeswax. Control chromatography on a thin layer of silica gel impregnated with silver nitrate revealed a considerable percentage of the unsaturated fraction. To be able to study the fractions separately we separated them by column chromatography on silica gel impregnated with silver nitrate. In this way, we obtained 80·2% saturated and 19·8% unsaturated fractions of esters 2. The IR spectra of both fractions showed absorption bands of esters (1178 and 1734 cm^{-1}) and, in the unsaturated fraction, also a band at 3000 cm^{-1}. The presence of a free hydroxyl group could not be demonstrated.

While the gas chromatography of simple alkyl esters[1] of beeswax (esters 1) on the silicone elastomer SE-30 yielded at 300°C finely separated peaks up to C_{52}, the two fractions of esters 2 did not give under the same conditions of chromatography the corresponding detector response as would be expected from the amount applied to the system. Hence one may assume that the individual esters in the group of esters 2 should contain in their chains a greater number of carbon atoms still (*cf.* also[4]).

For our further study of the individual fractions of esters 2 the saturated as well as the unsaturated fractions were saponified separately and the individual components

TABLE I

Overall Composition (%) of Esters 2 after Saponification
+ Stands for traces.

Group	Saturated esters 2			Unsaturated esters 2		
	of saturated esters 2	of esters 2	of beeswax	of unsaturated esters 2	of esters 2	of beeswax
A	0·23	0·18	0·024	+	+	+
B	33·2	26·6	3·42	35·2	6·97	0·89
C	0·27	0·22	0·028	+	+	+
D	1·83	1·47	0·188	+	+	+
E	29·8	23·9	3·07	27·9	5·52	0·71
F	21·3	17·1	2·19	21·1	4·18	0·53
G	1·47	1·18	0·151	+	+	+
H	12·0	9·62	1·24	15·8	3·13	0·40
Total	*100·1*	*80·2*	*10·3*	*100·0*	*19·8*	*2·53*

isolated by column chromatography (Fig. 1). Free acids had been esterified directly in the reaction mixture with diazomethane so that they could be separated from the alcohols more satisfactorily. Both the saturated and the unsaturated fraction contained always 8 groups of compounds (A—H), the R_F of which were practically identical in both fractions. Of these, the four most abundant components B, E, F and H were studied in detail, these fractions corresponding, respectively, to methyl esters of monocarboxylic acids, alcohols, methyl esters of monocarboxylic monohydroxy acids and to diols.

Further identification was done by gas chromatography, IR spectroscopy, mass spectrometry and nuclear magnetic resonance spectrometry. The participation of the individual groups of compounds in the saturated and unsaturated esters 2 is found in Table I.

Saturated Esters 2

Group B (fractions 3–6, Fig. 1) contains two homologous series of acids (Table II). The first series is represented by only 2·3% and was not studied further. According to the retention times we were dealing here probably with branched acids with a maximum at C_{17}. The second homologous series contains saturated straight-chain monocarboxylic acids from C_8-C_{22} with predominating C_{16}. Mass spectrometry showed that we are dealing here with palmitic acid which is also in agreement with the work of Tulloch[4].

Group D (fractions 9–12, Fig. 1) contains two homologous series of compounds A_D and B_D (Table III). The R_F value (0·57) on a thin layer of silica gel [chloroform–

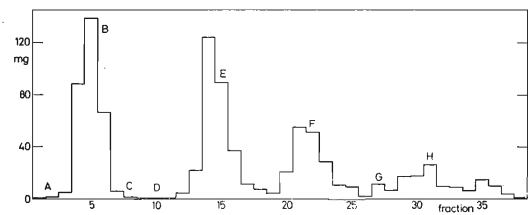

FIG. 1

Chromatographic Separation of the Individual Groups of Substances after Saponification of Saturated Esters 2 on Silica Gel

The symbols A—H designating the individual groups of compounds are explained in the text.

hexane (7 : 3)] is near the R_F value (0·65) of the mixture of compounds in fractions 4–7 obtained by column chromatography[1] of saponified unsaturated esters 1. According to IR spectroscopy we are dealing here with a mixture of esters (absorption bands at 1172, 1740 cm^{-1}). From the analogous behaviour observed in the similar type of compounds in the unsaturated esters 1 one may conclude that we have to do here with methyl esters of monocarboxylic acid with another oxygen function.

Group E (fractions 14–16, Fig. 1) are monoalcohols, as was demonstrated by comparing the R_F values during thin-layer chromatography on silica gel. Similarly, a comparison of the retention times during gas chromatography of free alcohols, their acetates and trimethylsilyl derivatives with synthetic standards showed that

TABLE II

Composition (%) of Monocarboxylic Acids (Group B) of Saturated und Unsaturated Esters 2
+ Stands for traces.

Number of C atoms	Saturated esters 2		Unsaturated esters 2	
	iso-acids	normal acids	saturated acids	unsaturated acids
8	–	+	–	–
9	–	+	–	+
10	–	+	–	+
11	–	+	–	+
12	+	0·1	+	+
13	+	–	–	–
14	–	0·2	0·1	–
15	+	+	+	–
16	–	96·0	24·6	0·5[a]
17	2·2	–	–	+
18	–	1·3	0·1	73·7
19	+	–	–	+
20	–	0·1	1·1	–
21	–	–	–	–
22	–	0·1	+	+
23	–	–	–	–
24	–	–	–	–
25	0·1	–	–	–
Total	*2·3*	*97·8*	*25·9*	*74·2*

[a]Does not belong to a homologous series of saturated or unsaturated acids.

we have to do here with a homologous series of aliphatic, straight-chain, monoalcohols from $C_{24}-C_{34}$ (see also Tulloch[4]) with a maximum at C_{30} (Table IV). Homologues $C_{22}-C_{25}$ are represented in a small amount, these alcohols being probably branched.

Group F (fractions 20–26, Fig. 1). Gas chromatography showed that two homologous series of compounds are present, *viz.* A_F and B_F (Table V). In addition, several homologues were found which did not belong to any homologous series. The last-named homologues and, at the same time, homologues of series A_F, are likely to possess a branched skeleton on the basis of the retention times. In view of their low

TABLE III

Composition (%) of Monocarboxylic Acids with Another Oxygen Function (Group D) of Saturated Esters 2

+ Stands for traces.

Number of C atoms	Series A_D	Series B_D
14	0·1	—
15	—	0·1
16	0·2	—
17	—	0·2
18	—	11·7
19	—	2·7
20	4·6	—
21	0·8	—
22	+	+
23	0·4	—
24	0·2	—
25	0·6	0·6
26	1·0	—
27	1·7	1·7
28	2·7	—
29	2·5	3·4
30	5·2	—
31	5·6	9·9
32	15·0	—
33	5·2	9·4
34	14·4	—
Total	*60·2*	*39·7*

TABLE IV

Composition (%) of Alcohols (Group E) of Saturated and Unsaturated Esters 2

+ Stands for traces.

Number of C atoms	Saturated esters 2	Unsaturated esters 2
22	+[a]	+[a]
24	6·8	6·5
25	1·1[a]	—
26	8·2	8·0
28	12·0	12·0
30	37·5	38·9
32	30·9	30·9
34	3·5	3·7
Total	*100·0*	*100·0*

[a]Does not belong to a homologous series.

concentration (2·6%) we did not pursue them further. Since one of the homologues of series B_F was clearly predominant (95%) the whole mixture could be considered as an individual for the purposes of further identification. According to IR spectroscopy it could be assumed that we are dealing here with a hydroxy acid (absorption bands for $-COOCH_3$ at 1173, 1439 and 1740 cm^{-1}, for $-OH$ at 3600 cm^{-1}). Mass spectrometry of the methyl ester of this hydroxy acid (*a*, with a free $-OH$ group; *b*, with trimethylsilylated $-OH$ group) revealed further that the acid in question has 16 carbon atoms with a hydroxyl group in position 15, *i.e.* 15-hydroxypalmitic acid (Fig. 2). The mass spectrum also shows the fundamental splitting of the molecule. Peaks at *m/e* 74, 87, 143, 199 and 243 are characteristic of methyl esters of higher aliphatic monocarboxylic acids[5] (Fig. 2*a*). The peak at *m/e* 73 (Fig. 2*b*) arose by splitting[6-8] of the ion $(CH_3)_3Si^+$. The identification of 15-hydroxypalmitic acid was further confirmed by nuclear magnetic resonance.

Indications of the presence of hydroxy acids in beeswax after saponification appeared in the literature several times[9-15]. It is probable that the isolated hydroxy acids originated directly from hydroxy esters which represent an appreciable part of beeswax[1,2,4]. The presence of 15-hydroxypalmitic acid in esters 2 was mentioned recently by Tulloch[4].

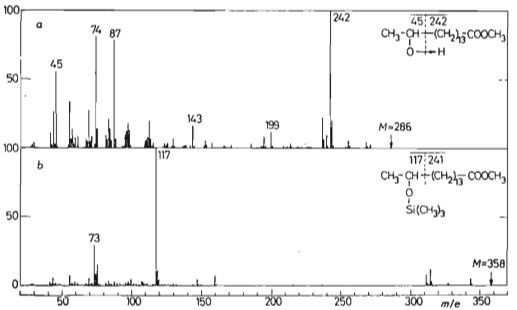

FIG. 2

Mass Spectrum of the Methyl Esters of 15-Hydroxypalmitic Acid (C_{16}) (Group F) of the Saturated Esters 2

a With free OH group, *b* with trimethylsilylated OH group.

Group H (fractions 29–35, Fig. 1). IR spectroscopy showed that the mixture of compounds contains only hydroxyl groups (absorption bands at 1052 and 3615 cm^{-1}). The character of the spectrum did not permit to determine reliably the position or the amount of hydroxyl groups present. Since during chromatography on a thin layer of silica gel this group showed a much lower R_F (0·16) than the monoalcohols (group E, R_F 0·49) we concluded that a mixture of diols might be involved. This was supported by comparing with R_F values of synthetic diols of different types. From chromatography on a thin layer of silica gel in chloroform–methanol (9 : 1) the R_F of the standard and that of the group H mixture were between 0·62 and 0·68.

TABLE V

Composition (%) of Monocarboxylic Monohydroxy Acids (Group F) of Saturated and Unsaturated Esters 2

+ Stands for traces.

Number of C atoms	Saturated esters 2		Unsaturated esters 2	
	series A_F	series B_F	series A_F	series B_F
10	+	–	–	–
11	+	+	+	+
12	–	+	–	–
13	–	+	–	–
14	–	0·3	+	0·1
15	+	+	–	–
16	0·4[a]	95·0	+	96·2
17	1·3	–	+	–
18	–	0·6	0·5	2·4
19	0·1	0·2	–	–
20	–	0·1	0·2	0·5
21	–	+	–	–
22	–	1·0	0·1	+
23	–	0·2	–	–
24	0·2[a]	+	–	–
25	0·1[a]	0·1	–	–
26	0·3[a]	0·1	–	–
27	–	–	–	–
28	0·2[a]	–	–	–
Total	*2·6*	*97·6*	*0·8*	*99·2*

[a] Does not belong to homologous series A_F or B_F.

Gas chromatography of compounds of group H revealed three homologous series A_H, B_H and C_H (Table VI). Since series A_H and C_H are represented by less than 4%, we did not pursue a closer identification of the individual homologues. We suspect them to be diols with different positions of hydroxyl groups than encountered in series B_H diols.*

More than 96% of the diols belonged to homologues of series B_H. To establish the number of carbon atoms in the individual homologues the whole mixture of diols was converted to hydrocarbons *via* iodides. The quantitative representation of the n-paraffins formed determined by gas chromatography, corresponded rather precisely to the representation of the individual homologues of the original diols. Since only a homologous series of n-paraffins was formed it proved that the individual homologues of series B_H diols have an unbranched skeleton.

A further proof for the assumption that the individual homologues of series B_H contain two hydroxyl groups was obtained directly from the GLC data, *viz.* from the ratio of the retention times of silylated and nonsilylated alcohol. While with the monoalcohols this ratio was found to be equal to 1·15, the alcohols of series B_H and synthetic 2,23-tetracosanediol gave ratios of 1·51 and 1·57, respectively.

The positions of the individual hydroxyl groups in three of the most represented homologues (C_{24}, C_{26} and C_{28}) of the B_H series were determined with the aid of mass spectra of trimethylsilyl derivatives of diols obtained on a mass spectrometer coupled with a gas chromatograph. The application of trimethylsilyl derivatives has the advantage that, on the basis of the ionic intensity by two mass units greater than the fragment under consideration one can determine the number of silicon atoms and hence also the number of hydroxyl groups in the fragment (the ^{28}Si is always accompanied by 3·1% isotope ^{30}Si). On the basis of some more abundant fragments one can determine rather reliably the position of the hydroxyl groups on the carbon chain[6-8,16]. Fig. 3*a* shows the mass spectrum of the C_{24} homologue of the B_H series. The molecular ion (*m/e* 514) is detectable but has a very low intensity. The most intense ion of the spectrum is the *m/e* 117. It contains a single Si atom and, on the basis of its fragmentation, corresponds to $CH_3—CH=\overset{(+)}{O}—Si(CH_3)_3$ whence it follows that one of the hydroxyl groups in the original diol is in position 2. Since the retention time during gas chromatography of synthetic 2,23-tetracosanediol where the hydroxyl groups are in position 2, (ω-1) was much shorter than the retention time of the C_{24} homologue of B_H series we reached the conclusion that the second —OH group of the compound studied wil be most probably terminal[17,18]. For this reason and because of the occurrence of the intense ion *m/e* 117 in the mass spectrum it follows that the second hydroxyl group must lie at the opposite end of the hydrocarbon chain rather than vicinally to the secondary hydroxyl group. In agreement with this fact the spectrum of the C_{24} homologue of the B_H series contains fragments

* See also group H of unsaturated esters 2.

characteristic for a terminal trimethylsilylated —OH group[16,19]. The ions in question are m/e 103, with the structure $CH_2{=}\overset{+}{O}{-}Si(CH_3)_3$ corresponding to a trimethylsilylated terminal —OH group (analogy with m/e 117), the ion m/e 483 (M − 31) formed from the $[M - 15]^+$ ion by fragmentation of the CH_4^* particle and the ion m/e 367 formed from the ion m/e 483 by splitting off 116* mass units. The fragments do not appear at all or have a very low intensity in the spectrum of bis-trimethylsilyl derivative of synthetic 2,23-tetracosanediol (C_{24}) (see Fig. 3*b*).

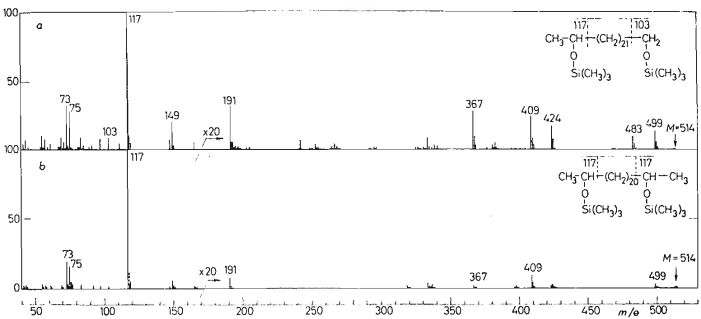

FIG. 3

Mass Spectra of Bis-trimethylsilyl Derivatives of Diols

a Homologue C_{24} of series B_H (Group H) of saturated esters 2 (1,23-tetracosanediol), *b* synthetic 2,23-tetracosanediol.

It follows from all the above data that the C_{24} homologue of the B_H series is 1,23-tetracosanediol. The nuclear magnetic resonance of the C_{24} homologue diacetate isolated by preparative gas chromatography showed also that the hydroxyl groups are in positions 1, (ω-1) (supported also by decoupling experiments). Another proof for this is the full agreement of the retention values of synthetically prepared 1,23-tetracosanediol with the values of the above quoted homologue, both with free diols and with their acetates and trimethylsilyl derivatives.

The other homologues of the B_H series (C_{26}, C_{28}) show an analogous mass-spectrometric behaviour as the C_{24} homologue. On the basis of this analogy they were identified as 1,25-hexacosanediol and 1,27-octacosanediol.

* Confirmed by metastable ions.

The saturated esters 2 may be seen to represent a very complicated mixture of compounds. Since the R_F established by means of thin-layer chromatography on silica gel for esters 2 (0·58) is much lower than for esters 1 (0·82) [in hexane–chloroform–ether (20 : 2 : 1)], esters 2 apparently contain two oxygen functions. Trimyristin had an R_F of 0·31 in the same solvent system. If it is taken into account that the main groups of compounds from which the saturated esters 2 of beeswax are formed are monocarboxylic acids (mostly palmitic), monocarboxylic monohydroxy acids (mostly 15-hydroxypalmitic), monoalcohols ($C_{24}-C_{34}$) and diols ($C_{24}-C_{32}$) two patterns may be offered for their mutual arrangement. The first of these, suggested by Tulloch[4], assumes that 15-hydroxypalmitic acid is acylated at its —OH group with palmitic

TABLE VI

Composition (%) of Diols (Group H) of Saturated and Unsaturated Esters 2
+ Stands for traces.

Number of C atoms	Saturated esters 2			Unsaturated esters 2		
	series A_H	series B_H	series C_H	series A_H	series B_H	series C_H
18	—	—	—	7·8	—	—
19	0·1	—	—	—	—	—
20	0·1	+	—	0·2	0·2	—
21	+	—	0·1	+	—	0·1
22	0·2	0·3	—	0·2	0·2	—
23	0·1	—	0·5	+	—	1·0
24	—	53·8	—	—	40·9	—
25	1·2	—	+	1·0	—	+
26	—	18·5	—	—	16·2	—
27	0·7	—	0·2	1·1	—	0·2
28	—	18·8	—	—	22·6	—
29	0·2	—	—	0·4	—	—
30	—	4·7	—	—	5·9	—
31	0·4	—	—	0·3	—	—
32	—	+	—	—	1·1	—
33	—	—	—	0·8	—	—
Total	*3·0*	*96·1*	*0·8*	*11·8*	*87·1*	*1·3*

acid and esterified at its —COOH group with monoalcohol. We assume that the saturated esters 2 contain another type of more complicated esters formed by acylation of both —OH groups of the diols with palmitic acid. The synthetically prepared

ester* of this second type (octadecanediol dimyristate, C_{46}) had an R_F of 0·54 in thin-layer chromatography on silica gel in the above solvent system, this value corresponding to the R_F of saturated esters 2. The two patterns are in agreement with the quantitative participation of the two above groups of compounds (Table I).

Unsaturated Esters 2

Group B contains two homologous series of monocarboxylic acids (Table II). The first series is formed by saturated acids from $C_{12}-C_{22}$ with a maximum at C_{16}. The second, better represented series is formed by unsaturated acids from C_9-C_{22} with a maximum at C_{18}. Both these types of acids in the form of their methyl esters were separated by chromatography on a thin layer of silica gel impregnated with silver nitrate. Saturated C_{16} acid was identified by mass spectrometry as palmitic acid.

Microhydrogenation of the mixture of unsaturated acids, in which the C_{18} acid represents almost 100%, consumed 1 mol hydrogen which supports the finding of one double bond in the molecule. IR spectroscopy showed further that the double bond is *cis* (absorption bands at 720, 1654 and 3000 cm^{-1}). The bands corresponding to the *trans* double bonds were completely absent. The position of the double bond was determined by mass spectrometry[8,20]. For this purpose, the acid was hydroxylated and the dihydroxy acid formed (containing two vicinal —OH groups) was silylated. It was found from the spectrum that the double bond is in position 9. The unsaturated acid was thus identified as *cis*-9-octadecenoic acid (oleic). This was confirmed by comparing the retention times from gas chromatography on a polar phase with that of standard oleic acid.

Similarly as with unsaturated esters 1 (ref.[1]) the question arose with the unsaturated esters 2 as to the explanation of almost 26% saturated acids. We assumed that like with the unsaturated esters 1, the unsaturated esters 2, a group of alcohols will contain an unsaturated fraction, corresponding roughly to 26% of saturated acids. However, this assumption was not borne out since chromatography on a thin layer of silica gel impregnated with silver nitrate yielded a single spot both with alcohols (group E) and with diols (H). A possible explanation here is that the individual types of esters forming together unsaturated esters 2, contain always oleic acid in their molecule. This acid, as the only bearer of unsaturation in the system may be responsible for the fact that during chromatography on silica gel impregnated with silver nitrate, a separation from the saturated esters takes place.

* Prepared by heating 1,8-octadecanediol (C_{18}) with myristoyl chloride (C_{14}) to 100°C for 1 h.

Group E are alcohols as was shown by chromatography on a thin layer of silica gel and by gas chromatography. We are dealing here again with a homologous series of aliphatic unbranched alcohols between C_{24} and C_{34} with a maximum at C_{30} (Table IV). Moreover, there may be a branched C_{22} homologue present in the system.

Group F contains again two homologous series of substances A_F and B_F (Table V) with predominating C_{16} in the B_F series. IR spectroscopy and mass spectrometry showed that the homologue in question is 15-hydroxypalmitic acid.

Group H is also formed by three homologous series of compounds with predominating B_H series (Table VI). The mass spectra of the three most abundant homologues of this series (C_{24}, C_{26} and C_{28}) are identical with the mass spectra of the same homologues of group H from saturated esters 2. Hence we have to do here again with diols of the 1, (ω-1) type.

Much more than in the saturated esters 2 the unsaturated esters 2 contain the homologous series A_H. The mass spectrum of the trimethylsilyl derivative dominating the C_{18} homologue is identical with the mass spectrum of the trimethylsilyl derivative of 9,10-dihydroxyoctadecanoic acid (C_{18}) as published by Capella and Zorzut[20]. The compounds are apparently identical. The dihydroxy acid represents 7·8% of group H, 1·25% unsaturated esters 2, 0·25% esters 2 and 0·032% beeswax. It is likely that also the other members of the homologous series A_H of unsaturated as well as saturated esters 2 might be dihydroxy acids not previously found in natural waxes.

EXPERIMENTAL

Material

Beeswax, silica gel and all the solvents were the same as described in the previous publication[1].

Separation of Saturated and Unsaturated Esters 2

1·30 g esters 2 obtained[1] from gradient elution chromatography of beeswax was adsorbed to 14 g silica gel and chromatographed on a column (3 × 60 cm) containing 250 g silica gel (0·10—0·25 mm) impregnated with 20% silver nitrate and deactivated with 15% water. The elution was done with a mixture of hexane (refined with H_2SO_4)–$CHCl_3$ (3 : 7), 50 ml fractions being collected. Fractions 1 and 2 contained saturated esters, fractions 5—12 unsaturated esters. The intermediate fractions 3 and 4 were combined, chromatographed in the same way and the individual fractions were combined with those of the main chromatography. A total of 1·029 g saturated esters (80·2%) and 254 mg unsaturated esters (19·8%) was obtained.

The IR spectra of the two groups of esters were recorded in a UR-10 apparatus in 0·01 cm cuvettes (6% solution in CCl_4). The peaks at 1178 (1178) and 1735 (1734) cm^{-1} are typical of the ester group. With unsaturated esters there was an absorption peak also at 3000 cm^{-1}. None of the groups showed absorption corresponding to a free hydroxyl group.

Saponification of Esters 2 and Separation of the Individual Components

800 mg saturated esters 2 were refluxed with 160 ml ethanol containing 1·6 ml water and 1·60 g KOH. The course of saponification was checked on a thin layer of silica gel. After 1 h of boiling, the mixture was cooled and made acid with 11 ml H_2SO_4 (1 : 20), 220 ml water was added and extracted with 1 × 160 ml and 5 × 80 ml ether. The combined extract was shaken with 3 × 100 ml water and dried with magnesium sulfate. The excess ether was distilled away and the remaining solution of acids and alcohols was mixed with a redistilled ether solution of diazomethane. The mixture was left to stand for 1 h. After distillation of the solvent the mixture of methyl esters of acids and free alcohols was dissolved in 20 ml elution solution and chromatographed on a column (2·5 × 102 cm) containing 220 g silica gel (0·10—0·25 mm), deactivated with 15% water. Chromatography on a thin layer of silica gel showed that the most suitable solvent system is one with chloroform–hexane–ether (13 : 6 : 1). Fractions 1—10 were 25 ml, fractions 11—25 were 50 ml, fractions 26—34 were 100 ml and fractions 35—38 were 250 ml in volume. The purity of the individual fractions was checked by chromatography on a thin layer of silica gel in the above solvent system and, at the same time, the R_F values of the individual groups of compounds A—H were established: A 1·00, B 0·93, C 0·78, D 0·62, E 0·49, F 0·35, G 0·28, H 0·16. The chromatography is shown in Fig. 1.

Like with the saturated esters 2, a total of 150 mg unsaturated esters 2 was processed and separated chromatographically.

Identification of Acids (Group B) from Unsaturated Esters 2

Preparative chromatography on a thin layer of silica gel impregnated with 20% silver nitrate separated the methyl esters of acids (group B) into a saturated and unsaturated fraction [chloroform–hexane (refined with H_2SO_4) 7 : 3]. The detection was done with Rhodamine B in UV light and the individual zones were extracted with absolute ether[21], the dye being trapped on a small column of silica gel.

The IR spectra of the unsaturated methyl esters of the acids showed in addition to a $-COOCH_3$ group (absorption maxima at 1438 and 1741 cm^{-1}) a *cis*-disubstituted double bond (absorption maxima at 720, 1654 and 3000 cm^{-1}); no *trans*-double bond was present. The measurement was carried out in a UR-10 apparatus in 0·01 cm cuvettes, with 6% solutions in carbon disulfide.

The consumption of hydrogen estimated by *microhydrogenation*[22] with the methyl esters of unsaturated acids corresponded to the presence of one double bond.

Hydroxylation was carried out according to Wolff and coworkers[23]. 19·7 mg OsO_4 dissolved in 2 ml of an ether–pyridine mixture (8 : 1) was added to 9·7 mg methyl esters of unsaturated acids. After 2 h of standing, 60 ml freshly prepared suspension of sodium sulfite in methanol (prepared by mixing 15 ml 16% aqueous Na_2SO_3 with 50 ml methanol) was added and, after 1 h, the precipitate was filtered through a S3 glass filter and the filtrate evaporated at reduced pressure practically to dryness. The residue was extracted with 3 × 5 ml of a mixture of hexane and ether (1 : 1). After evaporation, further 10 mg dihydroxy acid methyl esters were obtained. Five mg of these were converted to the bis-trimethylsilyl derivatives.

Preparation of trimethylsilyl derivatives[24]. 0·2—0·3 ml silylation mixture [trimethylchlorosilane–hexamethyldisilazane–pyridine (1 : 3 : 9)] was added to 5 mg dihydroxy acids (or monohydroxy acids, monoalcohols or diols). The reaction mixture was heated several times to about 60°C and well agitated. After 30 min, it was mixed with 5 ml water and the derivatives extracted with

4 × 5 ml hexane. The combined extract was shaken with 3 × 7 ml water, dried with magnesium sulfate and evaporated to dryness. Since the trimethylsilyl derivatives are relatively unstable, the gas–liquid chromatography and the mass spectrometry was done on the same or on the following day.

*Mass spectrometry** was done on a MCH 1303 (USSR) spectrometer with a sector magnetic analyzer and magnetic detector of the mass scale and with a device for direct injection of samples. The ionization chamber was maintained at a constant temperature of 200°C.

Identification of Hydroxy Acids (Group F)

The IR *spectra* of a mixture of compounds of group F of saturated as well as unsaturated esters 2 exhibited besides a —$COOCH_3$ group (absorption peaks at 1173, 1439 and 1740 cm^{-1}) also a —OH group (absorption maximum at 3600 cm^{-1}). The measurement was carried out in a UR-10 apparatus using a 0·01 cm cuvette and 5% solutions in CCl_4.

Mass spectrometry was done as described above. The mass spectra (see Fig. 2) of silylated and nonsilylated methyl esters of hydroxy acids (group F) of saturated esters 2 was identical with the corresponding mass spectra of hydroxy acid methyl esters of unsaturated esters 2.

Nuclear magnetic resonance was done in a Varian HA-100 apparatus. Solvent was $CDCl_3$, internal standard TMS. —$COOC\underline{H}_3$ 3·66 p.p.m. (s, 6 H); —$C\underline{H}(OH)—CH_3$ 3·76 p.p.m.; —O—CO—$CH_2—C\underline{H}_2$ 2·30 (t, 2 H); —$CH(OH)—C\underline{H}_3$ 1·16 (d, $J \cong 6{\cdot}5$ Hz).

Identification of Diols (Group H)

The IR spectrum of a mixture of compounds of group H of the saturated esters 2 showed the presence of a free hydroxyl group (absorption maxima at 1052 and 3615 cm^{-1}). The measurement was carried out in a UR-10 apparatus using a 0·01 cm cuvette (7% solution in $CHCl_3$).

Conversion to hydrocarbons was done according to Downing and coworkers[25]. Three mg compounds of group H of the saturated esters 2 were heated in a sealed tube with 3·5 mg red phosphorus and 15 mg iodine for 1·5 h at 100°C. The reaction mixture was extracted with 3 × × 5 ml hexane, the combined extracts were shaken with 3 × 10 ml water and dried with magnesium sulfate. After evaporation of the solvent the iodides were refluxed with 10 ml tetrahydrofuran (purified with $LiAlH_4$) and 50 mg $LiAlH_4$ for 2·5 h. After decomposition with HCl (1 : 20) the reaction product was extracted with 4 × 7 ml ether, the combined extracts were shaken with 3 × 10 ml water and dried with magnesium sulfate. The product obtained was purified by chromatography on a column (0·8 × 3 cm) of silica gel (0·025—0·063 mm) in hexane. A total of 2·1 mg of a mixture of hydrocarbons was obtained which was shown to contain a homologous series of n-paraffins during gas chromatography.

Preparation of trimethylsilyl derivatives was described above (see identification of acids of group B of the unsaturated esters 2).

Preparation of acetates[26]. 0·2 ml redistilled acetyl chloride was added to 1—2 mg mixture of diols (group H) or monoalcohols (group E), the mixture was heated to 60°C and excess acetyl

* The authors acknowledge the assistance with recording and interpreting the mass spectra by Dr L. Dolejš.

chloride was evaporated. The same procedure was used for the acetylation of 100 mg diols from saturated esters 2 for preparative gas chromatography.

Gas chromatography — mass spectrometry was done in a LKB 9000 apparatus with a monofocussing mass spectrometer and magnetic detection of the mass scale. The chromatographic column (0·25 × 250 cm) contained 1% SE-30 on Chromosorb W (80—100 mesh), the thermostat temperature was 220 and 250°C. The mass spectra of the individual components were recorded on reaching the top of the chromatographic wave. The ionization energy during chromatographic registration was 20 eV, during recording of mass spectra 70 eV, temperature of the source was 290°C.

Nuclear magnetic resonance was measured in a Varian HA-100 apparatus. Solvent was $CDCl_3$, internal standard was TMS. —OH(OAc)—CH_3: C$\underline{H}$ 4·88 p.p.m., C$\underline{H}_3$ 1·18 p.p.m. (d, $J = 6$ Hz); AcO—C$\underline{H}_2$—CH_2 4·05 p.p.m. (t, $J = 6{\cdot}5$ Hz); Ac$\underline{O}$ 2·01 and 1·99 p.p.m.

Gas Chromatography

Analytical gas chromatography was done on Pye Argon Chromatograph and on Perkin-Elmer F 11 with a dual system. The acid methyl esters were chromatographed on 10% SE-30 on Chromosorb W (100—120 mesh), on 3% SE-30 on Gas-Chrom P (100—120 mesh) and on 10% butanediol succinate on Chromosorb W (100—120 mesh). 3% SE-30 was used for the chromatography of the trimethylsilyl derivatives of monoalcohols and diols. The glass columns were 0·4 × 120 cm in size. The wax esters, alcohols, diols, their acetates and trimethylsilyl derivatives were chromatographed on 2·5% SE-30 on silanized Chromosorb G (100—120 mesh). The glass columns were 0·4 × 150 cm in size. The quantitative evaluation of the chromatograms was done in such a way as to compare the products of the retention times and of the corresponding wave heights (without using correction factors).

Preparative gas chromatography of diacetates of diols from the saturated esters 2 was done in an apparatus of original construction equipped with a catharometer as detector. A metal column (0·6 × 70 cm) contained 10% E-301 on Chromosorb W (100—120 mesh), thermostat temperature was 280°C, temperature of the injection chamber 290°C, flow of helium 30 ml/min. The individual homologues were condensed in glass traps containing a little column of silica gel 3 × 30 mm, elution was done with 4 ml tetrachloromethane. It was found by a control on an analytical apparatus that the prepared homologue C_{24} is 85% pure.

Synthesis of 2,23-Tetracosanediol

1,22-*Docosanedinitrile*. 2·00 g pure 1,22-docosanedioic acid was converted by gaseous ammonia at 300°C to its dinitrile by a modified method of Ralston and coworkers[27]. The dinitrile formed was dissolved in 20 ml chloroform and purified by chromatography on a column (2·5 × 35 cm) containing 80 g silica gel (0·10—0·25 mm) deactivated with 15% water. For elution we used a mixture of $CHCl_3$–ether (99 : 1). A total of 1·01 g very pure 1,22-docosanedinitrile was obtained. For $C_{22}H_{40}N_2$ (332·6) calculated: 79·45% C, 12·12% H, 8·42% N; found: 79·48% C, 11·96% H, 8·26% N.

2,23-*Tetracosanedione*. An ether solution of 0·50 g 1,22-docosanedinitrile was added dropwise and under intense stirring over a period of some 30 min to a Grignard reagent prepared from 365 mg magnesium and 2·5 ml methyl iodide. The reaction mixture was refluxed for 2·5 h. After decomposition with a saturated solution of ammonium chloride the reaction product was extracted with a mixture of hexane–ether (1 : 1) (4 × 30 ml), the combined extracts were shaken with water and dried with magnesium sulfate. A total of 0·53 g crude product was obtained. This was adsorbed to 2·5 g silica gel and purified by chromatography on a column (2 × 85 cm)

containing 120 g silica gel (0·10—0·25 mm). As elution agent we used a mixture of hexane–$CHCl_3$–ether (25 : 25 : 1). A total of 0·38 g pure 2,23-tetracosanedione was obtained. For $C_{24}H_{46}O_2$ (366·6) calculated: 78·63% C, 12·65% H, 8·73% O; found: 78·55% C, 12·55% H, 8·90% O.*

2,23-*Tetracosanediol*. An ether solution of 70 mg $LiAlH_4$ was added dropwise and under stirring to an ether solution of 50 mg 2,23-tetracosanedione. The reaction mixture was refluxed for another hour. After decomposition with sulfuric acid (1 : 20) the reaction product was extracted with ether (3 × 20 ml), the combined extracts were shaken with water and dried with magnesium sulfate. According to the results of gas chromatography the final product was at least 95% pure, which was acceptable for direct measurement by mass spectrometry.

Synthesis of 1,23-Tetracosanediol

100 mg methyl ester of 15-hydroxypalmitic acid isolated from beeswax was saponified as usual. The hydroxyl group of the acid was acetylated by heating with 2 ml acetyl chloride for 2 h to 100°C. After distillation of excess acetyl chloride the product formed (112 mg) was electrolyzed in 12 ml methanol together with the monomethyl ester of sebacic acid (C_{10}) (136 mg) with an addition of 5 mg sodium methylate for 30 min (Pt electrodes, 0·5 A, 25—60 V). After removing the solvent 100 mg reaction mixture dissolved in ether was reduced with 100 mg $LiAlH_4$. After decomposition of excess $LiAlH_4$ and extraction with ether a total of 90·6 mg product was obtained. According to its gas chromatography the peak corresponding to 1,23-tetracosanediol corresponded to about 20% of the mixture.

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* Oxygen was estimated by a modified method according to Unterzaucher using heat-conductivity detection of the CO formed. The authors are indebted to Dr K. Ubik for this determination.

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