The Di- and Triesters of the Lipids of Steer and Human Meibomian Glands

N. NICOLAIDES* and **EVELYN C. SANTOS**, *Department of Medicine (Dermatology), University of Southern California School of Medicine, 2025 Zonal Ave., Los Angeles, CA 90033*

ABSTRACT

Three groups of diesters have been isolated and identified in the lipids of steer meibomian glands. The first group, designated as αType I, with the abbreviated formula FA-αOHFA-FAlc, consisted of α-hydroxy fatty acids esterified to fatty acids and fatty alcohols in the approximate molar ratio 1:1:1. The second group, designated as ωType I-St, with the abbreviated formula FA-ωOHFA-St, consisted of ω-hydroxy fatty acids esterified to fatty acids and sterols in the approximate molar ratio 1:1:1. The third group, designated as α,ωType II, with the abbreviated formula FA-α,ωdiol-FA, consisted of α,ω-diols esterified to 2 moles of fatty acids. The sum of the different diesters comprised about 9% of total steer meibomian lipids.

Capillary GLC of the fatty acids of αType I diesters showed the fatty acids to be a family with a two-cluster profile, one at C_{12} to C_{20} and the other at C_{21} to C_{31}, with anteiso chains predominating. Fatty acids from ωType I-St and α,ωType II diesters gave mainly a one-cluster profile in the short chain region with prominent anteiso and $C_{18:1}$ peaks. Fatty alcohols of αType I diesters were mainly long chain, C_{23} to C_{30}, with anteiso chains predominating, while the α-hydroxy fatty acids were short chain C_{13} to C_{18} acids with C_{16} predominating. The sterols in diesters ωType I-St were cholesterol (~60%), Δ7 cholestenol (~35%) and an unidentified compound (~5%) with a GLC retention time slightly longer than Δ7 cholestenol on SE-30 phase. The ω-hydroxy fatty acids and α,ω-diols both were of exceedingly long chain lengths, C_{29}-C_{38}, and showed similar GLC profiles. Two types of triesters comprising approximately 1% of total steer meibomian lipids have been isolated but incompletely characterized. In terms of molar ratios, one group of triesters gave fatty acids:ω-hydroxy fatty acids:α-hydroxy fatty acids:sterols + fatty alcohols as approximately 1:1:1:1. The other contained fatty acids, α-hydroxy fatty acids and α,ω-diols in what appears to be a complex mixture of several triesters. Diesters ωType I and α,ωType II also were found in human meibum. Hitherto these two diesters have not been found in any animal tissue.

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INTRODUCTION

Within the eyelid of animals there is a row of a large sebaceous type of gland, the meibomian gland, which excretes an oily, waxy substance onto the edge of the eyelid. Some of this excreta then spreads out on the wet surface of the cornea, forming the lipid layer of what is known as the preocular tear film.

*To whom correspondence should be addressed.

Abbreviations: FA = fatty acid; FAlc = fatty alcohol; St = sterol; Chol = cholesterol; FAME = fatty acid methyl ester(s); SE = sterol ester(s); WE = wax ester(s); TG = triacyl glycerol(s); C = chloroform; M = methanol; HAc = acetic acid; TLC = thin layer chromatography; ANS = aniline naphthalene sulfonic acid; GLC = gas liquid chromatography.

In the literature, diesters of α-hydroxy fatty acids have been designated as Type I diesters and diesters of 1,2-diols as Type II diesters. In this paper we wish to generalize the term "Type I" diesters to include diesters of all hydroxy fatty acids no matter what position the hydroxyl group occupies. We also wish to extend the term Type II diesters to include diesters of any long chain diol regardless of the location of the two OH groups. When we want to be more specific as to the location of the OH groups for both types of diesters, we will use a prefix of a Greek letter(s) or Arabic numeral(s). To specify the alcoholic moiety of a di or triester, we will use the suffix FAlc to indicate fatty alcohols or St to indicate sterols.

Triesters will be similarly defined. Triesters Type I will be those containing only hydroxy fatty acids and Type II will be those containing a long chain diol plus a hydroxy fatty acid. We also wish to abbreviate structural formulas using a hyphen or line to signify an ester linkage. The following examples illustrate the usage:

diesters Type I — αType I = FA-α OHFA-FAlc; ωType I-ST = FA-ωOHFA-St

diesters Type II — α,βType II = FA-α,βdiol-FA′ or FA-α,βdiol FA′; 2,3Type II = FA-2,3diol-FA′ or FA-2,3diol FA′; α,ωType II = FA-α,ωdiol-FA′ or FA-α,ωdiol FA′

triesters Type I — FA-αOHFA-ωOHFA-St; FA-ωOHFA-αOHFA-FAlc

triesters Type II — FA-αOHFA-α,ωdiol-FA′; FA-ωOHFA-1,2diol-FA′

Although the meibomian gland is a sebaceous type of gland, its excreta, meibum, differs markedly in chemical composition from sebum, the excreta of the ordinary sebaceous gland. Meibomian lipids have a variety of special functions: They conserve tears by decreasing water evaporation from the tear film; they prevent eyelid skin from becoming overly wet and swelling, and they seal the lids during sleep, thus preventing the corneal surface from a damaging drying out.

In earlier work we have identified about 75% of the meibomian lipids of steer and human which were found to be remarkably similar (1). These lipids consist mainly of sterol esters (~30%), wax esters (~35%) and minor amounts of hydrocarbons, triacylglycerols, unesterified fatty acids and free cholesterol totalling about 10%. Approximately half of the remaining unidentified 25% are components migrating on silicic acid TLC into what we are calling the "diester region." This region lies between the nonpolar monoester region (i.e. where sterol esters and wax esters migrate) and the triester region (i.e. where triacylglycerols migrate). Borders of all these regions are not exact, and overlapping of regions due to differences in chain length, unsaturation and branching of the fatty chains can occur.

The saponified products of the total meibomian lipids from both the steer and the human yielded three lipid components, each with two esterifiable groups, i.e. OH or CO_2H. These difunctional group lipid components were α-hydroxy fatty acids (2), ω-hydroxy fatty acids (3) and long chain α,ω-diols (4). We have some preliminary evidence that still a fourth difunctional group lipid component is present in very small amounts in both the steer and human meibomian lipids, namely α,β-diols. If the hydroxyl group(s) in each of these substances esterifies with a fatty acid, and the carboxyl group esterifies with a fatty alcohol or sterol, we have a diester. If any of these esterifications take place with a second difunctional group lipid component, and the remaining hydroxyl or carboxyl groups esterify with a simple fatty acid, fatty alcohol or sterol, we would then have a triester. In like manner the possibility exists for the formation of tetraesters, pentaesters, etc. Partial hydrolysis of the diesters also could occur, yielding polar monoesters, the polar moiety being either an OH or a CO_2H group, depending upon whether an acid or an alcohol was removed. Similarly, partial hydrolysis of triesters could yield two polar monoesters or a polar diester plus a fatty acid or a fatty alcohol or a sterol.

Diesters of Types I and II (see Abbreviations) have been found in the skin surface lipids of many animals (5-10), in vernix caseosa (10-12) and in the preen glands of many birds (13,14). Triesters also have been found in the skin surface lipids of the rhino mouse (15,16) and the cow (10) and in beeswax (17). The present report describes the identification of three groups of diesters in meibomian lipids, here designated as αType I, ωType I-St and α,ωType II. To the best of our knowledge, this is the first report on the occurrence of the latter two diesters in any animal tissue. A complex group of triesters also has been incompletely characterized. A preliminary report of some of these findings has been made (18).

MATERIALS AND METHODS

In this study, lipids were obtained from two sources from the steer: from whole glands and from excreta (meibum). Lipids from both steer sources and meibum from humans were obtained as previously described (1). TLC of the lipids of both sources from the steer showed a great similarity. Furthermore, TLC of the steer lipids also were quite similar to the human lipids, although small differences, mainly in spot intensity, did exist. To distinguish between these two sources of lipids we shall call lipids derived from total meibomian glands "meibomian lipids," and lipids derived from excreta "meibum lipids." The latter are obtained by expressing the eyelids.

Dry column chromatography according to Loev et al. (19,20) was used to separate 768 mg of crude steer meibomian lipids. A nylon column 3.1 cm × 50 cm containing 258 g silica gel, Woelm (Waters Associates, Inc., Framingham, Massachusetts) was developed with benzene, then sectioned from the bottom upward into one piece 5 cm in length followed by 15 successive pieces 3 cm in length. Lipid from each successive section (designated here as Fractions Nos. 1 to 16, of our Column 210) was obtained by extracting the silica gel with benzene followed by a series of extractions with C/M (9:1, v/v) until negligible amounts of lipid were recovered.

Lipid from Fractions Nos. 1 to 4 weighed 487, 30.0, 4.6 and 1.1 mg, respectively, and TLC on SiO_2 of all fractions showed that only Nos. 1 to 4 had material migrating in the diester region. The bulk of Fraction No. 1, i.e. 480 mg, was then rechromatographed on another column of 52 g SiO_2, 100-200 mesh (Clarkson Chemical Co., Williamsport, Pennsylvania) with dimensions 2.3 cm × 22 cm (designated here as our Column 211). Hexane

eluted 2.7 mg of hydrocarbons (Fraction No. 1), and 20% benzene in hexane eluted 391.7 mg of sterol esters plus wax esters (Fractions Nos. 2 to 8 plus the bulk of No. 9). Since TLC showed no diesters in these fractions, except for a small amount in No. 9, nothing further was done with them. Fractions Nos. 10 through 24, eluted mainly with 40% benzene in hexane, contained the di- and triesters of this study. Fractions Nos. 25 and 26 (15.3 mg), eluted with benzene, and Fraction No. 27 (10.4 mg), eluted with 10% M in C, apparently were decomposition products as judged by the long tailing spots in TLC. Figure 1 shows the TLC of representative Column 211 Fractions.

To isolate and identify the di- and triesters, we used preparative TLC on Whatman LK5D plates (Whatman, Inc., Clifton, New Jersey) developed in hexane/benzene (50:50, v/v). These plates have a preadsorbent or leader portion on which the lipid is spotted. This allows the spotted lipid, during early development, to start as a narrow band when it reaches the adsorbent at the leader edge, thus enhancing resolution. We also used preparative TLC on $Mg(OH)_2$ plates, developed in 1.5 to 3% ethyl acetate in hexane, to separate esters containing sterols from those containing fatty alcohols. This separation will be discussed later. Components were made visible by spraying with ANS, a nondestructive spray, and viewing under UV. They were scraped off the plate and extracted with chloroform. Saponifications were carried out in test tubes with Teflon-lined screw caps, by heating the lipid for 3 hr at 90 C with a 20-fold excess of 10% KOH dissolved in ethanol/water (9:1, v/v). After the saponified products were acidified with HCl and extracted with

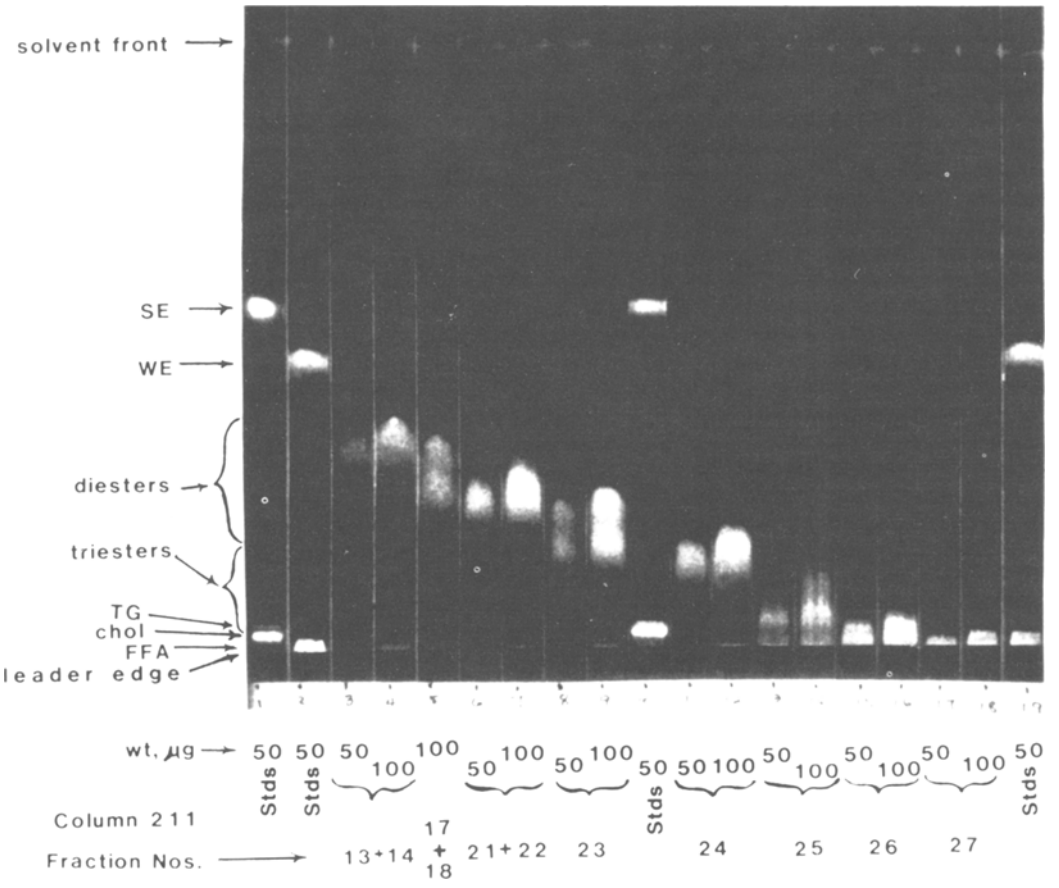

FIG. 1. TLC on SiO_2 of representative Column 211 fractions. Plate was developed with hexane/benzene 35:65, v/v. This plate and all subsequent SiO_2 plates were 250µ thick 20 cm × 20 cm Whatman LK5D (if 19-channel) or 5 cm × 20 cm Quanta/Gram LQD (if 4-channel) plates. ANS spray was used throughout for visualization under UV.

chloroform, they were subjected to TLC on a Quanta/Gram LQD four channel plate (Whatman, Inc.) developed linearly in 4 stages: first solvent C/M/water/HAc (90:10:0.9:0.3, v/v/v/v) to 2 cm above the leader edge, then dried 10 min on a hot plate at 55 C; second solvent C/M (96:4, v/v) to 7 cm above the leader edge, then dried 5 min at 55 C; third solvent C/M/conc NH_4OH (97:3:0.3, v/v/v), to 11 cm, then dried at 55 C for 5 min; fourth solvent, benzene to the score line at 14 cm above the leader edge. All plates were prewashed continuously overnight with ethyl acetate by placing the plate so that it protruded slightly out of the chamber, and placing the chamber cover against the plate so that when the solvent reached the point of contact of the cover it could evaporate into the hood, thus creating a continuous wash throughout the night.

Cholesterol and spots above cholesterol were scraped off the plate and extracted 3 times with chloroform. All remaining spots were extracted with C/M (2:1, v/v). All extracts were filtered through a Pyrex #36060 4.5-5.5 fine sintered glass filter, and the solvents were blown off with prepurified nitrogen. The lipid was then gently extracted with hexane, leaving behind in the flask any precipitated SiO_2. All lipid fractions then were weighed to 0.1 μg on a Cahn 25 Automatic Electrobalance (Ventron Corp., Cahn Instruments Div., Cerritos, California) except the different types of fatty acids, which first were converted to the methyl esters (21) before weighing. Weights of SiO_2 blanks taken from plates put through the 4-stage development system described above, then sprayed with ANS, rarely exceeded 1 or 2 μg. To calculate molar ratios, an average molecular weight for each lipid class was estimated from their GLC patterns. These estimated average molecular weights were 382, 386, 475, 277, 510 and 286 for the FAlcs, sterols, α,ω-diols, FAMEs, ωOHFAMEs and αOH FAMEs, respectively. A 5% correction was made to account for the increase in weight due to addition of the methyl group in the methyl esters of the fatty acids or α-hyroxy fatty acids; the corresponding methyl group correction made for the higher molecular weight ω-hydroxy fatty acids was 2.8%. Fatty alcohols and diols were acetylated by Farquhar's method (22). GLC was performed on a Varian 3700 instrument equipped with a flame ionization detector and an on-column injector (J & W Scientific Inc., Rancho Cordova, California) using a fused silica capillary column, 25 m × 0.25 mm, with chemically bonded SE-30 phase, 0.4μ film thickness (Chromapon Inc., Whittier, California). Temperature programming was from 85 C to 325 C at 7°/min with helium the carrier gas at a flow rate of 1.8 ml/min.

GC-MS was performed as previously described (3).

RESULTS AND DISCUSSION

Table 1 shows the portion of Column 211 which contained the diesters and triesters of steer meibomian lipids. These appeared in Fractions Nos. 9 through 24 (TLC of Fractions Nos. 9 through 12 are shown in Figure 2 and that of representative later eluting fractions are shown in Figure 1). Present were diesters α-Type I, ωType I and α,ωType II plus a mixture of triesters Type I and Type II. Before describing the isolation and identification of individual classes of diesters and triesters, we wish to comment on the general chromatographic behavior of these closely migrating substances on silicic acid. Referring to Figure 3, note that sterol esters (with one ester linkage) generally would have greater R_f values than wax esters (also with one ester linkage), and these classes are nearly completely separated from each other (compare a with b). Next, note that diesters of α-hydroxy fatty acids have greater R_f values than diesters of ω-hydroxy fatty acids of comparable chain lengths and unsaturation (compare c with e). Presumably, adsorptive sites are not as readily available for the two closely spaced ester groups of the diesters of α-hydroxy acids as they are for the widely separated ester groups of diesters of ω-hydroxy acids. But note again that when one of the ester groups of the ω-hydroxy fatty acid is with a sterol rather than a wax alcohol, again the diester containing the sterol migrates farther than one containing a wax alcohol (i.e. compare d with e). Note that e would have the same R_f as f, if all corresponding chains have the same length and degree of unsaturation, because the only difference in structure would be the direction of the ester linkage. The triesters (with three ester linkages) would be expected to adsorb to SiO_2 more strongly than the diesters with only two ester linkages, but here again one would expect ester linkages with sterols to be more weakly adsorbed than those with wax alcohols. Differences in chain length and unsaturation could further complicate the issue in that longer chain lengths would increase migration slightly while more unsaturation would decrease it slightly.

Diesters αType I FA-αOHFA-FAlc

Diesters αType I, the first of the diesters to elute from Column 211, were found mainly in

TABLE 1

Portion of SiO_2 Column Chromatogram of Total Steer Meibomian Lipids Containing Diesters and Triesters

Col. 211[a] Frac. No.	Wt. mg		Wt. of diesters αType I mg	Wt. of diesters ωType I-St mg	Wt. of diesters α,ωType II mg	Wt. of remaining unidentified "diesters"	Wt. of triesters[d] mg
9	7.80[b]		0.15				
10	1.99		1.11				
11	2.46		2.41				
12	3.76		3.61	0.12			
13	3.28	8.68	0.87	7.38	0.43		
14	5.40						
15	5.20	11.09		7.79	2.00	1.30	
16	5.89						
17	4.44	8.80		6.16	2.11	0.53	
18	4.36						
19	3.45	6.56		3.29	2.62	0.65	
20	3.11						
21	5.94	8.66		3.63	4.28	0.75	
22	2.72						
23	3.73				0.56		3.17
24	2.80						2.80
Total	66.3		8.2	28.4	12.0	3.2	6.0
Mg estimated from Fr. 2 & 3 Col. 210			1.0	7.0	5.0		2.0
Grand total			9.2	35.4	17.0	3.2	8.0
% of total steer meibomian lipids[c]			1.3	5.0	2.3	0.4	1.1

[a]These fractions were eluted mainly with 40% benzene in hexane. TLC shown in Figs. 1 and 2. Other fractions were eluted as described in Materials and Methods.

[b]The remainder of Fractions 9 through 12 were sterol and wax esters.

[c]Calculated on the basis of 725 mg total meibomian lipids recovered from Col. 210.

[d]Additional triesters may be present below the diester region. See text.

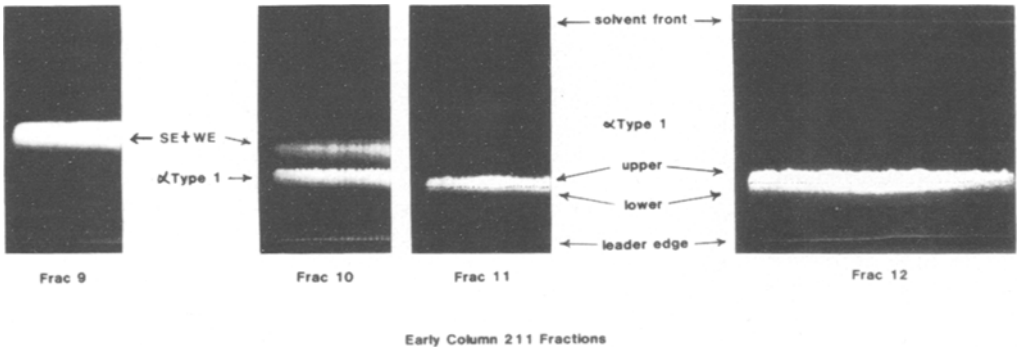

FIG. 2. Preparative TLC on SiO_2 of Column 211 Fraction Nos. 9-12. Plates were developed with hexane/benzene 50:50, v/v. Type of plate and spray used as in Fig. 1. Samples applied were: Frac. 9, 3.1 mg; Frac. 10, 1.2 mg; Frac. 11, 1.6 mg and Frac. 12, 3.0 mg. Plates show separation of αType I diesters from SE and WE and its purity from contamination with ωType I-St diesters.

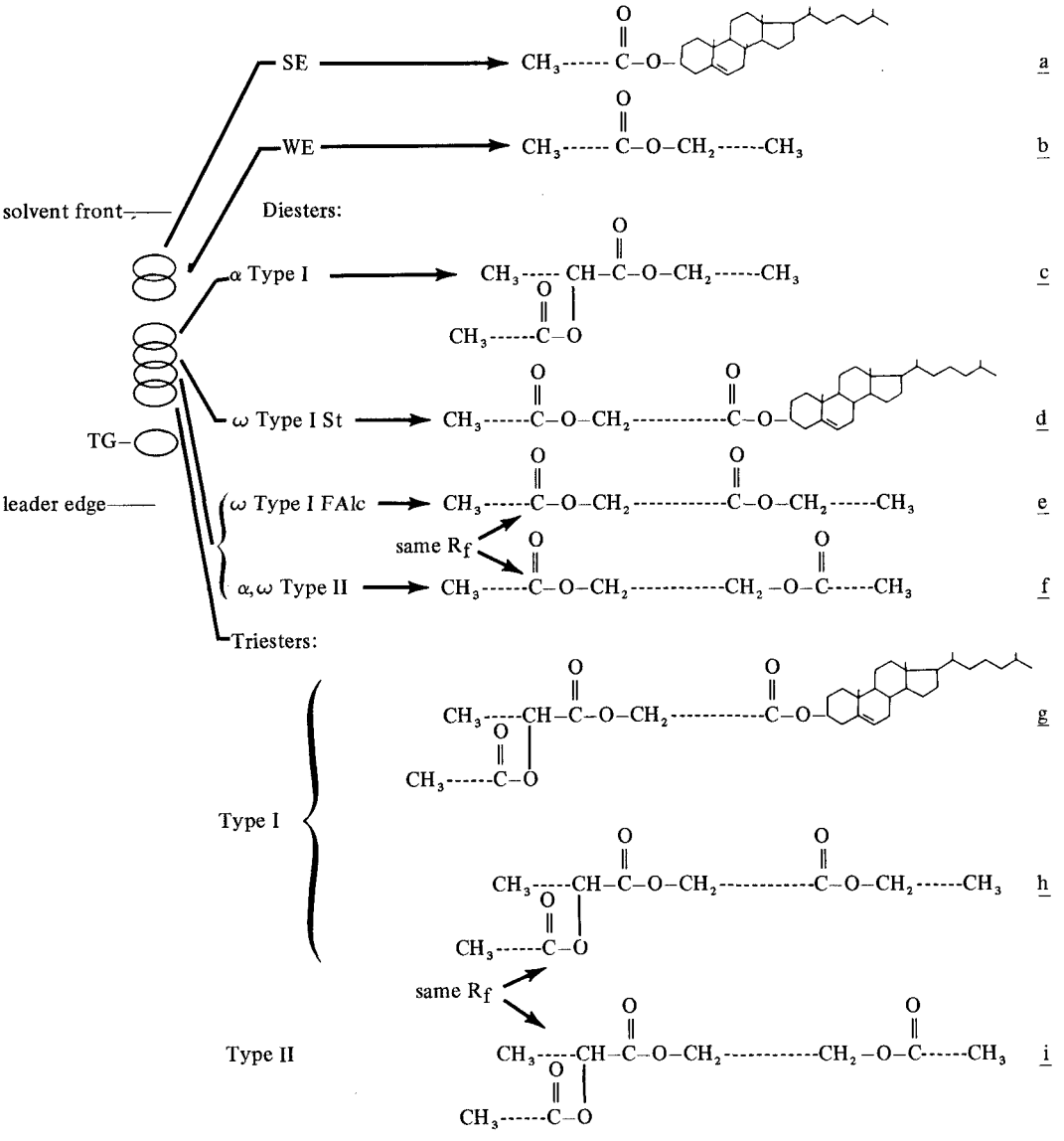

FIG. 3. Scheme showing the relative migration pattern of the mono, di and triesters on SiO_2. See text.

Fractions Nos. 10 to 14. Preparative TLC of these fractions on SiO_2 gave a diester band (Fig. 2) whose saponification products were FA, αOHFA and FAlc. GLC of the methyl esters of the fatty acids and the α-hydroxy fatty acids, and the acetates of the fatty alcohols, gave retention data matching those of standards. These data are listed in Table 2 and are discussed below.

To test for the possibility of another lipid class also being present in these diesters, the upper portion of the TLC band obtained from Fractions Nos. 11 and No. 12 was separated from the lower portion (Fig. 2) and individually saponified. Both portions gave similar results by TLC of the saponified products and GLC of the extracted fatty acids, α-hydroxy fatty acids and fatty alcohols, indicating homogeneity by this test. The weights recovered from the saponification products of these diesters were: α-hydroxy FAMEs, 170 μg, FAlcs, 201 μg and FAMEs, 187 μg. From the estimated average molecular weight of each component, a molar ratio of αOHFA:FAlc:FA of 1.0:0.93:1.1 was ob-

tained, which is approximately equimolar for all components. Within experimental error this supports the structure postulated.

Diesters ωType I and Possibly **FA-ωOHFA-St FA-ωOHFA-FAlc**

These diesters emerged from Column 211 in Fraction Nos. 12 to 22 (Table 1). If diesters FA-ωOHFA-FAlc occurred at all, they occurred in much smaller amounts than the amount of FA-ωOHFA-St. The latter diesters were separated from other material by preparative TLC on $Mg(OH)_2$ giving a broad band with an R_f of 0.1 when developed with 2.5% ethyl acetate in hexane. This adsorbent retains ring compounds with a flat surface more strongly than straight chain compounds of comparable molecular weight (23,24). Thus, molecules esterified to cholesterol or other similarly shaped sterols whose α side is relatively flat, are adsorbed more strongly on $Mg(OH)_2$ than those compounds esterified to straight chain fatty alcohols of comparable molecular weight. This chromatographic behavior of these two classes of esters on $Mg(OH)_2$ is quite the reverse of their behavior on SiO_2. TLC on SiO_2 of the material thus separated on $Mg(OH)_2$ as described above showed a single band, so the material was directly saponified without further purification. TLC of the saponified products by the 4-solvent linear development technique gave 3 bands: fatty acids, 163 μg, ω-hydroxy fatty acids, 307 μg (both as methyl esters), and sterols, 230 μg with respective R_f's of 0.36, 0.27 and 0.7. The sterols gave three peaks when they were subjected to capillary GC-MS and capillary GLC. The first to emerge was cholesterol, ~60%. The second was Δ7 cholestenol or 5α-cholest-7-en-3β-ol, ~35%. The third to emerge was present in too small an amount (~5% of the total sterols) to be identified. Authentic standards (Δ7 cholestenol from the Steroid Reference Collection, Medical Research Council, Westfield College, Hampstead, London) gave the same GLC retention times and nearly identical fragmentation patterns including a molecular ion at 386 by GC-MS. The molar ratio of FA:ωOHFA:St was 0.95:1.0:1.0, thus lending support, within experimental error, to the proposed structure. Compositions of the fatty acids of diesters FA-ωOHFA-St are listed in Table 2 and are discussed below.

Diesters α,ωType II **FA-α,ωdiol-FA**

These diesters were found in Fractions Nos. 13 to 23. They were separated from diesters FA-ωOHFA-St and other material also by the preparative TLC procedure on $Mg(OH)_2$ used above and were found in a broad band at R_f = 0.45. The saponification products of this band (which came from pooled Fraction Nos. 19 and 20) gave mainly fatty acids and α,ω-diols, but small amounts of α-hydroxy fatty acids, ω-hydroxy fatty acids and fatty alcohols also were present. The corresponding band at R_f = 0.45 from $Mg(OH)_2$ TLC of the earlier eluting pooled Fractions Nos. 15 and 16 also gave the same saponification products except that there were more α-hydroxy fatty acids, ω-hydroxy fatty acids and fatty alcohols present. The weights of these saponification products obtained from pooled Fractions Nos. 15 and 16 gave the following number of μmoles: FAlcs 0.12, α,ω-diols 0.37, FA 0.84, ωOHFA 0.24,

TABLE 2

Fatty Acids (as Methyl Esters) from Different Diester Fractions of Steer Meibomian Lipids (Column 211)

	Diesters αType I			
	Frac. 11			
	3 major homologs %			%
Short chain cluster (C12-C20)				
Saturates				
Normal	C16:0, 9.7	C14:0, 4.1	C18:0 2.0	16.3
Iso-branched	iC18:0, 7.2	iC14:0, 2.8	iC16:0 1.1	11.6
Anteiso-branched	aiC15:0, 18.8	aiC19:0, 5.9	aiC17:0 4.5	33.0
Monoenes				
Normal	C18:1, 10.1	C16:1, 3.3	C15:1 1.6	16.0
			Subtotal	76.9
Long chain cluster (C21-C31)				
Saturates				
Normal	C24:0, 0.5	C22:0, 0.4	C29:0 0.4	1.9
Iso-branched	iC26:0, 1.9	iC30:0, 1.4	iC24:0 0.9	5.0
Anteiso-branched	aiC25:0, 4.4	aiC27:0, 3.9	aiC29:0 1.5	11.7
Monoenes				
Normal	C27:1, 2.2	C28:1, 1.0	C26:1 0.7	4.5
			Subtotal	23.1
			Grand total	100.0

(Continued)

TABLE 2 (continued)

	Diesters ωType I-St							
	Frac. 15-16				Frac. 19-20			
	3 major homologs %			%	3 major homologs %			%
Short chain cluster (c12-C20)								
Saturates								
Normal	C16:0, 6.7	C18:0, 1.3	C15:0 0.8	10.4	C16:0, 5.0	C18:0, 1.6	C14:0 0.5	7.8
Iso-branched	iC18:0, 3.3	iC16:0, 1.4	iC14:0 0.7	5.6	iC18:0, 6.5	iC16:0, 0.8	iC20.0 0.6	8.3
Anteiso-branched	aiC15:0, 10.6	aiC17:0, 10.4	aiC19:0 1.2	22.3	aiC17:0, 6.4	aiC15:0, 5.8	aiC19:0 1.3	13.6
Monoenes								
Normal	C18:1, 34.5	C16:1, 21.7	C17:1 1.7	59.1	C18:1, 34.5	C16:1, 25.0	C17:1 1.7	59.5
			Subtotal	97.4				89.2
Long chain cluster (C21-C31)								
Saturates								
Normal	C24:0, 0.1	–	–	0.1	C24:0, 0.2	C26:0, 0.1	–	0.3
Iso-branched	iC26:0, 0.3	iC22:0, 0.3	iC24:0 0.1	0.8	iC22:0, 1.1	iC26:0, 0.8	iC21:0 0.7	2.6
Anteiso-branched	aiC25:0, 0.6	aiC27:0, 0.6	aiC29:0 0.3	1.7	aiC25:0, 2.7	aiC27:0, 1.4	aiC21:0 1.2	6.3
Monoenes								
Normal	–	–	–	–	C22:1, 0.8	C21:1, 0.8	–	1.6
			Subtotal	2.6				10.8
			Grand total	100.0				100.0

(Continued)

and αOHFA 0.24. The main diester present here was 0.37 μmoles of α,ωType II. If we assume that the FAlcs are 0.02 μmoles too high or the FAs are 0.02 μmoles too low we can account for all products. For example, assuming the former we could have the following products:

α,ωType II	FA-α,ωdiol-FA		
μmoles:	.37	.37	.37

STRUCTURE 1

αType I	FA-αOHFA-FAlc		
μmoles:	.05	.05	.05

STRUCTURE 2

ωType I	FA-ωOHFA-FAlc		
μmoles	.05	.05	.05

STRUCTURE 3

	αOHFA-ωOHFA	
μmoles:	.19	.19

STRUCTURE 4

Admittedly, Structure 4 is somewhat speculative, but there is no reason why it could not be formed, since the approximately 30 C-atoms in the chains of the ω-hydroxy fatty acids between the OH groups and CO_2H groups make these groups essentially independent. Molar ratios of the component diesters of pooled

TABLE 2 (continued)

	Diesters α,ω Type II							
	Frac. 15-16				Frac. 23			
	3 major homologs %			%	3 major homologs %			%
Short chain cluster (C12-C20)								
Saturates								
Normal	C16:0, 8.2	C18:0, 2.8	C15:0 1.5	13.7	C16:0, 3.7	C18:0, 1.2	C14:0 0.6	6.2
Iso-branched	iC16:0, 1.9	iC18:0, 1.5	iC14:0 0.7	4.7	iC18:0, 6.1	iC14:0, 1.0	iC16:0 0.7	7.8
Anteiso-branched	aiC15:0, 14.8	aiC17:0, 14.8	aiC19:0 2.1	31.7	aiC15:0, 9.5	aiC17:0, 5.1	aiC14:0 0.7	15.3
Monoenes								
Normal	C18:1, 33.6	C16:1, 8.1	C20:1 0.7	42.6	C18:1, 35.0	C16:1, 30.8	C14:1 2.5	70.4
			Subtotal	92.7				99.7
Long chain cluster (C21-C31)								
Saturates								
Normal	C21:0, 1.1	C23:0, 0.2	C26:0 0.1	1.4	–	–	–	–
Iso-branched	iC26:0, 0.6	iC28:0, 0.4	iC22:0 0.2	1.4	iC26:0, 0.1	iC24:0 0.1	–	0.2
Anteiso-branched	aiC27:0, 1.3	aiC25:0, 1.2	aiC29:0 0.5	3.2	aiC21:1 0.1	–	–	0.1
Monoenes								
Normal	C22:1, 0.8	C21:1, 0.5	–	1.3				
			Subtotal	7.3				0.3
			Grand total	100.0				100.0

Fractions Nos. 19 and 20 gave similar results. The sum of the weights of the diesters other than α,ω Type II are given in Table 1.

The purest preparation of α,ω Type II diesters that we were able to obtain was from Fraction No. 23, which gave a band at R_f = 0.51 on $Mg(OH)_2$ TLC. Saponified products from these diesters yielded 475 μg α,ω-diols and 201 μg fatty acids as methyl esters, giving a molar ratio of α,ω-diols to FA of 1.0:1.9 or approximately 1:2. Composition of the fatty acids of α,ω Type II diesters are given in Table 2 and are discussed below.

Triesters Type I

Triesters emerged from Colum 211 in Fractions Nos. 23 and 24. Preparative TLC of Fraction No. 23 on $Mg(OH)_2$ gave a broad band at R_f = 0.07 which, when further purified by preparative TLC on SiO_2, gave an intense upper band adjoined to a faint lower band. Saponification of the lipids from each band yielded the same products although in different amounts (Fig. 4). Corresponding substances from both lanes were pooled, all fatty acids methylated for GLC and weighed. Recovery of all substances gave the respective μmoles for FAlc, sterol, FA, ωOHFA and αOHFA as 0.05, 0.18, 0.24, 0.22 and 0.20. This gives a molar ratio of FA:αOHFA:ωOHFA:FAlc plus St of 1.2:1.0:1.1:1.1, or approximately 1:1:1:1. This molar ratio suggests the following two triester structues: FA-αOHFA-ωOHFA-St and FA-ωOHFA-αOHFA-FAlc, which would occur in the proportions 3.6:1, the molar ratio of sterols to wax alcohols. Interchanging the two hydroxy fatty acid moieties partially or totally may represent the actual structures, but the structures postulated would seem more plausible, because cleaving them at the middle ester linkage would

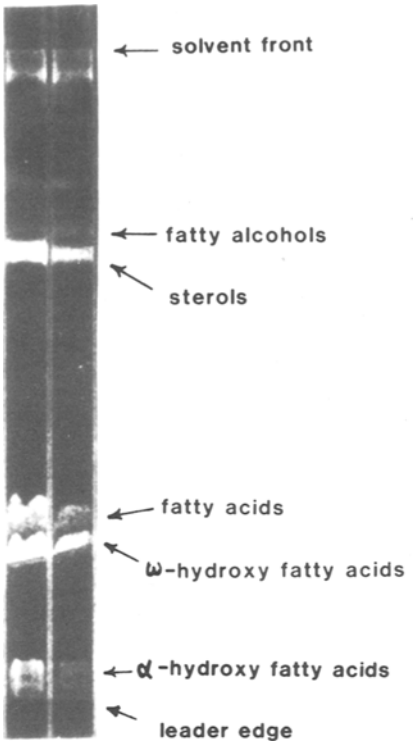

FIG. 4. TLC on SiO_2 of the saponified products of triesters Type I developed by the linear 4 solvent system described in the text. Fraction 23 Column 211 subjected to TLC on $Mg(OH)_2$; lipid from band with R_f = 0.07 subjected to TLC on SiO_2 yielding an intense band at R_f = .44 adjoining to a faint band at R_f = .41. Saponified products from intense band (250 μg) spotted in left lane and those from faint band (125 μg) spotted in right lane.

yield moieties already occurring in αType I and ωType I diesters. Analogously, one may argue that triesters with the structures FA-ωOHFA-αOHFA-St and FA-αOHFA-ωOHFA-FAlc are much less probable because the moiety αOHFA-St was not found in diesters αType I, and ωOHFA-FAlc occurs minimally if at all in diesters ωType I. Thus, in summary, the two most likely structures for triesters Type I are FA-αOHFA-ωOHFA-St and FA-ωOHFA-αOHFA-FAlc.

Triesters Type II

Fraction No. 23 also yielded another band (R_f = 0.23) when subjected to preparative TLC on $Mg(OH)_2$. The lipid from this band also gave an intense upper band adjoined to a faint lower band when subjected to preparative TLC on SiO_2. When the lipid from the intense band was saponified and the saponified products put through our TLC system (Fig. 5, Lanes 1 and 2) we obtained the following μmoles: FAlcs 0.24, sterols 0.05, α,ω-diols 0.10, FA 0.47, ωOHFA 0.16 and αOHFA 0.31. The total number of μmoles of all components with carboxyl groups was 0.94, and the total with hydroxyl was approximately equal at 0.96. The presence of significant amounts of α,ω-diols suggests the presence of some triesters Type II but does not exclude the presence of triesters Type I and possibly even some diesters. The following are some possibilities:

Type II triesters	FA-αOHFA-α,ωdiol-FA			
μmoles	.05	.05	.05	.05
Type II triesters	FA-ωOHFA-α,ωdiol-FA			
μmoles	.05	.05	.05	.05
Type I triesters	FA-αOHFA-ωOHFA-St			
μmoles	.05	.05	.05	.05
Type I triesters	FA-ωOHFA-αOHFA-FAlc			
μmoles	.06	.06	.06	.06

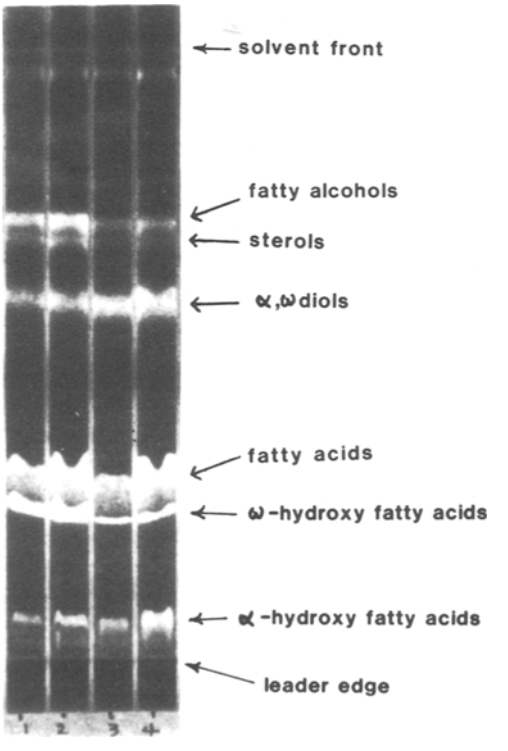

FIG. 5. TLC on SiO_2 of the saponified products of triesters Type II developed as in Fig. 4, obtained as described in text. Samples applied were 180 μg, 380 μg, 150 μg and 285 μg respectively for lanes 1 to 4. Structures postulated are tentative, see text.

This leaves 0.16 μmoles FA, 0.15 μmoles αOHFA plus 0.18 μmoles FAlc which is approximately correct for some α Type I diesters FA-αOHFA-FAlc, within experimental error. The difficulty is that αType I diesters should not elute this late in the Column 211. Perhaps it is a matter of selection of shorter chains for these compounds.

Fraction 24, when subjected to preparative TLC on $Mg(OH)_2$, gave a major band of R_f 0.23, the lipids of which were purified further by preparative TLC on SiO_2 to yield an intense upper band adjoined to a faint lower band as observed in Fraction No. 23. The saponified products of the intense band on SiO_2 of Fraction No. 24 (Fig. 5, Lanes 3 and 4) gave the following μmoles: FAlcs 0.09, sterols 0.02, α,ω-diols 0.18, FA 0.49, ωOHFA 0.11, and αOHFA 0.22. The total number of μmoles of carboxyl was 0.82 and hydroxyl was 0.80, suggesting the following possible substances:

Type I triesters	FA-αOHFA-ωOHFA-St			
μmoles	.02	.02	.02	.02
Type I triesters	FA-ωOHFA-αOHFA-FAlc			
μmoles	.09	.09	.09	.09
Type II triesters	FA-αOHFA-α,ωdiol-FA			
μmoles	.11	.11	.11	.11

This leaves 0.16 μmoles of FA and 0.07 μmoles of α,ωdiols which could be attributed to 0.07 μmoles of diesters FA-α,ωdiol-FA eluting with the triesters with an experimental error of 0.02 μmoles too many of FA.

The relative amounts of the different diesters present in the fractions of Column 211 were calculated from the weights recovered from TLC on SiO_2 and $Mg(OH)_2$, and are given in the totals of Table 1. Also given are estimates of the relatively small amounts of each diester present in Fraction Nos. 2 and 3 of Column 210, as judged by the intensity of TLC spots on SiO_2. Thus, steer meibomian lipids contain an estimated 1.3% αType I, 5% ωType I-St and 2.3% α,ωType II diesters. Table 1 also lists values obtained similarly for the triesters eluting with the more polar diesters. These comprise ~1% of steer meibomian lipids and do not include the triacylglycerols or other triesters that may migrate below them.

The fatty acids occurring in the three types of steer diesters isolated in this study are listed in Table 2. As mentioned earlier, when the different diesters emerge from the SiO_2 column, the fatty chains will change in that the more saturated chains will elute ahead of the more unsaturated chains, and the longer chains will elute ahead of the shorter chains. Perhaps a center cut or an average of an early and a late cut would give a fatty acid profile more representative of the total. Thus, for diesters ωType I-St the pooled Fractions Nos. 15 and 16 would be representative of the total. Considering these facts, there appear to be two fatty acid profiles: a one-cluster profile and a two-cluster profile. The first cluster is from C12 to C20, and the second from C21 to C31. The composition of the fatty acids of the αType I diesters is an example of the two-cluster profile; the fatty acids of the diesters ωType I and those of the α,ωType II belong largely to the one-cluster group. Anteiso chains are prominent in both profiles.

The wax alcohols of αType I diesters are quite similar to those of the wax monoesters (1), and the α-hydroxy fatty acids of αType I are similar to those of the total α-hydroxy fatty acids (2).

The very long chain difunctional group components, such as the ω-hydroxy fatty acids of ωType I diesters from pooled Fractions Nos. 15 and 16, Column 211, gave a profile similar to that of the total ω-hydroxy fatty acids reported earlier (2), and the α,ω-diols of α,ωType II diesters from pooled Fractions Nos. 19 and 20, Column 211, gave a profile similar to that of the α,ω-diols of the total unsaponifiables (4).

Human Meibum Polyesters

Human and steer meibum lipids when chromatographed alongside each other on the same TLC plate gave six bands in the diester and triester regions labeled DE-1 through DE-6 (Fig. 6). Each of these bands were scraped off from three such plates, pooled, extracted and the lipid obtained saponified. The saponified products were then chromatographed (Fig. 7) and the spots worked up and subjected to GLC as described above.

From our earlier data on the steer, we would expect that its DE-1, DE-2 and DE-3 bands would contain diesters αType I, ωType I-St and α,ωType II, respectively. In Figure 6, note that human DE-1 migrated farther than steer DE-1 but the migration of human DE-2 and DE-3 matched closely the respective migrations of DE-2 and DE-3 of the steer, although present in differing amounts.

If human DE-1 were αType I diesters as in the steer, we should have obtained α-hydroxy fatty acids. GLC analysis of the ultra small sample obtained from the human DE-1 band gave inconclusive evidence of this. This fact, coupled with the difference in migration be-

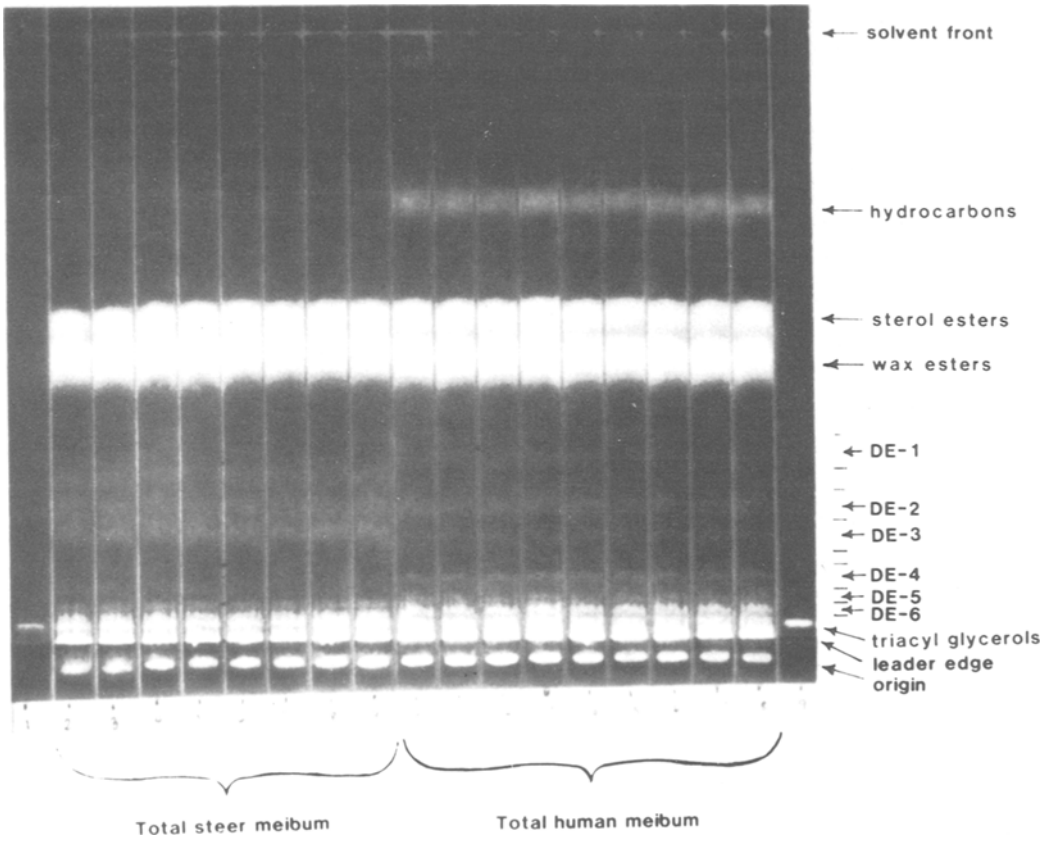

FIG. 6. TLC on SiO_2 of total steer and human meibum lipids. Plate was developed in hexane/benzene 55:45, v/v. Type of plate and spray used as in Fig. 1. Lanes 1 and 19 were 30 μg each of cholesterol and triolein standards. Lanes 2-9 and 10-18 are 300 μg each of total steer and human meibum lipids respectively.

tween the human and steer DE-1, makes it still an open question as to whether any αType I diesters occur in human meibum.

The saponified products of human DE-2 matched rather closely those of the steer DE-2. The amounts of sterol, FA and ωOHFA recovered (Fig. 7) and their GLC patterns are consistent with the expected structure for diesters ωType I-St. Capillary GLC of the sterols showed a chromatogram very similar to that of the steer except that there was only ~10% Δ7 cholestenol as determined by GC-MS. Similarly, human DE-3 showed amounts of α,ω-diols and fatty acids consistent with the structure of α,ωType II diesters. However, there was some contamination of DE-3 with DE-2 and vice versa.

The saponified products from DE-4, DE-5 and DE-6 from both the steer and the human showed enough similarity to lead one to believe that the triesters of both species also bear close resemblances to each other. Human meibum lipids also appear to contain small amounts of still a fourth difunctional group lipid component, namely, α,β-diols (see DE-4 and DE-6 of Fig. 7), and very faint traces of these diols also seem to be present in steer meibum lipids.

CONCLUSION

Approximately 90% of the lipids of steer and human meibum are esters of some 8 or 9 types, a large part of the remainder being free alcohols, sterols, acids and hydrocarbons. Each ester class in turn consists of a very large number of molecular species. It has been shown that the esterification of fatty acids with fatty alcohols for the human wax esters was a random process (25). Thus, 69 fatty acids of all fatty chain types times 40 fatty alcohols of all chain types form 2760 different molecular species of wax ester. If we add a third component such as the family of 11 α-hydroxy fatty acids, as found in diesters αType I, we get over

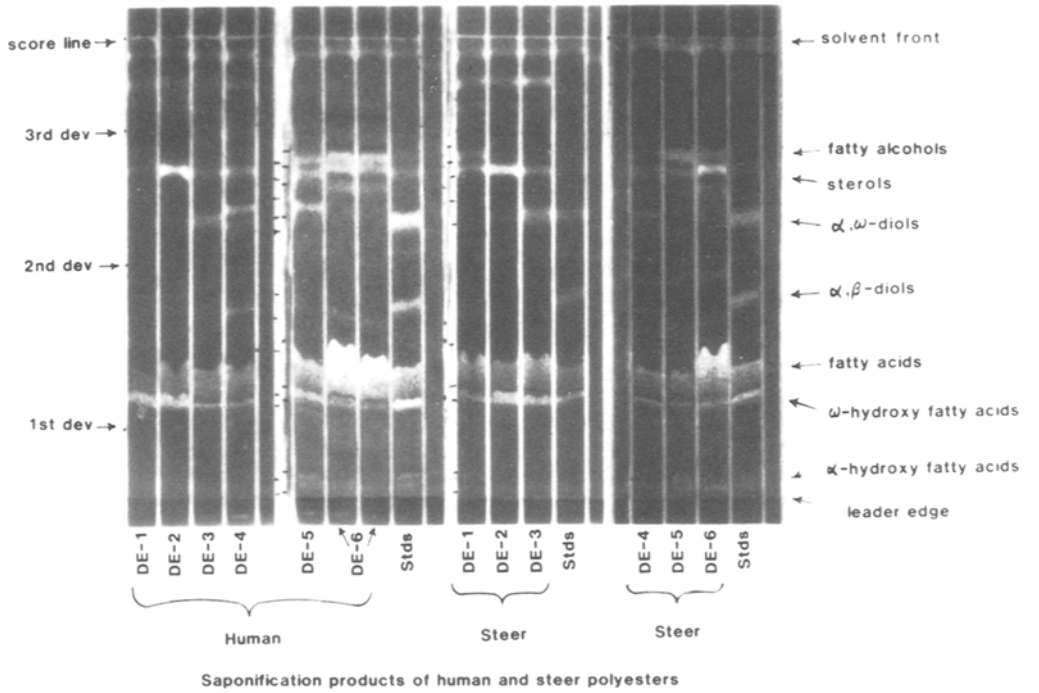

FIG. 7. TLC on SiO_2 of saponified products of bands in the diester region of total steer and human meibum lipids. Plates were developed as in Fig. 4. Type of plate and spray used as in Fig. 1. Samples applied on each lane from left to right were: Human meibum, DE-1 85 μg, DE-2 160 μg, DE-3 100 μg, DE-4 185 μg, DE-5 150 μg, DE-6 200 μg and 160 μg each; Steer meibum, DE-1 145 μg, DE-2 150 μg, DE-3 200 μg, DE-4 95 μg, DE-5 130 μg, DE-6 270 μg. Stds., 100-150 μg each, contained fatty alcohols, sterols, α,ω-diols, α,β-diols, fatty acids, ω-hydroxy fatty acids and α-hydroxy fatty acids.

30,000 molecular species, and a fourth component as in the triesters would bring the total number of molecular species to many hundreds of thousands. It would appear that no one of these molecules is more important than any of the others, but that all of them synergistically endow the mixture with those properties that enable the meibum to perform all of its functions.

One important physical property that this semi-solid lipid mixture must have is the correct viscosity (or fluidity) to enable it to flow out of the gland orifice onto the edge of the eyelid at the temperature of the eyelid. A second essential physical property is the right spreading characteristics over the wet tear surface so that it can keep the preocular tear film covered at all times by expanding and contracting with every blink and thus retarding evaporation of tear moisture. A third essential physical property is the correct adhesion to the outer skin of the eyelids so that a hydrophobic wall forms, thus preventing the skin from getting wet and swelling. Another function of this semi-solid wall is to direct the tears to flow into the puncta at the side of the nose and prevent them from overflowing onto the cheek. Finally, a fourth physical property required of this semi-solid lipid is that it be cohesive or sticky enough that the lipids of the upper and lower lids form a water tight seal when the eyes are closed during sleep, thus preventing the corneal surface from drying out. Whether meibum lipids have any useful chemical properties, such as toxicity to some pathogenic microorganism, remains yet to be determined.

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REFERENCES

1. Nicolaides, N., Kaitaranta, J.K., Rawdah, T.M., Macy, J.I., Boswell, F.M., and Smith, R.E. (1981)

Invest. Ophthalmol. Vis. Sci. 20, 522-536.
2. Nicolaides, N., Santos, E.C., and Papadakis, K. (1984) *Lipids 19*, 264-277.
3. Nicolaides, N., and Ruth, E.C. (1982) Current Eye Res. 2, 93-98.
4. Nicolaides, N., Santos, E.C., Papadakis, K., Ruth, E.C., and Muller, L. (1984) Lipids 19, 990-993.
5. Nikkari, T. (1969) Comp. Biochem. Physiol. 29, 795-803.
6. Nicolaides, N., Fu, H.C., and Ansari, M.N.A. (1970) Lipids 5, 299-307.
7. Logani, M.K., Austin, W.A., Nhari, D.B., and Davies, R.E. (1975) Biochim. Biophys. Acta 380, 155-164.
8. Logani, M.K., Nhari, D.B., Forbes, P.D., and Davies, R.E. (1977) Lipids 12, 626-628.
9. Nikkari, T., and Haahti, E. (1968) Biochim. Biophys. Acta 164, 294-305.
10. Downing, D.T., and Lindholm, J.S. (1982) Comp. Biochem. Physiol. 73 B, 327-330.
11. Karkkainen, J., Nikkari, T., Ruponen, S., and Haahti, E. (1965) J. Invest. Derm. 44, 333-338.
12. Fu, H.C., and Nicolaides, N. (1969) Lipids 4, 170-175.
13. Haahti, E., Lagerspetz, K., Nikkari, T., and Fales, H.M. (1964) Comp. Biochem. Physiol. 12, 435-437.
14. Jacob, J. (1976) Bird Waxes, Chemistry and Biochemistry of Natural Waxes, Chap. 4, Elsevier Pub. Co., New York, NY.
15. Logani, M.K., Nhari, D.B., and Davies, R.E. (1975) Biochim. Biophys. Acta 388, 291-300.
16. Logani, M.K., Nhari, D.B., and Davies, R.E. (1977) Lipids 12, 283-287.
17. Tulloch, A.P. (1971) Chem. Phys. Lipids 6, 235-265.
18. Nicolaides, N., Santos, E.C., and Smith, R.E. (1984) Arvo abstract, Supp. to Invest. Ophthal. and Vis. Sci. 25/4, 320.
19. Loev, B., and Snader, K.M. (1965) Chem. Ind. (London) 15.
20. Loev, B., and Goodman, M.M. (1967) Chem. Ind. (London) 2026.
21. Metcalfe, L.D., and Schmitz, A.A. (1961) Anal. Chem. 33, 363-354.
22. Farquhar, J.W. (1962) J. Lipid Res. 3, 21-30.
23. Nicolaides, N. (1970) J. Chromatog. Sci. 8, 717-720.
24. Stewart, M.E., and Downing, D.T. (1981) Lipids 16, 355-359.
25. McFadden, W.H., Bradford, D.C., Eglington, G., HajIbrahim, S.K., and Nicolaides, N. (1979) J. Chrom. Sci. 17, 518-522.

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