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Identification by gas chromatography and mass spectrometry of lipids from the rat Harderian gland

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ABSTRACT

Lipids from rat Harderian glands were extracted with ethyl acetate, hydrolysed with base and examined by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) as trimethylsilyl (TMS), [$^2H_9$]TMS, methyl ester–TMS, picolinyl, nicotinate and nicotinylidene derivatives. The latter three derivatives were used to reveal the structures of the alkyl chains of fatty acids, alcohols and glycerol ethers, respectively. Forty-eight compounds were identified, representing about 97% of the total extracted lipids as measured by GC peak areas. The major constituents were fatty acids with chain lengths from 12 to 22 carbon atoms (mainly C_{18} and C_{20}) and fatty alcohols (C_{16} to C_{26}) derived from wax esters. Most of these acids and alcohols were unsaturated in the ω-7 position and were accompanied by smaller amounts of the saturated and ω-5 monounsaturated analogues. Glycerol ethers were also identified for the first time in this secretion; the ether chains contained from 14 to 19 carbon atoms (mainly 16) and were straight-chain saturated, unsaturated (ω-5 and ω-7) and branched (*iso*). The only sterol found was cholesterol amounting to 1.24% of the total extract.

INTRODUCTION

The Harderian glands are large, lipid-secreting glands found in the orbit of the eye in species possessing a nictitating membrane or third eyelid. The composition of the secretion, which consists mainly of long-chain esters, has received some attention in recent years and has been shown to be very species-dependent [1–15]. The rabbit (for example refs. 1–6) produces mainly esters of glycerol ethers in wich the acids have predominently straight chains and the glycerol ethers can have straight, branched or hydroxylated chains. The guinea pig [7–11], on the other hand, produces a very complex mixture of glycerol ethers in which both acid and glycerol moieties are highly branched. The mouse [12] also produces esterified glycerol ethers, but the secretion from the rat [13] has been reported to consist mainly of wax esters in which both acid and alcohol components are unsaturated. As a continuation of our studies into the composition of these secretions, we have reinvestigated the composition of the rat secretion using gas chromatography–mass spectrometry (GC–MS) and show here that, as well as containing wax esters as the main component, it also contains esterfied glycerol ethers.

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EXPERIMENTAL

Materials

Straight-chain saturated (10:0–24:0), monounsaturated (18:1,δ-9 and 18:1,δ-11) and diunsaturated (18:2,δ-9,11) acids were obtained from Sigma (Poole, U.K.). The corresponding alcohols were obtained by reduction of the acids with lithium aluminium hydride in diethyl ether. 1-O-Hexadecylglycerol and 1-O-octadecylglycerol were obtained from Sigma. Nicotinic acid, 3-pyridylcarbinol and pyridine-3-carboxaldehyde were from Aldrich (Gillingham, U.K.).

Preparation of lipid extracts

The Harderian glands were removed from three adult male Wistar rats, cut into about ten pieces and compressed under ethyl acetate (5 ml) to extract the lipid. The solution was filtered, dried over anhydrous magnesium sulphate, and the ethyl acetate was removed under reduced pressure. The residue was hydrolysed by heating it at 80°C for 8 h with 1 ml of a 1:2 (v/v) mixture of 1 *M* aqueous potassium hydroxide solution and acetonitrile. After cooling and acidification with 1 *M* sulphuric acid, the lipid constituents were extracted with ethyl acetate (3 × 1 ml), washed with water (2 ml) and saturated sodium chloride solution (2 × 2 ml) and dried with anhydrous magnesium sulphate. Samples, in ethyl acetate, were stored at −20°C until required for derivatization and analysis.

Preparation of derivatives

TMS derivatives. An aliquot of the sample (0.1 ml) was evaporated to dryness with a nitrogen stream and heated at 80°C for 10 min with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, 10 μl). Samples (1 μl) of this solution were injected directly into the equipment.

[$^2H_9$]TMS derivatives [16]. These were prepared as above by the use of [$^2H_{18}$]bis(trimethylsilyl)acetamide in place of the BSTFA.

Methyl ester–TMS ethers. The dried sample was dissolved in a freshly prepared ethereal solution of diazomethane (0.5 ml, prepared from 'Diazald', Aldrich) and allowed to stand at room temperature for 10 min. The solvent and reagents were removed with a nitrogen stream and the residue was converted into TMS derivatives as described above.

Nicotinate esters [5,17,18]. A saturated solution of nicotinoyl chloride in acetonitrile was prepared as follows: nicotinic acid (0.5 mg) was heated at 80°C for 1 h with thionyl chloride. The reagent was removed with a nitrogen stream and the residue was washed three times with anhydrous benzene, the benzene being removed with a nitrogen stream. Acetonitrile (about 1.0 ml) was added to the solid residue and shaken to prepare a saturated solution. The derivatives were prepared by adding 0.5 ml of this solution to the residue from 0.1 ml of the dried sample solution and heating for 1 h at 80°C. The products were extracted from the cooled solution with ethyl acetate after dilution with water (1 ml), washed

with water (1 ml) and saturated sodium chloride solution (2 × 1 ml) and dried over anhydrous magnesium sulphate. The organic solution was then separated and evaporated to dryness with a nitrogen stream. BSTFA (10 μl) was added and the mixture was heated at 80°C for 10 min to form TMS derivatives of compounds with acid groups.

Picolinyl esters [19,20]. These were prepared by conversion of the acids in the residue from 0.1 ml of the sample solution to their acid chlorides and reaction of these with 3-pyridylcarbinol as described earlier [20]. Any underivatised functional groups were converted into TMS derivatives as described above.

Nicotinylidene derivatives [21]. The dried sample was dissolved in acetonitrile, *p*-toluenesulphonic acid (about 500 μg) and pyridine-3-carboxaldehyde (500 μg) were added and the mixture was heated at 90°C overnight. Water (1 ml) was added to the cooled solution and the products were extracted with ethyl acetate (3 × 1 ml). The combined extracts were washed with water (1 × 1 ml) and saturated sodium chloride solution (2 × 1 ml) and dried over anhydrous magnesium sulphate. The solvent was then removed with a stream of dry nitrogen and the residue was reacted with BSTFA as described above.

Gas chromatography

GC data were recorded with a Hewlett-Packard 5890A gas chromatograph (with flame ionization detector) fitted with a 25 m × 0.3 mm bonded-phase OV-1 fused-silica capillary column, film thickness 0.52 μm (Hewlett-Packard). Helium at 2.0 ml/min was used as the carrier gas and the split–splitless injector was used in the split mode with a split ratio of 15:1. Injector and detector temperatures were both 300°C and the column oven was temperature programmed from 130 to 380°C at 2°C/min. Results were recorded with a Servoscribe chart recorder and quantitative data were recorded with a Hewlett-Packard 3390A recording integrator.

Gas chromatography–mass spectrometry

GC–MS data were recorded with a VG 70/70F mass spectrometer interfaced to a Varian 2440 gas chromatograph fitted with a SGE split–splitless injection system operated in the split mode with a split ratio of 10:1. The column was a 25 m × 0.2 mm OV-1 bonded-phase fused-silica capillary, film thickness 0.33 μm terminating 10 mm inside the mass spectrometer ion source. Helium at 1 ml/min (measured in the absence of the mass spectrometer vacuum) was used as the carrier gas. Operating conditions were: injector, transfer line and ion source temperatures, 300, 300 and 280°C, respectively; column oven, temperature-programmed from 150 to 350°C at 2°C/min; accelerating voltage, 4 kV; electron energy, 70 eV; trap current, 1.0 mA; scan speed, 1 s/decade. Spectra were processed with a VG 11/250 data system.

RESULTS

Fig. 1 shows a gas chromatogram of the lipids from the rat Harderian glands separated as TMS derivatives on a 25-m OV-1 fused-silica capillary column. No compounds were found in the 280–380°C region. The traces obtained from the other rats were very similar with only minor differences in the relative peak areas. Peaks were identified as described below and are listed in Table I. Forty-eight compounds were identified, amounting to about 97% of the total extracted lipid as estimated from GC peak areas. Four types of compound were found, fatty acids, fatty alcohols, glycerol ethers and steroids (cholesterol). Identified compounds are summarised by lipid class in Table II.

Fatty acids

These compounds were identified as TMS, [$^2H_9$]TMS and methyl esters by comparison of GC–MS properties with those of reference samples and with published data. Positions of structural features of the aliphatic chain were confirmed by use of the picolinyl esters [19,20]. The major acids (C_{18}) and (C_{20}) were monounsaturated with ω-7 double bonds. Lower concentrations of ω-5 and unsaturated acids were also found together with trace amounts of two branched-chain acids, *iso*-16:0 (peak 8) and *iso*-17:0 (peak 14). Chain lengths varied from 12 to 22 carbon atoms with the majority of acids having an even number of carbons. The distribution of the various acid types is shown in Fig. 2.

Alcohols

The alcohols were identified in a similar way to the acids with the chain structure being revealed by preparation of nicotinate esters using the modified preparative method published earlier [5]. The profile of alcohols (Fig. 3) was similar to that of the acids in that the major compounds were monounsaturated with ω-7 double bonds and minor constituents were found with saturated chains and ω-5

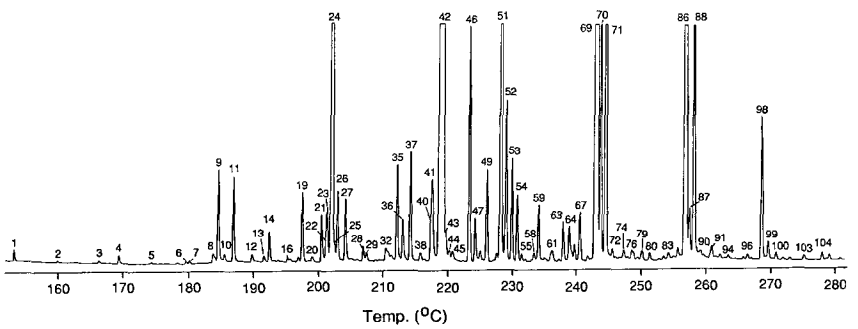

Fig. 1. Gas chromatogram of the TMS derivatives of the constituent lipids extracted from hydrolysed rat Harderian glands. The separation was made with a 25 m × 0.2 mm OV-1 fused-silica capillary column operated under the conditions listed in the Experimental section.

TABLE I

IDENTIFIED COMPOUNDS FROM HYDROLYSED RAT HARDERIAN GLANDS (TMS DERIVATIVES)

Peak No.[a]	Compound type	Carbon atoms	Longest chain	Chain type	Percent of total[b]	Elution time (min)
1	Acid	12	12	Straight	0.06	12.75
3	Acid	14	14	Straight	0.02	19.29
7	Alcohol	16	16	Straight	0.01	25.96
8	Acid	16	15	*iso*	0.09	28.01
9	Acid	16	16	δ-9 (ω-7)	0.67	28.41
10	Acid	16	16	δ-11 (ω-5)	0.05	28.89
11	Acid	16	16	Straight	0.62	29.95
12	Acid	17	16	δ-? *iso*	0.06	31.03
14	Acid	17	16	*iso*	0.23	32.36
16	Alcohol	18	18	δ-11 (ω-7)	0.01	
17	Acid	17	17	Straight	0.01	
19	Alcohol	18	18	Straight	0.54	34.88
21	Acid	18	18	δ-9,12	0.35	36.35
24	Acid	18	18	δ-11 (ω-7)	7.45	37.18
26	Acid	18	18	δ-13 (ω-5)	0.45	37.57
27	Acid	18	18	Straight	0.47	38.18
28	Acid	19	18	δ-?, ?	0.12	39.57
29	Glycerol-ether	14	14	Straight	0.20	41.29
35	Alcohol	20	20	δ-13 (ω-7)	0.77	42.16
36	Alcohol	20	20	δ-15 (ω-5)	0.33	42.61
37	Alcohol	20	20	Straight	0.90	43.18
42	Acid	20	20	δ-13 (ω-7)	34.01	45.67
43	Acid	20	20	δ-15 (ω-5)	0.05	
46	Glycerol-ether	16	15	*iso*	1.92	47.67
47	Glycerol-ether	16	16	δ-9 (ω-7)	0.35	48.11
48	Glycerol-ether	16	16	δ-11 (ω-5)	0.06	48.50
49	Glycerol-ether	16	16	Straight	0.76	49.03
51	Alcohol	22	22	δ-15 (ω-7)	4.88	50.12
52	Alcohol	22	22	δ-17 (ω-5)	1.29	50.51
53	Alcohol	22	22	Straight	0.81	50.96
54	Glycerol-ether	17	16	*iso*	0.59	51.38
58	Glycerol-ether	17	17	Straight	0.08	52.71
59	Acid	22	22	δ-15 (ω-7)	0.47	53.06
61	Alcohol	23	23	Straight	0.02	
63	Glycerol-ether	18	18	δ-11 (ω-7)	0.31	54.99
64	Glycerol-ether	18	18	δ-13 (ω-5)	0.38	55.46
67	Glycerol-ether	18	18	Straight	0.45	56.30
69	Alcohol	24	24	δ-17 (ω-7)	16.93	57.67
70	Alcohol	24	24	δ-19 (ω-5)	2.06	57.92
71	Alcohol	24	24	Straight	3.97	58.32
74	Glycerol-ether	19	19	Straight	0.07	59.67
76	Alcohol	25	25	δ-18 (ω-7)	0.07	60.31
80	Alcohol	25	25	Straight	0.06	61.68
86	Alcohol	26	26	δ-19 (ω-7)	9.62	64.52
87	Alcohol	26	26	δ-21 (ω-5)	0.37	64.76
88	Alcohol	26	26	Straight	2.52	65.11
98	Cholesterol				1.24	70.36
	Unidentified				3.17	

[a] Peaks in Fig. 1. Peaks not listed in the table were not identified.

[b] Expressed as a percentage of the total peak area as measured by GC.

TABLE II

MAJOR LIPID TYPES FOUND IN THE RAT HARDERIAN GLANDS LISTED BY CLASS

Compound type	Chain	Position of structural feature	Chain lengths
Acid	Straight	–	12, 14, 16, 17, 18
	Branched	*iso*	16, 17
	Unsaturated	ω-5	16, 18, 20
		ω-7	16, 18, 20, 22
Alcohol	Straight	–	16, 18, 20, 22, 23, 24, 25, 26
	Unsaturated	ω-5	20, 22, 24, 26
		ω-7	18, 20, 22, 24, 26
Glycerol ether	Straight	–	14, 16, 17, 18, 19
	Branched	*iso*	16, 17
	Unsaturated	ω-5	16, 18
		ω-7	14, 16, 18

unsaturation. Chain lengths were generally longer than those of the acids with the major alcohol having 24 carbon atoms. Chain lengths varied from 16 to 26 carbon atoms.

Glycerol ethers

These were identified by comparison with authentic standards and by preparation of both nicotinylidene [21] and bis(nicotinate) derivatives [18] to reveal the chain structure. They were present in generally low concentration and had saturated, branched and unsaturated (ω-5 and ω-7) chains (Fig. 4). Chain lengths varied from 14 to 19 carbon atoms.

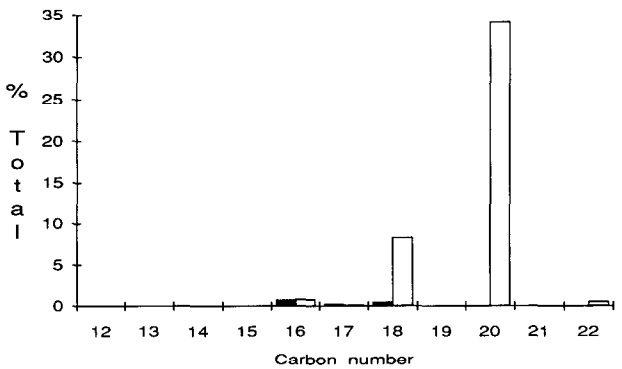

Fig. 2. Histogram showing the relative concentrations of the various acid types in the rat Harderian glands. Concentration is expressed as a percentage of the total peak area as measured by GC. (■) Saturated; (□) unsaturated.

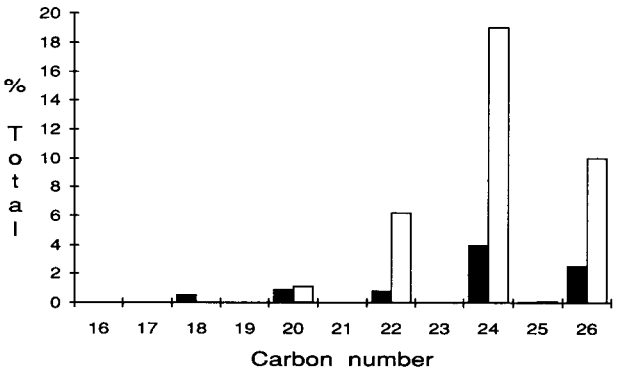

Fig. 3. Histogram showing the relative concentrations of the various monohydric alcohols in the rat Harderian glands. Concentration is expressed as a percentage of the total peak area as measured by GC. (■) Saturated; (□) unsaturated.

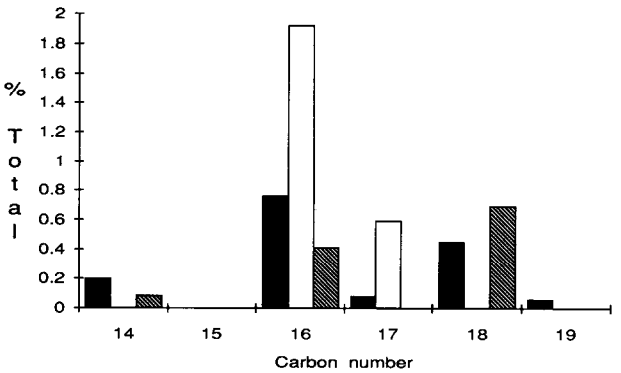

Fig. 4. Histogram showing the relative concentrations of the various glycerol ethers in the rat Harderian glands. Concentration is expressed as a percentage of the total peak area as measured by GC. (■) Straight; (□) branched; (▨) unsaturated.

Steroids

The only steroid found was cholesterol (peak 98, Fig. 1).

DISCUSSION

These results confirm that the rat produces wax esters as the major constituents with individual acids and esters having ω-7 unsaturation. The range of chain lengths was essentially the same as that found by Murawski [13], but several new, minor constituents were identified. In addition, it was found that the rat, in common with other species, does produce glycerol ethers in the Harderian extract, albeit in relatively low relative abundance. The major glycerol ethers had

branched (*iso*) chains, but unsaturated ethers, again having ω-5 and ω-7 chains were also present. The three pyridine-containing derivatives (picolinyl, nicotinate and nicotinylidene derivatives) successfully gave the structures of most compounds, with only two peaks being too weak for positive identification (12 and 28).

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