Occurrence of ungulic acid in some epidermal tissues

E. LEIKOLA, ELNA NIEMINEN,* and ANNA-MAIJA TEPPO

Department of Pharmacy, University of Helsinki, Helsinki, Finland

ABSTRACT The lipids isolated from different animal tissues have been studied qualitatively, by TLC, for the occurrence of the ungulic acid fraction. This fraction was found in considerable amounts only in epidermal tissues and its keratinized derivatives. In the present study it was isolated from human keratinous epidermis, hair, and nails, pig bristles, wool, and feathers. The analytical results indicated that a lipid fraction from all of these sources contained ceramide, galactose, galactosamine, sulfate, and sialic acid in equimolar amounts, and that the fractions were similar to the ungulic acid isolated earlier from a horse's hoof.

SUPPLEMENTARY KEY WORDS keratinous epidermis · hair · nail · wool · feather · pig bristles · ganglioside sulfate · fatty acids · thin-layer chromatography

In a previous paper (1) we described the properties of ungulic acid, which we had isolated from horse hooves. This material contained ceramide, galactose, galactosamine, sulfate, and sialic acid in equimolar amounts.

Since then, we have used TLC to study lipid extracts from various mammalian organs and tissues for the presence of this new sulfolipid. We were unable to detect this lipid fraction in liver, lung, spleen, pancreas, thymus, or lymph nodes. All tissues and structures found to contain the fraction in question were of epidermal origin.

This paper describes the results of analyses of the sulfolipid fractions isolated from human keratinous epidermis, human hair and nails, pig bristles, lamb's wool, and feathers.

MATERIALS

Owing to the difficulty of obtaining epidermis directly from healthy volunteers in amounts sufficient for structural analyses of the sulfolipid, keratinous epidermis was used. Keratinous skin products were collected from patients with leg fractures when the plaster cast was removed; the skin which could be lightly scraped off was used for analysis. Hair of female volunteers was washed in the ordinary way and rinsed several times with water before cutting. Nails without any artificial coloring were obtained from toes of cadavers submitted for medico-legal autopsy. Pig bristles were taken in a slaughterhouse immediately after the animal was killed, and were washed with water. Unwashed lamb's wool and unwashed feathers were used. All the samples were lyophilized and stored in a deep freezer until they were to be used.

The following reference compounds were used: fatty acid methyl esters (Sigma Chemical Co., St. Louis, Mo.); D (+)-galactose (British Drug Houses, Ltd., Poole, England); sialic acid concentrate (Nutritional Biochemicals Corp., Cleveland, Ohio); beef brain sphingomyelin (Mann Research Labs, Inc., New York); synthetic *dl*-sphingosine (Miles-Yeda, Ltd., Kiryat Weizmann, Rehovoth, Israel); beef spinal cord sulfatides (Applied Science Laboratories Inc., State College, Pa.); bovine brain gangliosides, type II and D(+)-galoctosamine hydrochloride (Sigma Chemical Co.). The methyl esters of hydroxy fatty acids were a generous gift from Dr. A. P. Tulloch, National Research Council, Saskatoon, Canada, and the *O*-methyl sphingosines were from Dr. A. Kisic, University of Illinois, Urbana, Ill.

Abbreviations: TLC, thin-layer chromatography; TMSi, trimethylsilyl.

*Present address: Control Laboratory for Pharmaceutical Preparations, Helsinki, Finland.

All solvents were of analytical grade and were used without further purification.

METHODS

The extraction of lipids was carried out with chloroform–methanol mixtures in the ratios 2:1 and 1:2 (1). The combined lipid extracts were evaporated to a small volume and filtered. An aliquot of the filtrate was evaporated to dryness and weighed. The content of ungulic acid was determined by the charring method (2) after its separation by TLC.

The ungulic acid fractions were isolated from the lipid extracts on an Al_2O_3 column and were further purified by TLC, as described in our previous paper (1). To avoid oxidation during the isolation procedure, an antioxidant, 2,6-di-*tert*-butyl-*p*-cresol (BHT), was added to the solvents (3). The fractions were crystallized from methanol, and their homogeneity was confirmed by melting point determinations and by TLC on silica gel plates in the following solvent systems: chloroform–methanol–water 24:7:1; chloroform–methanol–2.5 N NH_4OH 60:35:8; propanol–water 7:3; and propanol–concentrated NH_4OH 7:3.

The analytical methods employed for characterization of the isolated fractions have been described previously (1). Galactosamine and sphingosines were determined by GLC on a column packed with 2.5% SE-30 on Chromosorb W. The sulfate content was measured by the benzidine method (4), and neutral sugars were determined by the orcinol method (5). In the present study, the aliquot used for sialic acid determination was first passed through an anionic Dowex 2 column as recommended by Svennerholm (6). The sialic acid was determined both by the resorcinol method according to Miettinen and Takki-Luukkainen (7) and by the thiobarbituric acid method (8).

Samples for fatty acid analysis were treated with anhydrous methanol–HCl, and the fatty acid methyl esters were extracted from the hydrolysate with petroleum ether (1). The petroleum ether extracts were concentrated and divided into two parts. One part was used for quantitative determination of fatty acid methyl esters by GLC on a column packed with 10% DEGS on Chromosorb W. The remainder was used for the separation of normal and hydroxy fatty acid methyl esters by preparative TLC with diethyl ether–hexane 15:85 as described by Siddiqui and McCluer (9). The detection was carried out by spraying the partly covered plates with iodine in methanol. The fatty acid methyl esters were eluted from the adsorbent with diethyl ether. Normal fatty acid esters were identified by GLC on a DEGS column. Hydroxy fatty acid esters were identified by GLC as such and as their TMSi ether derivatives, using both DEGS and SE-30 columns.

RESULTS

The total lipid contents of the various samples which were examined, that is human keratinous epidermis, human hair, human nails, pig bristles, lamb's wool, and feathers, varied from 1.2 to 5.1% of the fresh weight. The fraction under investigation constituted 3.5–13.8% of total lipids and 0.10–0.23% of the fresh weight (Table 1). This compares well with the unidentified sulfate-containing fraction found by Nieminen et al. (10) in normal human epidermis, which comprised about 7.1–9.2% of the total lipids and about 0.11% of the fresh epidermis.

There were small variations in the melting points of the fractions after they were crystallized from methanol. This may have been due to the presence of impurities (Table 1). The thin-layer chromatograms of the isolated fractions are presented in Fig. 1. The fractions gave positive resorcinol reactions and had the same R_f values as ungulic acid using the solvent systems described above. With chloroform–methanol–water in the ratio 24:7:1, all these fractions migrated slightly ahead of the cerebroside sulfate esters. With the other solvent systems, the two sulfolipids had the same R_f values.

The IR spectra showed an absorption peak around 1240 cm^{-1}, which is characteristic of sulfatides.

The analytical results obtained from the hydrolysis products are presented in Table 2. The content of sialic acid was determined by the resorcinol method (7). The presence of sialic acid was confirmed by the thiobarbituric acid method (8), which gave values about 20% lower. Sphingosine, galactose, galactosamine, sulfate, and sialic acid constituted 76.7–80.9% of the fractions isolated. The fatty acid constituted an additional 20.8% (calculated as stearic acid).

The TLC of fatty acid methyl esters revealed that human keratinous epidermis, hair, and nails, and feathers contained hydroxy and normal fatty acids, whereas pig bristles and lamb's wool contained only

TABLE 1 TOTAL LIPID AND UNGULIC ACID CONTENTS IN DIFFERENT TISSUES

Material	Total Lipids	Ungulic Acid	Ungulic Acid	Melting Points of Ungulic Acid Fractions
	% of fresh weight	*% of total lipids*	*% of fresh weight*	°C
Human keratinous epidermis	1.2	13.8	0.17	191–192
Human hair	3.1	3.9	0.12	189–190
Human nails	2.1	10.2	0.21	185–187
Pig bristles	3.1	3.5	0.11	191–192
Lamb's wool	5.1	4.5	0.23	190–192
Feathers	2.0	7.0	0.14	188–192

TABLE 2 COMPOSITIONS OF UNGULIC ACIDS ISOLATED FROM HUMAN KERATINOUS EPIDERMIS, HAIR, AND NAILS, PIG BRISTLES, LAMB'S WOOL, AND FEATHERS

Material	Aliquot	Sphingosine	Galactose	Galactosamine	Sulfate	Sialic Acid	Total
				weight %			
Human keratinous epidermis	1	22.1	10.7	12.1	9.4	27.4	
	2	23.1	11.3	12.9	9.2	23.6	
		22.6	11.0	12.5	9.3	25.5	80.9
				molar ratios			
		1.00	0.81	0.93	1.28	1.09	
				weight %			
Human hair	1	24.7	14.7	10.7	7.4	22.2	
	2	23.3	11.2	11.5	8.8	20.9	
	3	24.0		11.1	7.4		
	4				8.6		
		24.0	12.9	11.1	8.1	21.5	77.6
				molar ratios			
		1.00	0.89	0.77	1.05	0.87	
				weight %			
Human nails	1	21.4	10.4	17.3	6.8	21.0	
	2	23.8	10.7	16.3	6.8	20.2	
	3	24.3			6.4		
	4				6.0		
		23.2	10.6	16.8	6.5	20.6	77.7
				molar ratios			
		1.00	0.76	1.21	0.87	0.86	
				weight %			
Pig bristles	1	24.9	12.6	14.8	7.3	20.8	
	2	20.4	12.0	15.6	7.1	20.8	
	3	24.6		15.8			
	4	20.5					
		22.6	12.3	15.4	7.2	20.8	78.3
				molar ratios			
		1.00	0.91	1.14	0.99	0.89	
				weight %			
Lamb's wool	1	21.7	10.5	12.0	7.4	24.1	
	2	21.5	10.3	12.8	7.9	25.5	
	3	22.5			7.2		
		21.9	10.4	12.4	7.5	24.8	77.0
				molar ratios			
		1.00	0.79	0.95	1.07	1.10	
				weight %			
Feathers	1	21.6	10.7	12.8	9.0	22.2	
	2	21.6	12.9	12.8	9.0	20.9	
		21.6	11.8	12.8	9.0	21.5	76.7
				molar ratios			
		1.00	0.91	0.99	1.30	0.96	

normal fatty acids. The methyl esters of normal and hydroxy fatty acids were separated by preparative TLC and examined by GLC. The ratio of hydroxy to nonhydroxy fatty acid varied from 0.07 to 0.09 in these samples. The TMSi ether of the hydroxy acid had the same retention time as the TMSi ether of 4-hydroxy stearic acid both on DEGS and on SE-30 columns. In all samples the normal ester fraction contained palmitic and stearic acid as the main components (Table 3).

The GLC of the TMSi ethers of sphingosines showed two peaks, the first having the same retention time as *O*-methyl sphingosine and the second having the same as that of C_{18}-sphingosine. To clarify whether the *O*-methyl sphingosine was a by-product or previously existed in

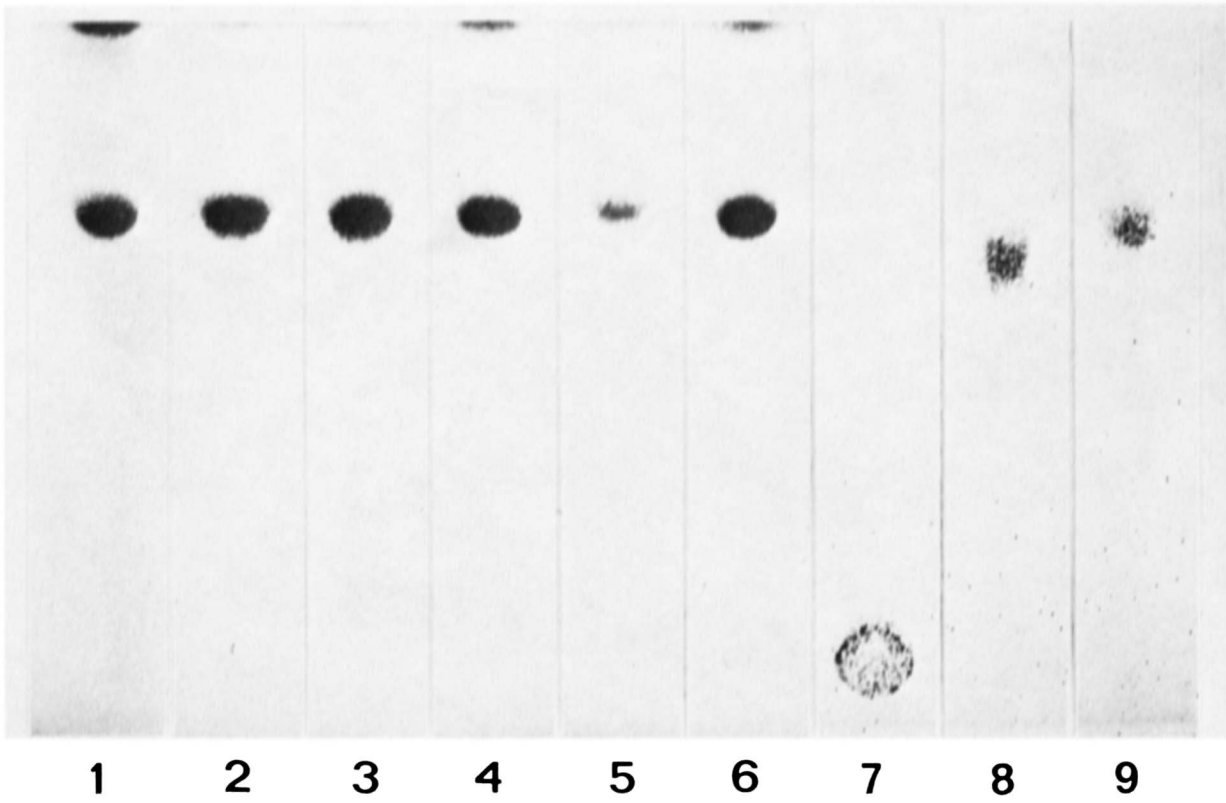

FIG. 1. TLC of ungulic acid isolated from 1, wool; 2, pig bristles; 3, human nails; 4, human keratinous epidermis; 5, feathers; 6, human hair and reference standards; 7, gangliosides; 8, sulfatides; and 9, ungulic acid isolated from horses' hooves. The TLC plates were developed with chloroform–methanol–water 23:8:1 (v/v/v). Detection was carried out by spraying the plates with $K_2Cr_2O_7$ dissolved in 70% aqueous sulfuric acid.

the molecule, the samples were also submitted to methanolysis with the modified reagent recommended by Gaver and Sweeley (11). This methanolysis reduced the amount of *O*-methyl sphingosine in every sample to a minimum.

According to the present analyses the fractions isolated from human keratinous epidermis, hair, and nails, pig bristles, wool and feathers contain about equimolar amounts of ceramide, galactose, galactosamine, sulfate, and sialic acid, and their composition is thus identical with that of the ungulic acid isolated from horse hooves.

DISCUSSION

Attempts to find the sulfate-containing glycolipid fraction in tissues other than epidermal ones have so far given negative results. With regard to the human kidney, however, the results are somewhat uncertain; the amounts of the fraction observed have been so small that no definite conclusion can be drawn.

The actual function of ungulic acid in the epidermis and its appendages remains obscure, but it is possible that it exerts a sort of protective action. In fact, preliminary results concerning the antimicrobial activity

TABLE 3 FATTY ACID COMPOSITIONS OF UNGULIC ACID FRACTIONS ISOLATED FROM VARIOUS SOURCES

Material	16:0*	16:1	18:0	18:1	Hydroxy Acid	20:0	Others
Human keratinous epidermis	53.2	7.9	28.5	—	6.6	3.8	—
Human hair	49.2	—	32.0	—	7.4	6.0	5.4
Human nails	38.7	1.9	50.6	—	8.4	traces	—
Pig bristles	39.1	—	42.2	7.6	—	5.1	6.0
Lamb's wool	32.8	10.2	41.5	10.1	—	—	5.4
Feathers	29.8	25.5	30.5	—	7.3	4.6	2.3

* Number of carbon atoms:number of double bonds.

of this fraction revealed certain bacteriostatic and even bactericidal activity, for instance against streptococcus and staphylococcus species. Further studies are still in progress.

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References

1. Leikola, E., E. Nieminen, and A.-M. Teppo. 1969. *J. Lipid Res.* **10:** 440.
2. Marsh, J. B., and D. B. Weinstein. 1966. *J. Lipid Res.* **7:** 574.
3. Wren, J. J., and A. D. Szczepanowska. 1964. *J. Chromatogr.* **14:** 405.
4. Spencer, B. 1960. *Biochem. J.* **75:** 435.
5. Radin, N. S., J. R. Brown, and F. B. Lavin. 1956. *J. Biol. Chem.* **219:** 977.
6. Svennerholm, L. 1958. *Acta Chem. Scand.* **12:** 547.
7. Miettinen, T., and I. T. Takki-Luukkainen. 1959. *Acta Chem. Scand.* **13:** 856.
8. Warren, L. 1959. *J. Biol. Chem.* **234:** 1971.
9. Siddiqui, B., and R. H. McCluer. 1968. *J. Lipid Res.* **9:** 366.
10. Nieminen, E., E. Leikola, M. Koljonen, U. Kiistala, and K. K. Mustakallio. 1967. *Acta Dermato-Venereol.* **47:** 327.
11. Gaver, R. C., and C. C. Sweeley. 1965. *J. Amer. Oil Chem. Soc.* **42:** 294.