New sialic acid-containing sulfolipid: "ungulic acid"

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ABSTRACT Human epidermis, hair, nails, and kidney as well as bovine and horses' hooves were found to contain a lipid fraction, which on thin-layer chromatography migrated slightly ahead of the cerebroside sulfate esters and gave the color reaction specific for sialic acid. This fraction was isolated from horse hoof, in which it constituted nearly half of the total lipids. The purified fraction contained sulfur, but no phosphorus. The IR spectrum revealed the presence of a sulfate group, which was also determined by the benzidine method. Thin-layer and gas–liquid chromatography of the products of acid hydrolysis revealed the presence of sphingosine, galactose, galactosamine, and sialic acid. Fatty acid analysis showed that stearic acid was the major component, with minor amounts of palmitic and arachidic acids.

The fraction isolated contained ceramide, sialic acid, galactose, galactosamine, and sulfate in equimolar amounts. We conclude that the new lipid is a ganglioside sulfate, which we have called "ungulic acid" because it was first separated and identified from a horse's hoof (Latin, *ungula*).

SUPPLEMENTARY KEY WORDS hoof · ganglioside sulfate · fatty acids · thin-layer · gas–liquid chromatography · mass spectrum · epidermis · hair · nail · kidney

THE SULFOLIPIDS (sulfatides) found in human and animal organisms are generally characterized as sulfate esters of cerebrosides. They were first obtained from nervous tissue (1, 2), but in recent years the presence of these compounds has been demonstrated in other tissues too (3, 4). According to Mårtensson (5), human kidney contains two different types of sulfolipids, one with the same chemical composition as brain sulfatides, galactose being the sole carbohydrate component, the other containing glucose and galactose in about equimolar amounts.

In a previous investigation on human epidermis, Nieminen et al. (6) found a sulfur-containing lipid fraction which in thin-layer chromatography migrated slightly ahead of the cerebroside sulfate esters. Since then, a lipid fraction with similar behavior has been isolated from human kidney, hair, and nails, and from the walls of bovine and horses' hooves. Surprisingly, the fractions gave the color reaction specific for sialic acid, which is a characteristic component of gangliosides. The purpose of the present study was to clarify the chemical composition of this fraction.

MATERIALS AND METHODS

Materials

The specimens of epidermis, hair, and nails were taken from young, healthy volunteers. The epidermis was separated from the dermis as previously described (6). The kidney specimens were obtained at autopsy. The hooves were taken in a slaughterhouse immediately after the horse had been killed. After washing, the walls of the hooves were separated and ground. The samples were stored in a deep-freeze until 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, phosphatidyl choline, and phosphatidyl ethanolamine (Nutritional Biochemical Corp., Cleveland, Ohio); beef brain sphingomyelin (Mann Research Laboratories, Inc., New York); dihydrosphingosine and synthetic *dl*-sphingosine (Miles-Yeda, Ltd., Kiryat Weizmann, Rehovoth, Israel); and beef spinal core sulfatides (Applied Science Laboratories, Inc., State College,

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Pa.). The mixture of brain gangliosides was a generous gift from Dr. K. Puro, Helsinki, Finland. All solvents were of analytical grade and were used without further purification.

Lipid Extraction

The tissue samples were extracted by the method of Folch, Lees, and Sloane Stanley (7). The hair, nail, and hoof samples were first refluxed for 1 hr with chloroform–methanol 2:1 and then for 1 hr with chloroform–methanol 1:2. The combined extracts were dried with anhydrous Na_2SO_4 and evaporated to dryness in vacuo.

Isolation of Polar Lipids

50 g of Al_2O_3 (activity grade II–III Brockmann, E. Merck A. G., Darmstadt, Germany) was suspended in chloroform and the slurry was poured into a column (1.5 × 60 cm). The crude total lipid from 50 g of horse hoof was applied to the column in 30 ml of chloroform. Neutral lipids were eluted with 500 ml of chloroform, after which the polar lipids were eluted with 500 ml of methanol. The methanol fraction was dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue of polar lipids was further purified by TLC.

Thin-Layer Chromatagraphy

Ascending TLC was performed on Silica Gel G, 0.25 mm thick, which had been activated at 110°C for 1 hr. Before use the plates were developed with diethyl ether to remove contaminants from the adsorbent.

For preparative TLC, 10–20 mg of polar lipids in chloroform was applied on a 20 × 20 cm plate as a 17-cm-long streak. The chromatograms were developed with chloroform–methanol–water 23:8:1. The areas corresponding to the unknown ("fraction X") were located by spraying partly covered plates with iodine in chloroform. The adsorbent in these areas was scraped off and extracted three times with methanol. The solution was evaporated to dryness and the residue recrystallized three times from methanol.

The homogeneity of fraction X was confirmed with the following solvent systems: chloroform–methanol–water in various proportions (24:7:1, 65:25:4, 60:35:8); chloroform–methanol–2.5 N NH_4OH 60:35:8; propanol–water 7:3; and propanol–concentrated NH_4OH 7:3. The plates were sprayed with a saturated solution of potassium dichromate in 70% aqueous sulfuric acid and heated for 3–10 min at 180°C, or they were sprayed with molybdenum reagent to detect phospholipids (8), or with resorcinol–HCl reagent to detect gangliosides (9).

Sugars obtained after hydrolysis of fraction X (see below) were subjected to TLC on silica gel in ethyl acetate–isopropanol–water 130:45:25 for 12 hr, or in ethyl acetate–isopropanol–pyridine–water 70:30:20:20, and detected by spraying with diphenylamine–aniline reagent (10) and with ninhydrin reagent (11).

Gas–Liquid Chromatography

General. We used a Varian Aerograph model 204 chromatograph equipped with a flame ionization detector and Servo Riter integrator. As carrier gas, nitrogen was used. For analysis of fatty acid methyl esters the column, 1.7 m × 4 mm, was packed with 10% diethyleneglycol succinate on silanized Chromosorb W. For sugar analyses the column was packed with 2.5% SE-30 (methylpolysiloxy gum) on the same support.

Carbohydrates and Sphingolipid Bases. About 10 mg of fraction X was hydrolyzed in sealed tubes with 3 ml of 2 N methanolic HCl at 70°C for 20 hr. After cooling, the methyl esters of fatty acids were removed by two successive extractions with 3 ml of petroleum ether saturated with methanolic HCl. The combined petroleum ether extracts were used for fatty acid analysis, described later. The methanolic hydrolysate was applied to an anion-exchange resin (Amberlite IR-45) column and eluted with 50 ml of methanol. The solution was evaporated to dryness in vacuo. The mixture was trimethylsilylated as follows (12): 2 ml of pyridine and 0.3 ml of hexamethyldisilazane–trichlorosilane 2:1 were added successively and the mixture was kept at 20°C for 20 min. The excess of silylating reagents was evaporated off at 40°C and the TMSi ethers were dissolved in 2 ml of *n*-hexane for GLC.

This solution was also employed for the mass spectrum and for quantitative determination of the sphingosine moiety, the TMSi ether of sorbitol being used as internal standard and the TMSi ether of sphingosine, isolated from bovine brain sphingomyelin, as reference standards.

Hexosamines. 5 mg of fraction X was hydrolyzed in 2 N HCl in methanol–water 1:1 at 100°C for 40 hr. The hexosamines were separated from the hydrolysate on a cationic Dowex-50 column according to the method of Boas (13). The hexosamine fraction was mixed with an internal standard (0.50 mg of sorbitol) and trimethylsilylated as described above. A mixture containing 0.50 mg of galactosamine and 0.25 mg of sorbitol in 1 ml of *n*-hexane was used as a standard.

Preparation of Fatty Acid Methyl Esters. The petroleum ether extract obtained after methanolysis of fraction X was washed several times with equal volumes of water, dried with anhydrous Na_2SO_4, and evaporated to dryness in vacuo. 1 mg of methyl behenate was added and the residue was dissolved in 1 ml of petroleum ether. This mixture was used for both qualitative and quantitative analysis of fatty acids by GLC.

Determination of Neutral Sugars, Sialic Acid, and Sulfate

15 mg of fraction X was hydrolyzed in 5 ml of ethanol–chloroform–concentrated HCl 7.4:6:3 at 70°C for 3 hr. The cooled mixture was evaporated to dryness in vacuo and the nonlipids were separated from the residue with Sephadex (G-25, fine) as described by Wells and Dittmer (14). The nonlipid fraction was evaporated to dryness and the residue was dissolved in an appropriate amount of water. Nonlipids were analyzed qualitatively by TLC (see above). Neutral sugars were determined by the orcinol method, as described by Radin, Brown, and Lavin (15), and sialic acid by Svennerholm's resorcinol method (9) as modified by Miettinen and Takki-Luukkainen (16). Reference standards were also subjected to these vigorous hydrolysis conditions.

1.0 mg of fraction X was hydrolyzed with 200 μl of 1 N HCl in a sealed tube overnight at 70°C. Sulfate in the hydrolysate was determined according to Spencer (17).

Spectra

IR spectra were obtained with a Perkin–Elmer model 257 spectrophotometer equipped with a NaCl prism.

The mass spectrum of the sphingosine isolated from fraction X was obtained on an LKB model 9000 combination gas chromatograph–mass spectrometer. The gas chromatographic inlet system was equipped with a 2-m glass column, packed with 2.2% SE-30 on acid-washed and silanized 100–120 mesh Gas Chrom P. A column temperature of 200° C, a temperature of molecular separation of 260–275°C, an ionizing potential of 70 ev, and an ionizing current of 60 mA were used.

RESULTS AND DISCUSSION

When thin-layer chromatograms of hoof lipids were sprayed with potassium dichromate–H_2SO_4 reagent, fraction X appeared as a purple spot after heating for as little as 3 min at 180°C, whereas the other polar lipids appeared only after 5–10 min. A corresponding fraction was observed in chromatograms of lipids from human kidney, epidermis, hair, and nails, but could not be found in lipids from human brain or liver. In chloroform–methanol–water 24:7:1, fraction X had an R_f value of 0.31 and was clearly separated from the reference sulfatides, which migrated slightly more slowly, R_f 0.27. In this system the gangliosides remained at the starting point and sphingomyelin, phosphatidyl choline, and phosphatidyl ethanolamine had R_f values 0.12, 0.26, and 0.46, respectively. When chromatograms were sprayed with the resorcinol–HCl reagent and heated at 110°C for 15 min, fraction X gave the same bluish red color as the sialic acid used as reference compound. It was not detected with molybdenum blue phospholipid reagent. These color reactions seemed to indicate that fraction X probably was a glycolipid belonging to the ganglioside group.

Qualitative TLC showed that fraction X constituted the main part of the horse hoof lipids, and it was therefore isolated from this source. The total lipids of the hoof constituted about 2% of the fresh weight. The polar lipids obtained from the Al_2O_3 column made up about 50% of the total lipids, and according to TLC consisted mainly of the fraction under study, with only traces of neutral lipids. However, the loss due to TLC purification and recrystallization was considerable; the recovery was about 25% of the total lipids. The purified fraction consisted of white crystals resembling snowflakes which were rather soluble in methanol and slightly soluble in water; mp 190–192°C. On TLC, it gave only one spot with each of the different solvent systems described under Methods. The IR spectrum (Fig. 1) of fraction X showed absorption around 1240 cm^{-1}, which is characteristic of sulfatides.

After hydrolysis of fraction X with methanolic HCl, galactose and galactosamine were demonstrated by TLC. The GLC pattern of TMSi derivatives of the carbohydrate moiety confirmed the presence of these

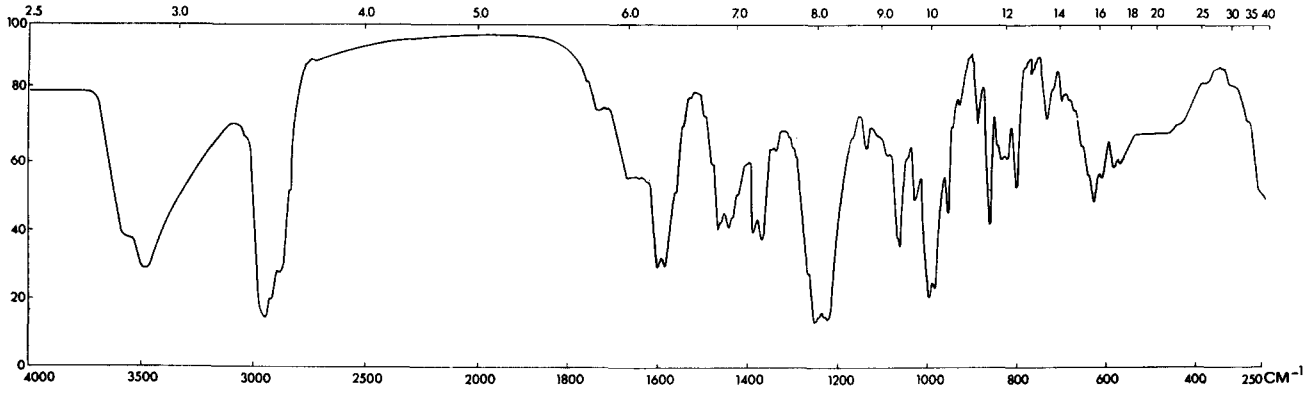

FIG. 1. IR spectrum of ungulic acid (KBr disc).

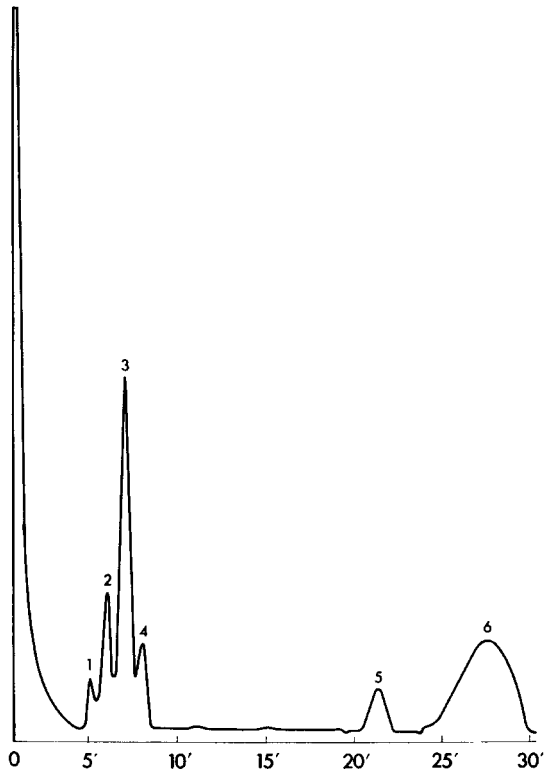

FIG. 2. Gas chromatogram of TMSi derivatives of carbohydrates and sphingolipid bases from fraction X. 1, γ-galactose. 2, galactosamine. 3, α-galactose. 4, β-galactose. 5 and 6, sphingosine. On SE-30 at 165°C.

sugar components (Fig. 2). The retention times of the peaks appearing after the carbohydrates corresponded to those of the TMSi derivatives of the sphingolipid base isolated from sphingomyelin. GLC did not reveal sialic acid, either because it was destroyed during this hydrolysis or because it was retained by the anion exchanger. The mass spectrum of the major component of the TMSi derivative of the sphingosine moiety (peak 6 of Fig. 2) gave a molecular ion at m/e 443. Fragment ions at m/e 428 $(M-15)^+$, m/e 353 $(M-90)^+$, m/e 311 $(M-132)^+$, and m/e 263 $(M-180)^+$ were observed. These values showed that the major component consists of C_{18}-sphingosine. By GLC the material of peaks 5 and 6 was found to contain 27.0% of sphingosine (Table 1).

GLC of the fatty acid methyl esters showed that stearic acid was the major component (63.7%) with small amounts of palmitic acid (19.7%), arachidic acid (12.0%), and others (4.6%); no evidence of the presence of hydroxy fatty acids could be detected. This fatty acid composition has been found to be typical of gangliosides (18). In brain gangliosides, stearic acid constitutes 80–95% of the total fatty acids (19, 20).

The nonlipid fraction eluted from a Sephadex column after strong acid hydrolysis was found to contain 26.9% of sialic acid (calculated as *N*-acetylneuraminic acid) and

TABLE 1 COMPOSITION OF FRACTION X

Aliquot	Galactose	Galactosamine	Sialic Acid	Sphingosine	S	N	Sulfate
				weight %			
1	14.4	11.6	25.6	27.4	2.4	3.8	7.9
2	11.6	10.8	28.7	26.6			7.1
3	12.8	11.2	26.6				
Mean	13.1	11.2	26.9	27.0	2.4	3.8	7.5
				molar ratios			
	0.9	0.8	1.1	1.1	1.0	3.6	1.0

S, sulfur; N, nitrogen.

13.1% of galactose (Table 1). TLC of the carbohydrate fraction obtained with a Dowex-50 column revealed the presence of galactosamine. Quantitative analysis of galactosamine was attempted, using the Elson–Morgan color reaction (21), but it gave nonreproducible results. On the other hand, with sorbitol as internal standard, good reproducibility was obtained in GLC. Fig. 3 shows the gas chromatogram of the TMSi derivatives of the amino sugar component and of the internal standard. On 2.5% SE-30 at 165°C only one peak was obtained from galactosamine. The same finding has been made by Kärkkäinen, Lehtonen, and Nikkari (12). The

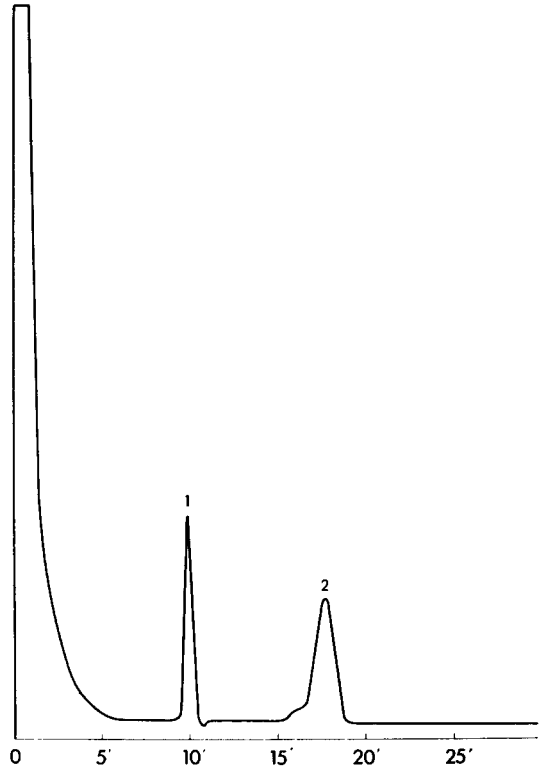

FIG. 3. Gas chromatogram of TMSi derivatives of amino sugars from fraction X, with sorbitol (peak 2) as internal standard. Peak 1 corresponds to galactosamine. On SE-30 at 150°C.

galactosamine content of fraction X was found to be 11.2% (Table 1).

Elemental analysis gave nitrogen 3.8% and sulfur 2.4%. The benzidine method revealed 7.5% of sulfate to be present. No phosphorus could be detected by the ascorbic acid method (22). By the osmometric method, using methanol as solvent, the mol wt was shown to be 1280. Fraction X thus contained ceramide, sialic acid, galactosamine, galactose, and sulfate in approximately equimolar amounts (Table 1). A notable feature was the unusually strong acid hydrolysis needed to release the sialic acid from this compound. It seems that there is some difference in the general structure of this compound compared with known gangliosides. Since sialic acid is considered to be a component specific to the gangliosides, it can be concluded that this new sulfolipid is a sulfate ester of a ganglioside.

The corresponding fraction from human kidney, nails, hair, and epidermis seems to belong to the same lipid group, but studies to establish the point are still in progress.

The mass spectrum was recorded at the Department of Clinical Chemistry, University of Helsinki. We are indebted to Professor H. Adlercreuz, M. D., for valuable advice.

Elemental analyses were performed at Ilse Beetz Mikroanalytisches Laboratorium, Kronach, West Germany and the osmometric determination of molecular weight was carried out at the Alfred Bernhard Mikroanalytisches Laboratorium, Max Planck-Institute, West Germany.

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