J. Lipid Research, October 1963
Volume 4, Number 4

Purification and partial characterization of sphingomyelin from human plasma*

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[Manuscript received March 15, 1963; accepted June 4, 1963.]

SUMMARY

Isolation of pure sphingomyelin from human plasma on a preparative scale is described. Analyses of fatty acids by gas-liquid chromatography on two polar polyester columns indicate the presence of all even- and odd-carbon, saturated fatty acids from C_{14} to C_{25} and most of the corresponding monoenoic acids as well. Palmitic acid is the predominant component; seven of the acids constitute over 90% of the total fraction. A previously unassigned trace constituent of the fatty acid fraction has been tentatively identified as a C_{24} dienoic acid on the basis of relative retention times, microhydrogenation, and reaction with mercuric acetate.

Of the polar lipids that are constituents of serum lipoproteins, the sphingolipids are perhaps the least well characterized. Until recently, it was difficult to obtain precise information on the sphingomyelin fatty acids in tissues and fluids containing low concentrations of sphingolipids. However, the application of newer analytical methods (notably silicic acid and thin-layer chromatography (TLC) for quantitative separations of mixed polar lipids, and gas-liquid chromatography (GLC) for analyses of volatile components) has made possible such structural investigations. Nelson and Freeman (1) have reported fatty acid compositions of phospholipids of human serum lipoproteins including that of sphingomyelin. Hanahan et al. have studied the composition of fatty acids of sphingomyelin isolated from human and bovine blood (2).

In previous papers (3, 4), we described a method for the qualitative inspection of sphingolipid bases by gas chromatography. Sphingomyelin, isolated from human plasma on a small scale, contained several unknown bases in addition to sphingosine and dihydrosphingosine. Although some conclusions about structure may be drawn from GLC studies, it is necessary that a pure sample of sphingomyelin be obtained in sufficient quantity for more definitive structural studies of these bases. The present communication describes the preparative isolation of sphingomyelin from human plasma, determination of fatty acid:base:phosphorus ratio in the fraction, and examination of the fatty acid moiety. The methods used for the preparation of sphingomyelin follow those of Hanahan et al. (2) and Böttcher and van Gent (5).

METHODS

Materials. Outdated ACD (acid citrate dextrose) human blood was made available through the generosity of the blood banks of Presbyterian-University Hospital, Montefiore Hospital, and the Veterans Administration Hospital, Pittsburgh.

After centrifugation of the blood, plasma was withdrawn and lyophilized. Unisil, an activated, size-graded silicic acid, was obtained from Clarkson Chemical Co., Inc., Williamsport, Pa. Silica Gel G for TLC was supplied by Brinkmann Instruments, Inc., Great Neck, Long Island, N.Y. Synthetic N-palmitoylsphingomyelin was the generous gift of Dr. D. Shapiro of the Weizman Institute of Science, Rehovoth, Israel. All solvents were reagent grade and were used as supplied unless specially noted.

Analytical Procedures. Esters were determined colorimetrically by the method of Rapport and Alonzo (6). Total long-chain base was estimated by the method of Lauter and Trams (7), modified somewhat as described in the text. Phosphorus assays were made by the procedure of Bartlett (8). Preliminary separations

* This study was supported in part by Research Grant A-4307 from the U.S. Public Health Service.

of mixed lipids into simple and polar classes were carried out by silicic acid chromatography as described originally by Borgström (9).

Thin-layer chromatography was used as a monitoring assay during various steps in the isolation. The solvent system employed for separations of polar lipids was chloroform–methanol–water 100:42:6 (v/v) contained in a lined glass tank. The plates were coated with Silica Gel G by dipping them into a bottle containing a well-shaken mixture of 400 g of gel, 800 ml of chloroform, and 400 ml of methanol (10). By the dipping technique, uniform and reproducible plates were made with a capacity somewhat lower than those made with a standard commercial spreading device (11).

Extraction of Lipids. Lyophilized human plasma (650 g, representing approximately 15 liters of blood from 30 donors) was extracted in portions of about 150 g in a Soxhlet extractor, using 3 liters of anhydrous acetone for the initial extraction of 24 hr. The powder was wetted with about 500 ml acetone-ethanol 9:1 (v/v) before beginning this extraction, on the assumption that ethanol would aid in the disruption of lipoprotein complexes. The extraction was continued for an additional 36–48 hr with 3 liters of anhydrous (reagent) ethanol. These extracts were re-used with each new batch of powder. After all of the lyophilized plasma had been extracted, the solutions of crude lipids were taken to dryness in vacuo in a rotary evaporator. The separate residues were dissolved in about 200 ml of chloroform–methanol 2:1 (v/v), and the solutions were washed with 0.2 volume of distilled water according to the procedure of Folch, Lees, and Sloane Stanley (12). The lower layers were mixed with ethanol (0.1 volume) and evaporated in vacuo. Crude lipids obtained from acetone are referred to as Batch 1 and those from ethanol as Batch 2.

The protein-containing residues from the Soxhlet extractor were further extracted in portions with 5 liters of chloroform-methanol-water 2:1:0.05 (v/v) in a large blendor, the homogenate was filtered through fluted paper, and the filtrate was used for the following extractions. The final extract was washed with 0.2 volume of water, and lipids were isolated as described above. Crude lipids obtained in this way are referred to as Batch 3.

Silicic Acid Chromatography of Crude Lipids. The combined lipids from Batches 1 (12.5 g) and 2 (6.5 g) were dissolved in 200 ml of chloroform and applied to a column (2.5 x 110 cm) containing 150 g of Unisil (100–200 mesh). The column was washed with chloroform until negative tests for lipid were obtained by spotting 5–10 μl of eluate on a ferrotype plate, as described by Lands and Dean (13). A total of 1,500 ml of choroform was necessary for complete elution of neutral lipids. The column was eluted next with 1,000 ml of chloroform–methanol 4:1 (v/v) to remove a "cephalin" fraction that contains cerebroside and perhaps other relatively nonpolar sphingolipids (14). Crude sphingomyelin was obtained by elution with 2 liters of chloroform-methanol 1:3 (v/v), the eluate being collected in 250-ml fractions. Those fractions containing sphingomyelin, with an R_F of 0.12 on TLC, were pooled and taken to dryness in vacuo. The yield of crude sphingomyelin was 1.18 g. This fraction, as expected, was grossly contaminated with lecithin (R_F 0.28) and also contained some lysolecithin (R_F 0.06).

The crude lipids of Batch 3 (8.8 g) were chromatographed in the same manner as described above, yielding 1.13 g of crude sphingomyelin.

Mild Alkaline Hydrolysis of Crude Sphingomyelin. Mild alkaline hydrolyses were carried out by Dawson's method (15) as modified by Hübscher, Hawthorne, and Kemp (16). Crude sphingomyelin (2.31 g) was dissolved in 50 ml of chloroform and mixed with 50 ml of 0.6 N sodium hydroxide in methanol. The reaction mixture was allowed to stand at room temperature for 1 hr, after which were added in the following order 2.6 ml of 12 N HCl, 50 ml of chloroform, and 40 ml of water, with mixing after each addition. The biphasic system, which approximated the conditions of a Folch wash, was allowed to settle and the lower phase was separated and washed twice with methanol-water 1:1 (v/v). The clear chloroform layer was mixed with 0.1 volume of ethanol and was taken to dryness in vacuo, yielding 2.0 g of residue.

Final Purification of Sphingomyelin by Silicic Acid Chromatography. The chloroform-soluble fraction obtained after mild alkaline hydrolysis contained sphingomyelin, methyl esters of lecithin, and a small quantity of an unknown artifact of the hydrolysis with an R_F (0.57) on TLC similar to that of phosphatidyl ethanolamine. The mixture (2.0 g) was chromatographed on Unisil (50 g), the methyl esters being eluted with 500 ml of chloroform, the artifact with 500 ml of chloroform-methanol 5:1 (v/v), and sphingomyelin with 500 ml of chloroform-methanol 1:3 (v/v). After evaporation of the solvent from the last fraction, 1.1 g of product was obtained in the form of a pale yellow glass. The purified sphingomyelin was dissolved in 10 ml of glacial acetic acid, with warming, and the solution was diluted to about 100 ml with benzene. Lyophilization gave a low-density white powder, which was dried for several days in a vacuum desiccator over sodium hydroxide pellets. The final yield of sphingomyelin was 0.94 g.

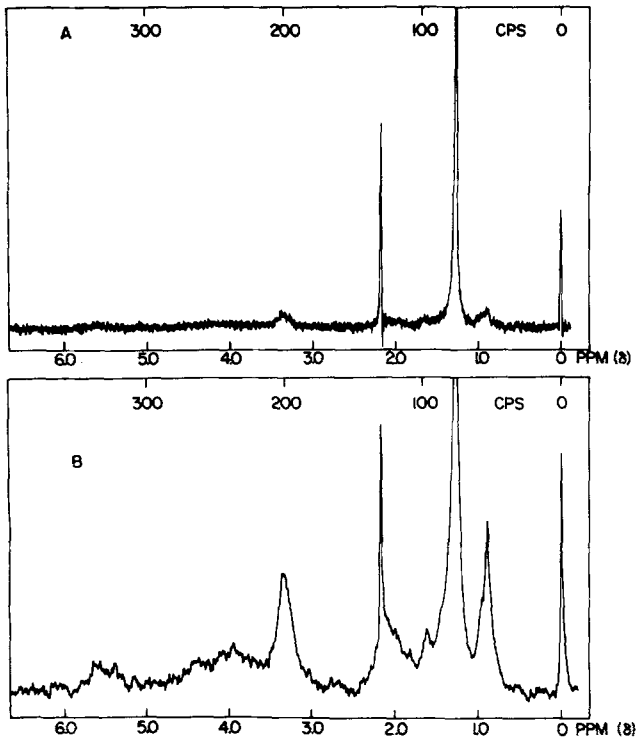

FIG. 1. Nuclear magnetic resonance spectra of plasma sphingomyelin (10.0 % in deuterochloroform) at spectrum amplitudes 12.5 (A) and 125 (B).

Elemental Analysis. Found: C 65.77, H 11.92, N 3.42, P 3.84. Calculated for $C_{42}H_{85}N_2O_6P \cdot H_2O$: C 66.10, H 11.49, N 3.67, P 4.06.

Determination of Long-Chain Base and Amide. Sphingomyelin (8.8 mg, 10.9 μmoles P) was dissolved in 5 ml of dry 3 N methanolic HCl. The solution was heated at 75° for 24 hr in a screw-cap tube with Teflon liner. The mixture was cooled, transferred quantitatively to a volumetric flask, and diluted to 25 ml with methanol.

Methyl esters were determined on 3-ml aliquots. Complete removal of the HCl was ensured by removing the solvent at the temperature of a warm water bath in a stream of nitrogen. Chloroform (1–3 ml) was added and evaporated in the same way, and this step was repeated. The final residue was dissolved in diethyl ether, and the analysis was completed in the usual manner. The results, in terms of micromoles of ester, may be interpreted as amide, provided that the sphingolipid sample contains no ester or free fatty acid before methanolysis. Duplicate analyses indicated 10.3 μmoles of amide per 10.9 μmoles P.

Total long-chain base was determined on 0.1-ml aliquots by a modification of the method of Lauter and Trams (7). The aliquot was evaporated under a stream of nitrogen, and excess HCl was removed in the manner just described. To the residue, consisting of base hydrochlorides, were added 5 ml of ethyl acetate, 3 ml of 0.1 M acetate buffer, pH 3.65, and 0.1 ml of methyl orange solution (7). After vigorous mixing and centrifugation, methyl orange in the organic layer was extracted into 1 N sulfuric acid and determined in a Coleman spectrophotometer at 515 mμ. The present modification avoids extraction of free bases from aqueous alkali prior to assay, a step that easily leads to small losses due to incomplete extraction. These analyses gave 11.1 μmoles of sphingosine and related bases in the original sample.

Analysis of Fatty Acids. Methyl esters were prepared from sphingomyelin by direct methanolysis of the lipid with dry 3 N methanolic HCl, as described above. Methyl esters were isolated by three extractions with redistilled hexane. Analyses were made by GLC on 6-ft x $^1/_4$-in. polyester columns (15% polyethylene glycol succinate on 80–100 mesh Chromosorb W prepared by Applied Science Laboratories, State College, Pa., and 15% polyethylene glycol adipate on 80–100 mesh Chromosorb W) in a Barber-Colman Model 10 instrument. Identifications were based on comparisons of some peaks with standards, calculations of predicted retention times from semilogarithmic plots of retention time versus chain length for saturated and monounsaturated acids, and by analysis of the sample after microhydrogenation. Calculation of the composition was based on area percentage of each component, using peak height times width at half height as a measure of area. Linearity of the argon detector was checked with standard mixtures of methyl esters obtained from the National Heart Institute.

To confirm the assigned identifications, methyl esters (10 mg) were separated into saturated and unsaturated fractions by silicic acid chromatography after converting the unsaturated esters to acetoxymercuri-methoxy compounds by the procedure of Jantzen and Andreas (17), as described by Mangold (11). The reaction mixture was evaporated to dryness in vacuo. The residue was dissolved in a small volume of hexane–chloroform 1:1 (v/v) and chromatographed on a small column containing 5 g Unisil (200–325 mesh)

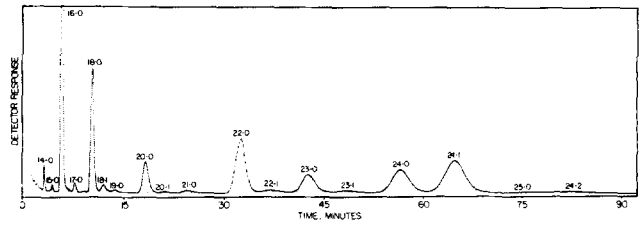

FIG. 2. Fatty acids of plasma sphingomyelin, analyzed on polyethylene glycol succinate, 175°, 15 psi argon inlet pressure.

packed with the same solvent. Saturated methyl esters were eluted with 50 ml of hexane–chloroform 1:1 (v/v), and the adducts of unsaturated esters with 50 ml of chloroform-methanol-acetic acid 2:1:0.03 (v/v). Unsaturated methyl esters were recovered as described previously (11). The fractions were analyzed by GLC on the adipate polyester.

RESULTS AND DISCUSSION

Direct extraction of plasma is not practical on a large scale, and studies were therefore made of procedures for extraction of lyophilized plasma. Continuous extraction with acetone and with ethanol was effective on a small scale and resulted in the accumulation of total lipids in a relatively small volume of solvent. However, in the preparative run, the recovery of sphingomyelin and other polar lipids was low. Fortunately, subsequent extraction of the residue with wet chloroform-methanol provided complete recovery of the remaining polar lipids, including sphingomyelin, as judged by comparing the yield of polar lipids with that obtained by direct extraction of plasma with chloroform-methanol.

Chromatography of the crude total lipids on large columns of silicic acid provided useful initial separations of the mixture into various classes. It was not possible, however, to obtain a highly pure sphingomyelin in this way because of overlapping with lecithin and lysolecithin. Selective mild alkaline hydrolysis of the crude product removed these contaminants, and a second chromatographic treatment on silicic acid yielded a product of high purity. Sphingomyelin thus prepared was free of other common phospholipids and glycolipids, as shown by TLC; the product was chromatographically indistinguishable from synthetic N-palmitoylsphingomyelin (R_F 0.12).

Elemental analyses were in close agreement with those predicted for $C_{42}H_{85}N_2O_6P \cdot H_2O$, the arbitrarily selected formula of N-nonadecanoylsphingomyelin. The average chain length of the fatty acids, calculated from gas chromatographic data, was 19.4 and thus agreed reasonably well with the same empirical formula. In accordance with the results of Baer (18) and Shapiro, Flowers, and Spector-Shefer (19), the analyses suggested a "hydrated" form of the zwitterionic structure. The molar ratio of N to P, calculated from the elemental analyses, was 1.98, and the molar ratio of sphingosine to fatty acid to P was 1.02:0.95:1.00. These data eliminate the possibility of contamination by lysoplasmalogen, an alkali-stable compound with properties similar to those of sphingomyelin on silicic acid and thin-layer chromatography.

TABLE 1. FATTY ACIDS OF SPHINGOMYELIN FROM HUMAN PLASMA

Acid*	Proportion
	%
14:0	0.7
15:0	0.2
16:0	41.7
17:0	0.6
18:0	9.4
18:1	0.7
19:0	0.3
20:0	3.9
20:1	0.2
21:0	0.3
22:0	12.1
22:1	0.4
23:0	5.1
23:1	0.5
24:0	8.9
24:1	14.1
24:2	0.7
25:0	0.4

*C:X indicates carbon chain length and degree of unsaturation.

The infrared spectrum, obtained in chloroform, was identical with those previously published for synthetic N-stearoylsphingomyelin (19) and sphingomyelin from beef brain (20). Complete absence of a peak at 5.76 μ (aliphatic carboxylic ester) was indicative of the absence of glycerolipids as contaminants and confirmed the results of direct ester analyses and TLC.

The specific rotation (1.29% in ethanol) was +9.9° as compared with a value of +8.9° reported by Hanahan et al. (2) and somewhat lower values by other investigators (21, 22). It was interesting to investigate the optical rotatory dispersion[1] of sphingomyelin in the hope that a higher rotation might be observed in the ultraviolet region. The results were disappointing in this respect; little variation in rotation was noted over a range of wavelengths from 350 to 750 mμ.

Analysis of sphingomyelin by nuclear magnetic resonance[2] was carried out in deuterochloroform; Fig. 1 shows the spectrum at two amplitudes. At a normal amplitude (A), peaks at 2.16, 1.3, and 0.9 δ have been assigned tentatively to —CH_3 (choline), —CH_2—, and —CH_3 (sphingosine and fatty acids), respectively. At high amplitude (B), additional peaks were observed; of these, a barely visible signal at 5.5 δ is prob-

[1] We are indebted to Dr. William Harrington, Johns Hopkins University, for determinations of optical rotatory spectra.

[2] NMR spectra were taken with the Varian Associates A-60 Spectrometer. The data are expressed as ppm shift from tetramethylsilane as internal standard. We wish to thank Dr. John R. Dyer, Georgia Institute of Technology, for these analyses.

ably due to the olefinic protons of sphingosine and unsaturated fatty acids. Other peaks at about 4.0, 3.3, and 2.0–1.5 δ, have not been assigned.

The observed pattern of fatty acids (Fig. 2) was characteristic of sphingolipids in general, with relatively high concentrations of C_{20} to C_{24} acids. There were seven major fatty acids (Table 1), of which palmitic acid was predominant, but the mixture contained many additional components at lower levels, as was found also in the case of brain cerebrosides (23, 24). In contrast to previous studies of the fatty acids of sphingomyelin from human plasma, we found little evidence for the presence of linoleic acid or arachidonic acid, which together comprised 7 and 28% of total fatty acids as reported by Hanahan et al. (2) and Nelson and Freeman (1), respectively. Oleic acid, a major component of the preparation of Nelson and Freeman, was a trace constituent of this sample. These discrepancies are almost certainly to be attributed to the presence of some lecithin in the sphingomyelin previously described. In keeping with this view, samples of sphingomyelin prepared from sera on an analytical scale, without removal of alkali-labile phospholipids, always contained 3–5% of oleic and linoleic acids.[3] In addition, the mixture of fatty acids is much more complex than was indicated previously (1, 2), since evidence has been obtained for the presence of C_{15} to C_{25} odd-numbered acids in addition to a number of monoenoic acids. In general, these results agree more closely with the reported compositions of fatty acids from brain spingolipids (23–25), except, of course, that no evidence was found for the presence of hydroxy acids. We have made the tentative assignment of one new fatty acid, a trace component in Fig. 2 and Table 1, as a C_{24} dienoic acid. This component was recovered entirely in the fraction of mercuric acetate adducts. Its retention time on adipate was that of 25:0, while it was eluted from succinate (Fig. 2) later than 25 : 0 but earlier than expected for 25 : 1 (unless the latter were to have an unusually placed double bond). Its carbon number (26) on succinate was 25.33, whereas that expected for 25:1 is 25.44.

[3] E. C. Horning, C. C. Sweeley, B. G. Creech, and M. E. DeBakey, unpublished studies.

The author wishes to acknowledge the competent technical assistance of Mrs. Susan Thorpe, Miss Louise Switkes, and Miss Melissa Haynes.

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