Sphingosine Bases of Normal Human White Matter*

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ABSTRACT: The sphingosine bases of the nonganglioside polar lipids of two areas of human white matter were analyzed by means of gas-liquid chromatography. Sphingosine accounted for 94–99% and dihydrosphingosine 1–5% of the total. Little difference was found between the two areas analyzed, the corpus callosum and the pons-medulla oblongata portion of the brain stem. C_{16} dihydrosphingosine was found at levels up to 2%, and C_{14} dihydrosphingosine was found in trace quantities. No C_{20} dihydrosphingosine or C_{20} sphingosine was present. Other compounds were noted in minor quantities, one of which may be C_{16} sphingosine.

In recent years various investigators have reported the presence in mammalian tissues of long-chain bases other than sphingosine. Dihydrosphingosine was earlier isolated and characterized by Carter *et al.* (1947) from beef brain and spinal cord. Since then Sweeley and Moscatelli (1959) presented evidence for the presence of an unknown base in human plasma sphingolipids; the compound is believed to be a dehydrosphingosine. Proštenik and Majhofer-Oreščanin (1960) found the 20-carbon homolog of sphingosine, or C_{20} sphingosine, in horse and beef brain sphingolipids. Klenk and Gielen (1961) noted its presence in bovine but not in human gangliosides. Quantitative data were reported by Stanacev and Chargaff (1962), who concluded that C_{20} sphingosine (which they named icosisphingosine) is present in approximately the same proportion as sphingosine in bovine mucolipids (ganglioside-like preparations) and that these two compounds account for the large majority of sphingosine base in that source. Most recently, in the course of an analysis of lipid components of gangliosides, Sambasivarao and McCluer (1964) have described both C_{20} sphingosine and C_{20} dihydrosphingosine as components of the gangliosides of several species, including the human. Since these compounds were found in ganglioside but not in other sphingolipid preparations these authors named them gangliosphingosine and dihydrogangliosphingosine.

In this paper we wish to present the results of a study of the distribution of sphingosine bases in nonganglioside polar lipids of two general areas of human white matter with a particular view to detecting the presence of shorter chain homologs of sphingosine and dihydrosphingosine.

Materials and Methods

Dissection of White Matter. The white matter used in these studies was obtained from normal adult human brains which were placed in ice and dissected as soon after autopsy as possible. Two areas of white matter were used, the pons-medulla oblongata portion of the brain stem and the corpus callosum. These were removed by gross dissection, freed of meninges and traces of gray matter, rinsed in cold water, and stored in a freezer at −12°.

Preparation of Polar Lipids. The pons-medulla oblongata structure was sliced carefully along the medial-sagittal plane to provide mirror-image halves, one of which was used for the studies reported here. Such a section generally weighed about 15 g. In the case of corpus callosum material an arbitrary amount approximating 15 g was removed for extraction. Total lipids were obtained by the method of Folch *et al.* (1957), using 0.74% KCl to wash the lipid extract. Gangliosides were thereby removed in the upper phase, which was discarded. Concentration of the ganglioside-free lower phase to dryness was accomplished by heating at 35–40° *in vacuo* using a rotary evaporator. Absolute ethanol and dry benzene were sequentially added and evaporated off to ensure removal of water. After being subjected to high vacuum for 2 hours at 35–40° yields of total lipid were obtained equivalent to 18–23% of the wet weight of white matter.

Approximately 600 mg of lipid was used for the next step, which was separation of polar from neutral lipids by means of a 10- × 2.3-cm silicic acid column. Mallinckrodt No. 2847 silicic acid, 100 mesh, was used, slurried first with chloroform-methanol (4:1), then washed with chloroform until a translucent column was obtained. The lipid was moistened with several drops of chloroform-methanol (2:1), warmed, then dissolved and dispersed into 5 ml of chloroform with the aid of a Teflon pestle in a Potter-Elvehjem homogenizer. After allowing the 5-ml charge to run onto the column, neutral lipids were removed by elution with an excess (about 450 ml) of chloroform. The desired polar lipids were eluted with 250 ml of chloroform-methanol (1:1) followed by 600 ml of methanol. It was found necessary to use pressure and to occasionally stir the top 4–5 cm. of the column. The combined eluates containing

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polar lipids were evaporated to dryness *in vacuo* in the manner described above. Yields representing 49–69% of the total lipid were obtained.

Isolation of Sphingosine Bases and Conversion to Aldehydes. The crude sphingosine base fraction was isolated after methanolysis of the entire polar lipid fraction. Methanolysis and subsequent solvent extraction and silicic acid chromatography steps were performed as described by Sweeley and Moscatelli (1959). The yield of crude sphingosine base from 300 to 400 g of polar lipids varied from 10 to 25 mg. All of the base fraction was converted to aldehydes by the sodium periodate method (Sweeley and Moscatelli, 1959).

Gas-Liquid Chromatographic Analysis. Analyses were performed at 160 and 130°, using an F & M Model 400 instrument equipped with a hydrogen flame ionization detector. A 183-cm × 4-mm (i.d.) glass column packed with 8% diethylene glycol succinate polyester on acid- and solvent-washed, silanized Gas-Chrom P (resieved to 115–150 mesh) was employed. The column packing was prepared by the filtration method (Sweeley *et al.*, 1959). Both the polyester and the Gas-Chrom P were obtained from Applied Science Laboratories, State College, Pa. Helium was used as the carrier gas, at a flow rate of 55 ml/min.

Compositions of the sphingosine base mixtures were calculated on the basis of the area under each aldehyde peak. Areas were determined by the product of the peak height and the width at half-height (Horning *et al.*, 1964). The areas of the peaks representing sphingosine and *O*-methylsphingosine were added together to give a corrected value for sphingosine. Standard retention times were obtained using saturated aldehyde standards as well as authentic samples of triacetylsphingosine and *O*-methylsphingosine and a sample of purified gangliosides known to contain a high percentage of C_{20} sphingosine.

Catalytic Hydrogenation. Aliquots of aldehyde mixtures, corresponding to several mg of total aldehyde, were evaporated to dryness in a stream of nitrogen, dissolved in 3–4 ml of absolute ethanol, and hydrogenated to saturated aldehyde with 2 mg of 10% palladium-charcoal catalyst in a Teflon-lined screw cap tube filled with hydrogen at 1 atm. The hydrogenation mixture was subjected to rapid swirling for 10 minutes using a Vortex Jr. mixer (Scientific Industries, Inc., Queens Village, N.Y.) during which time the temperature rose slightly to 40–45°. Hydrogenation did not quite go to completion in this time but the reaction was deliberately stopped at 10 minutes because a time study revealed the production of alcohols after 20 minutes. The catalyst was removed by filtration on a solvent-washed Celite pad and the filtrate was evaporated to dryness *in vacuo*. The residue was then dissolved in chloroform for gas-liquid chromatography.

Sodium Bisulfite Removal of Aldehydes. Chloroform solutions of up to 10 mg of mixed aldehydes, in some cases containing methyl palmitate as a marker, were evaporated to dryness in a stream of nitrogen, redissolved in 1 ml of methanol, and shaken vigorously with 2 ml of saturated aqueous sodium bisulfite. The water-methanol solution was extracted with two volumes of petroleum ether, the extract (containing methyl palmitate and traces of aldehydes) was filtered and evaporated to dryness in a stream of nitrogen, and the residue was redissolved in chloroform for gas-liquid chromatography.

Results

The sphingosine base composition of seven polar lipid preparations is listed in Table I. Composition in each case is calculated to total 100% without regard to aldehydes individually present at less than 1% of the total of all aldehyde peaks. The total of such minor components rarely exceeded 2%.

TABLE I: Sphingosine Base Composition of Human White Matter.

Source of Polar Lipids	Base Composition[a] C_{16} Dihydrosphingosine (%)	Dihydrosphingosine (%)	Sphingosine (%)
Pons-Medulla Oblongata:			
I[b]	1	3	96
Ia	1	4	95
II[c]	<1	2	98
IV		3	97
V	<1	1	99
Corpus Callosum:			
IICC	2	4	94
IIICC	1	5	94

[a] Compositions are calculated to total 100% without regard to components present at less than 1%. Where such components were clearly present as traces they are reported as <1%. [b] Roman numerals refer to individual brains; CC indicates corpus callosum. I and Ia represent the same sphingosine base preparation. [c] This preparation contained a trace of C_{20} sphingosine, i.e., less than 1%.

Typical chromatograms at 130 and 160° are shown in Figure 1. All peaks are numbered which were well defined in the appropriate chromatograms and which therefore appear to be relevant to sphingosine base composition.

Semilogarithmic plots of retention time versus chain length were used to partially identify the saturated aldehydes representing the dihydrosphingosine series of bases. In all chromatograms examined, the retention times for peaks 1, 3, and 6 yielded a straight line identical

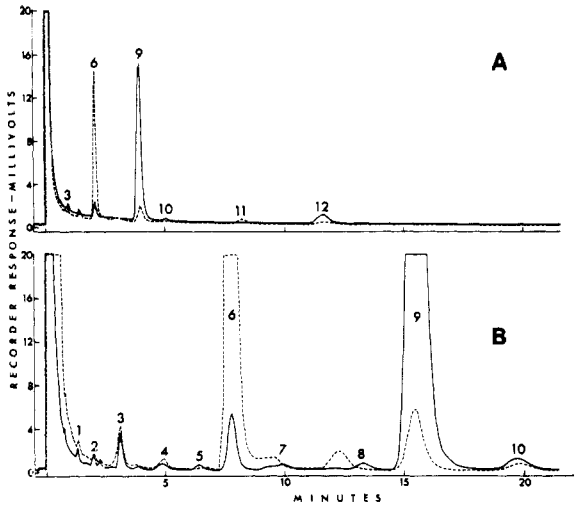

FIGURE 1: Gas-liquid chromatographic analyses of sphingosine bases as aldehydes derived from periodate oxidation. Analyses on an 80-cm × 4-mm id column packed with 8% diethylene glycol succinate polyester on 115–150 mesh acid- and solvent-washed silanized Gas-Chrom P. Helium flow rate, 55 ml/min. Temperatures: chromatogram A, 160°, chromatogram B, 130°. Dotted line represents analysis after catalytic hydrogenation. Peak identification: 1, dodecanal; 2, unidentified; 3, tetradecanal; 4, unidentified; 6, hexadecanal; 8, unidentified; 9, hexadecenal; 10, unidentified; 11, *O*-methylhexadecanal; 12, *O*-methylhexadecenal. Peaks 5 and 7 may represent C_{12} and C_{14} homologs of hexadecenal.

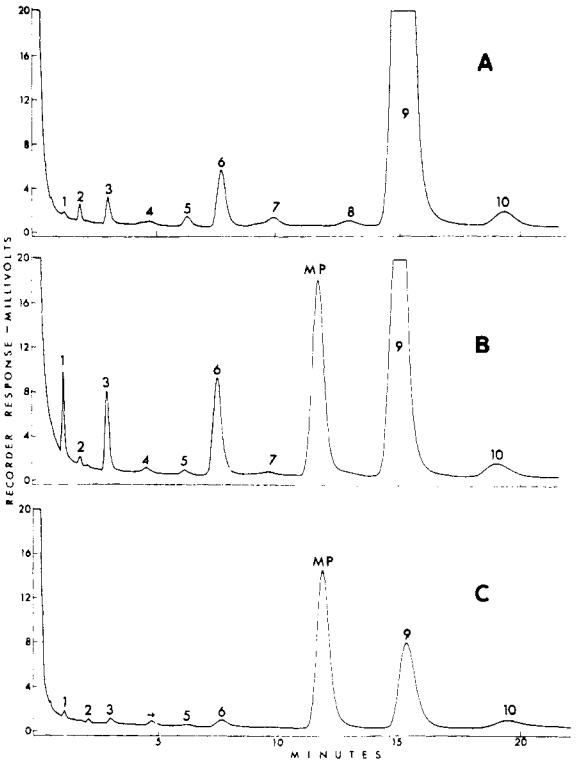

FIGURE 2: Gas-liquid chromatographic analyses of sphingosine bases as aldehydes derived from periodate oxidation. Column as in Figure 1. Helium flow rate, 55 ml/min., temperature, 130°. Chromatograms: A, typical mixture of aldehydes; B = A after addition of dodecanal, tetradecanal, hexadecanal, and methyl palmitate (MP); C = B after shaking with aqueous sodium bisulfite. Peak identification is same as in Figure 1.

with one obtained using authentic dodecanal, tetradecanal, and hexadecanal. The retention time for the saturated aldehyde obtained from gangliosides containing the C_{20} sphingosine bases also fell on the straight line and was, as expected (Sambasivarao and McCluer, 1964), identical with that of authentic octadecanal.

The retention times at 130° for peaks 5, 7, and 9 were plotted to give a straight line nearly parallel to that obtained for the saturated aldehydes. In this case, however, the homologs were indicated to differ by only one carbon. Peaks 5 and 7 would therefore be expected to represent tetradecenal and pentadecenal, respectively. Appropriate unsaturated aldehydes were not available to confirm the existence of this series. The retention times for peaks 4 and 10 did not fit any of these straight lines.

Relative retention times calculated relative to hexadecanal were also used to identify unknown peaks. When aldehydes were obtained from authentic triacetylsphingosine and *O*-methylsphingosine, gas-liquid chromatography yielded peaks with relative retention times identical with those of peaks 9 and 12. Table II shows the relative retention times at 160° of the various peaks identified, with the exception of dodecanal, which occurred too close to the large solvent peak for optimal measurement. The dodecanal peak, although present in almost all preparations chromatographed at 130°, was generally too small to allow accurate measurement of area.

It should also be noted that addition of authentic dodecanal, tetradecanal, and hexadecanal to a typical mixture of unknown aldehydes, followed by rechromatography, caused peaks 1, 3, and 6 to increase in height with no distortion. This is demonstrated in chromatograms A and B of Figure 2.

The relative retention times for hexadecenal and octadecanal (see Table II) are sufficiently different in this gas-liquid chromatography system to allow differentiation between sphingosine and C_{20} dihydrosphingosine (separation factor = 1.14). Although no C_{20} dihydrosphingosine was found in any of the white matter preparations, this compound accounted for approximately 2% of the sphingosine bases in human gangliosides, thus confirming the data Sambasivarao and McCluer (1964) obtained by permanganate studies.

Further evidence toward identification of the various peaks of interest was provided by catalytic hydrogenation of the aldehyde mixtures followed by identification

TABLE II: Relative Retention Times.[a]

Sphingosine Base	Aldehyde	Relative Retention Time[b]
C_{16} dihydrosphingosine	Tetradecanal	0.47
Dihydrosphingosine	Hexadecanal	1.00
Sphingosine	Hexadecenal[c]	1.90
C_{20} dihydrosphingosine	Octadecanal	2.17
O-Methyldihydrosphingosine	*O*-Methylhexadecanal	3.95
C_{20} sphingosine	Octadecenal	4.04
O-Methylsphingosine	*O*-Methylhexadecenal	5.70

[a] Gas-liquid chromatography on an 80-cm × 4-mm id column packed with 8% diethylene glycol succinate polyester on 115–150 mesh acid- and solvent-washed silanized Gas-Chrom P. Helium flow rate, 55 ml/min. Temperature, 160°. [b] These are average values from 4 to 15 chromatograms. Variation was ±1.0% or less in all cases except that of *O*-methylhexadecenal, whose relative retention time varied ±1.7%. [c] Compounds are not further specified as to location of double bond or location of *O*-methyl substituent because this column does not discriminate among positional isomers.

of the saturated aldehydes by gas-liquid chromatography. Comparison of chromatograms obtained before and after hydrogenation strengthened identifications made by the retention time studies. The dotted lines in Figures 1A and 1B represent analyses after hydrogenation of the mixture represented by the solid line. The persistence or increase in size of peaks 1, 3, and 6 is consistent with their identification as saturated compounds, while the decreases in size of peaks 5, 7, and 9 is consistent with unsaturation. As expected, peak 9 (hexadecenal) was converted almost entirely to peak 6 (hexadecanal). Only a small decrease was noted in the height of peak 10.

In an attempt to deal with the possibility that minor peaks in aldehyde mixtures produced by the methanolysis-periodate oxidation method might be persistent traces of fatty acid esters, experiments were conducted in which a typical unknown mixture was supplemented with a "marker" ester plus known aldehydes, then shaken with aqueous sodium bisulfite to remove aldehydes without removing esters. The results of such an experiment are shown in Figure 2. As previously noted, addition of authentic aldehydes caused an increase in height but no distortion of peaks 1, 3, and 6 (Figure 2B). The new peak between peaks 7 and 8 represents methyl palmitate, added in such quantity as to yield a peak approximately the same height as the aldehyde peaks. After treatment with sodium bisulfite, the solvent extract of the reaction mixture was concentrated and analyzed at a level which yielded a methyl palmitate peak of approximately the same magnitude as in Figure 2B. It is apparent from Figure 2C that under these circumstances peaks 1, 3, and 6 were decreased to less than 5% of the height of the methyl palmitate peak. The relative decrease in peak 10 was not significant.

Discussion

It is clear that in nonganglioside polar lipids of human white matter sphingosine itself accounts for the great majority of the sphingosine base composition, while dihydrosphingosine accounts for 1 to 5% of the total. These are very similar to the results obtained by Sambasivarao and McCluer (1964) for a similar lipid preparation from human gray matter. There appears to be little difference in sphingosine base composition between the two areas of white matter studied.

Small quantities of homologs of dihydrosphingosine are present in the lipids studied. Since the gas-liquid chromatography column used clearly distinguishes between sphingosine and C_{20} dihydrosphingosine it is possible to state that none of the latter compound was found in any of the chromatograms. On the other hand the shorter-chain homologs were generally found present in diminishing quantities. C_{16} dihydrosphingosine is present in a small quantity not exceeding 2% of total sphingosine bases present, as can be seen from the data presented in Table I. Figures 1 and 2 reveal the characteristic presence of a trace amount of C_{14} dihydrosphingosine.

A trace of the C_{16} homolog of sphingosine also appears to be present in nonganglioside lipids of human white matter. Although the retention time for peak 7 is consistent with the retention time expected for pentadecenal, the existence of C_{17} sphingosine in mammalian systems is unlikely enough so that more data are required before its presence can be inferred.

These data may help to elucidate the specificity of the enzyme system presumably responsible for the biosynthesis of dihydrosphingosine and sphingosine (Brady and Koval, 1958). Results are compatible with the observation by Radin and Akahori (1961) that cerebroside sphingosine base samples from four different sections of human brain consisted of 95% or more sphingosine and about 2% dihydrosphingosine. These investigators also noted the presence of a trace of an unknown sphingosine base. In the present study peaks 2 and 8 may represent traces of unknown sphingosine bases according to the hydrogenation and sodium bisulfite treatment data. Peaks 4 and 10 are unaccounted for. They are not significantly affected by the hydrogenation and sodium bisulfite procedures, yet do not meet retention time criteria for any of the normal series of saturated fatty acid methyl esters. More rigorous methods will be required for the identification of these peaks.

It should be noted that the gas-liquid chromatography column used in this investigation does not discriminate among positional isomers. This is evident from the presence of a single *O*-methylsphingosine peak, whereas

it has recently been reported that methanolysis under similar conditions results in the formation of four stereoisomers (Weiss, 1964).

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8,9,13-Triacetoxydocosanoic Acid, an Extracellular Lipid Produced by a Yeast*

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ABSTRACT: An extracellular lipid produced by the yeast NRRL YB-2501 was shown to be the triacetate of 8,9,13-trihydroxydocosanoic acid.

The structure of the trihydroxy acid was established by its conversion to suberic acid and 5-ketotetradecanoic acid.

In previous publications (Wickerham and Stodola, 1960; Stodola and Wickerham, 1960; Stodola *et al.*, 1962; Maister *et al.*, 1962) we described the formation of the extracellular lipids tetraacetylphytosphingosine and triacetyldihydrosphingosine by the yeast *Hansenula ciferrii*. When this work was extended to other species, we found that a new yeast NRRL YB-2501 produced more than a gram of extracellular lipd per liter of culture liquor. An examination of this lipid has shown it to be composed largely of 8,9,13-triacetoxydocosanoic acid (compound I). In this paper we report the production, isolation, characterization, and determination of the structure of this compound.

The species used in this work is a yeast or yeastlike organism of indefinite taxonomic position. It was isolated from frass of white spruce, *Picea glauca* (Moench) Voss. A description of the species will be published elsewhere.

The lipid was produced in a yield of 1.42 g/liter by growing the yeast for 7 days at 25° in shaken flasks on a medium containing 5% malt extract and 1% glucose. The crude product, obtained as an oil by extraction of the culture liquor with hexane, was shown by thin-layer chromatography to consist largely of a single component. Silicic acid chromatography gave a pure liquid having the composition $C_{28}H_{50}O_8$, the infrared absorption spectrum of which showed strong bands at 5.75 (ester or δ-lactone) and 5.85 μ (carboxyl). Further bands at 3.45 and 6.85–6.96 μ corresponded to CH and CH_2 stretching frequencies in saturated aliphatic compounds, and a 7.30 μ band indicated the presence of $C-CH_3$ groups.

Saponification of the pure C_{28} acid yielded a $C_{22}H_{44}O_5$ acid (compound II) (mp 157–158°) showing strong infrared absorption bands at 3.15 (OH) and 5.85 μ (COOH). Hydroxyl analyses of the C_{22} acid and the loss of six carbons on saponification suggested that the

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