# Lipid components of gangliosides

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SUMMARY The fatty acids and the long-chain bases of gangliosides from several sources were analyzed by means of gas-liquid chromatography. Stearic acid was found to be the major fatty acid component while palmitic and arachidic acids were found in small amounts, confirming previous observations. Hydroxy acids were not found in any significant amounts. In addition to sphingosine and a small amount of dihydrosphingosine, two additional bases containing 20 carbon atoms each were found. Since these $C_{20}$ bases were found only in gangliosides and not in other sphingolipids, the names gangliosphingosine and dihydrogangliosphingosine are proposed.

GANGLIOSIDES, WHICH were first isolated by Klenk (1), contain sphingosine, fatty acids, glucose, galactose, N-acetylgalactosamine, and N-acetylneuraminic acid (NANA) (2). Several structures have been proposed for this group of glycolipids (3–6). Stearic acid has been reported as the major fatty acid in all samples examined (1, 7, 8). The presence of a long-chain base with 20 carbon atoms in addition to the normal sphingosine in mucolipids from bovine brain has been reported by Stanacev and Chargaff (9). This $C_{20}$-sphingosine, which was first described by Proštenik and Majhofer-Oreščanin (10) as a component of horse and beef brain lipids, was reported by Klenk and Gielen (11) to be present in gangliosides from beef brain but not in gangliosides from human brain.

The present investigation concerns the nature of the lipid components of gangliosides. Data on the fatty acid composition, with particular reference to the minor components, are presented. Evidence for the occurrence of $C_{20}$ homologues of dihydrosphingosine and sphingosine in gangliosides from several species is given. The absence of the $C_{20}$ bases in non-ganglioside sphingolipids from brains of several species is also demonstrated.

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## METHODS

*Preparation of Gangliosides.* Lyophilized brains (mostly gray matter in the case of human tissues) or acetone powders of other brains were twice extracted with 95% acetone followed by two or more extractions with diethyl ether to remove most of the neutral lipids and phospholipids. The residue was then extracted with chloroform–methanol 1:2 (all solvent ratios given are v/v) in a Soxhlet apparatus. After removal of the solvent, the lipids were subjected to a 4-tube double withdrawal countercurrent distribution using chloroform–methanol–0.1% aqueous NaCl 8:4:3 (12). Resorcinol assay indicated only trace amounts of gangliosides in the lower phase. The combined upper layers, which were concentrated to a small volume under vacuum at low temperature, were dialyzed against distilled water and passed through a Dowex 50 W-X1 ($H^+$) column. The eluate from the resin column was lyophilized to obtain a white powder. When the phosphorus content of the product was higher than 0.15%, the material was redistributed as before with Folch solvents using 3 tubes. When fresh brain tissues were used, the phosphorus content was found to be low. Sulfur was not detected by a qualitative test and fatty acid ester was not detectable by the hydroxamic acid test (13) in a typical preparation from human brain. On the basis of phosphorus content and analysis by thin-layer chromatography (TLC) (14), the preparations were estimated to be better than 95% pure. Gangliosides were isolated by this procedure using as little as 20 g (wet tissue) of formalin-fixed human brain (Tay-Sachs). Calf brain gangliosides were further purified by precipitating them as barium salts (15). To obtain phosphorus-free gangliosides, a human brain preparation in pyridine was placed on an alumina column and eluted with pyridine. When no more ganglioside was eluted, the solvent was changed to methanol to obtain a second fraction of gangliosides.

Phosphorus was estimated by a perchloric acid oxidation procedure (16). A resorcinol method (17) as modified

TABLE 1 FATTY ACID COMPOSITION OF GANGLIOSIDES

| Ganglioside Source | NANA | P | Ganglioside Fatty Acids | | |
|---|---|---|---|---|---|
| | | | Palmitic | Stearic | Arachidic |
| | % | % | % | % | % |
| Human brain (mostly grey matter) | | | | | |
| I | 30.9 | 0.13 | 1.0 | 89.2 | 9.8 |
| Ia* | 26.6‡ | 0.05 | 0.9 | 88.8 | 10.3 |
| Ib† | 21.0‡ | 0.03 | 1.9 | 85.9 | 12.2 |
| II | 30.0 | 0.12 | 1.4 | 88.1 | 10.5 |
| Human brain (Tay-Sachs) | 19.6 | 0.38 | 1.7 | 93.1 | 5.2 |
| Calf brain | 29.2 | 0.08 | 2.5 | 95.2 | 2.3 |
| Rabbit brain | 33.1 | 0.08 | 1.3 | 92.0 | 6.7 |
| Dog brain | 31.3 | 0.14 | 1.6 | 91.7 | 6.7 |
| Rat brain | 29.6 | 0.20 | 2.0 | 88.8 | 9.2 |

* Pyridine eluate from alumina column.
† Some NANA is cleaved from the ganglioside by the alumina column.
‡ Methanol eluate from alumina column.

by Miettinen and Takki-Luukkainen (18) was used to determine NANA.

*Preparation of Non-Ganglioside Sphingolipids.* These were obtained by evaporation of the lower phase obtained during the ganglioside preparation. Analysis of a human brain lower phase material on TLC according to Muldner, Wherrett, and Cumings (19) showed sphingomyelins, cerebrosides, and cerebroside sulfate esters among the major products.

*Preparation of Methyl Esters of Fatty Acids from Gangliosides.* Gangliosides were methanolized with methanol–sulfuric acid 95:5 under reflux for 8 hr (10 mg gangliosides/ml). Fatty acid esters were extracted from the hydrolysate with petroleum ether. The petroleum ether extract was washed with water and dried over anhydrous sodium sulfate, and the solvent was removed at reduced pressure.

*Separation of Hydroxy Esters from Normal Fatty Acid Esters.* Hydroxy esters were separated from normal esters on Florisil (Floridin Co., Tallahassie, Florida) (20). Elution with Skellysolve B was continued until no further material was obtained from the column. Hydroxy esters were then eluted with Skellysolve B–diethyl ether 9:1.

*Isolation of Sphingosine Bases from Gangliosides and Sphingolipids.* Gangliosides were hydrolyzed with 2 N HCl in methanol under reflux for 8 hr and sphingosine bases isolated and purified by silicic acid chromatography (21). Sphingosine bases were prepared from non-ganglioside sphingolipid fractions after refluxing with methanol–sulfuric acid 95:5 for 8 hr.

*Conversion of Sphingosine Bases into Aldehydes and Fatty Acid Esters.* Sphingosine bases were converted to aldehydes by a sodium periodate oxidation method (21). For conversion to fatty acids, 20–30 mg of the bases were dissolved in 1 ml glacial acetic acid and 40–60 mg of powdered $KMnO_4$ was added. After standing at room temperature with occasional shaking for 1 hr, 5 ml of xylene was added and the solvents were removed at 45° under vacuum in a rotary evaporator. Equal volumes of 10% sodium bisulfite and 2 N $H_2SO_4$ were added to the residue and the fatty acids were extracted with ethyl ether. The ether extract was washed with water and dried over anhydrous sodium sulfate, and the solvent was removed. Fatty acids were then converted into methyl esters with methanol–boron trifluoride (22).

*Hydrogenation.* Hydrogenation of aldehydes and sphingosine bases was carried out in an ether solution at room temperature at 10 psi for 1 hr using 5% palladium on charcoal as catalyst.

*Gas-Liquid Chromatography (GLC).* Methyl esters of fatty acids were analyzed at 200° with an F and M Model 500 instrument equipped with a flame ionization detector and a 10 ft × 1/4 in. o.d. column (F and M Scientific Corp., Avondale, Pa.) packed with 20% LAC 728 coated on 30–60 mesh Chromosorb P. The instrument used for the analyses of aldehydes was an Aerograph A-350-B fitted with a thermal conductivity detector. A 10 ft × 1/4 in. o.d. column (Applied Science Laboratories, State College, Pa.), packed with 10% ethylene glycol succinate polyester on 60–80 mesh Chromosorb W, was employed. Analyses were carried out at 180° with a helium pressure of 60 psi and a flow rate of 100 ml/min.

*Thin-Layer Chromatography (TLC).* Sphingosine bases were separated by TLC using a chloroform–methanol–2 N ammonia system described previously (23). Hydroxy esters were separated from normal esters with a petroleum ether–diethyl ether solvent (24).

## RESULTS

*Fatty Acid Composition.* The fatty acid composition as well as the phosphorus and NANA contents of the various preparations are shown in Table 1. In order to determine whether the palmitic acid was contributed by the impurities in the gangliosides, one of the human preparations was fractionated on an alumina column as described under Methods. Even though the phosphorus content was considerably reduced, the fatty acid composition remained essentially the same. Thin-layer chromatography of fatty acid methyl esters (24) indicated the presence of small amounts of hydroxy esters. Methyl ricinoleate and fatty acid esters of a sphingolipid preparation (known to contain hydroxy esters) were used as standards. In this system, monohydroxy esters have an $R_F$ value of 0.2.

In order to isolate the hydroxy esters, approximately 340 mg of methyl esters (pooled from several hydrolysates

of human and calf brain gangliosides) were separated on a Florisil column (20). Approximately 31 mg of a hydroxy ester fraction, contaminated with normal esters, was obtained. This fraction was again fractionated on a smaller column to obtain approximately 10 mg of a hydroxy ester fraction, which still contained some normal esters. In addition, several minor spots above and one below the hydroxy ester spot were observed on TLC. These were not identified. It can, however, be concluded that the fatty acid esters of gangliosides contain not more than 1–2% hydroxy esters.

*Characterization of Sphingosine Base Fraction.* The sphingosine bases obtained by ethyl ether extraction of the sphingolipid hydrolysates were examined by TLC (23). Spots corresponding to 3-O-methyl-, *erythro*-, and *threo*sphingosine were observed in the samples derived from gangliosides. In samples from non-ganglioside sphingolipids, a spot corresponding to dihydrosphingosine could also be discerned. Small quantities of contaminating fatty acid esters were removed from these preparations by chromatography on silicic acid (21) prior to gas chromatographic examination.

(a) *GLC of periodate oxidation products:* The aldehydes obtained after periodate oxidation of sphingosine bases from non-ganglioside sphingolipids and gangliosides were analyzed by GLC and typical recordings are shown in Figs. 1 and 2. *trans*-2-Hexadecenal and *trans*-2-octadecenal, derived from sphingosine and $C_{20}$-sphingosine, respectively, were identified after hydrogenation by comparison with known saturated aldehydes. The aldehyde derived from the oxidation of 3-O-methylsphingosine was identified with a pure sample of 3-O-methylsphingosine prepared previously (23). The large amount of 2-methoxy aldehyde obtained from non-ganglioside sphingolipids (Fig. 1) as compared to that obtained from gangliosides (Fig. 2) is due to the use of methanol–sulfuric acid 95:5 and 2 N HCl in methanol, respectively, for hydrolysis. One of the two unidentified peaks (peak 4 in Fig. 2) found in the case of gangliosides disappeared upon hydrogenation and corresponded to the unidentified base of Sweeley and Moscatelli (21). They believed that this may be a base that is more unsaturated than sphingosine. As can be seen from Fig. 2, hexadecenal and octadecanal (peaks 3 and 7) have closely similar retention times and cannot be separated. Hence a saturated base with 20 carbon atoms cannot be identified in this system. However, permanganate oxidation studies discussed later afforded good evidence for its occurrence. The compositions of the sphingosine bases of human and calf brain gangliosides as analyzed by GLC are given in Table 2. The values for sphingosine include sphingosine and any saturated base with 20 carbon atoms, since the aldehydes from these two compounds were not separated. A small amount of dihydrosphingosine was found in gangliosides. Sphingosine and $C_{20}$-sphingosine occur in approximately equal proportions in human brain gangliosides. The bases from the non-ganglioside sphingolipids of human and calf brain did not contain $C_{20}$-sphingosine.

(b) *Permanganate oxidation studies:* In order to locate the double bond, sphingosine bases from human and calf brain lipids were oxidized with permanganate and the resulting fatty acids were analyzed by gas chromatography. The results could not be quantified since the fatty acids were somewhat further degraded to lower

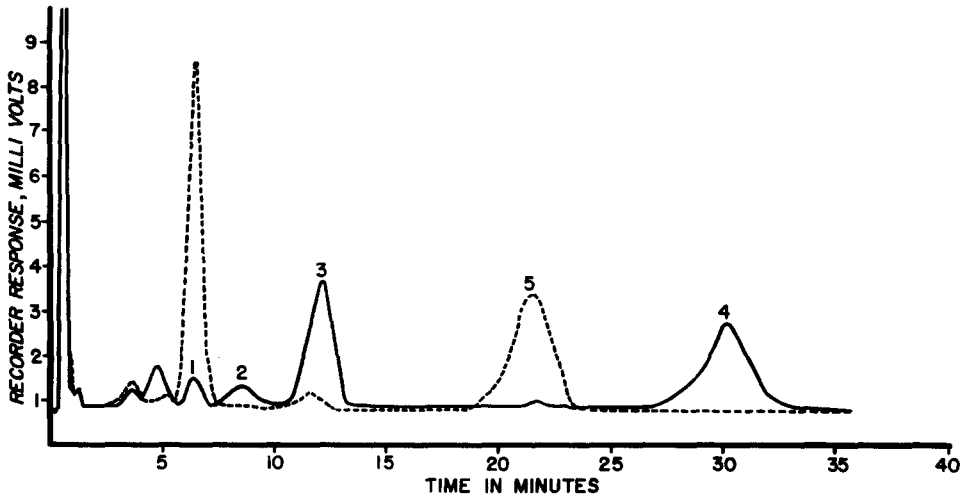


FIG. 1. Gas chromatographic analysis of the aldehydes derived from the periodate oxidation of sphingosine bases of human brain sphingolipids. Dotted line represents the analysis after hydrogenation of aldehydes. Peak identification: 1, hexadecanal; 2, unidentified; 3, hexadecenal; 4, 2-methoxy heptadecenal; 5, 2-methoxy heptadecanal.

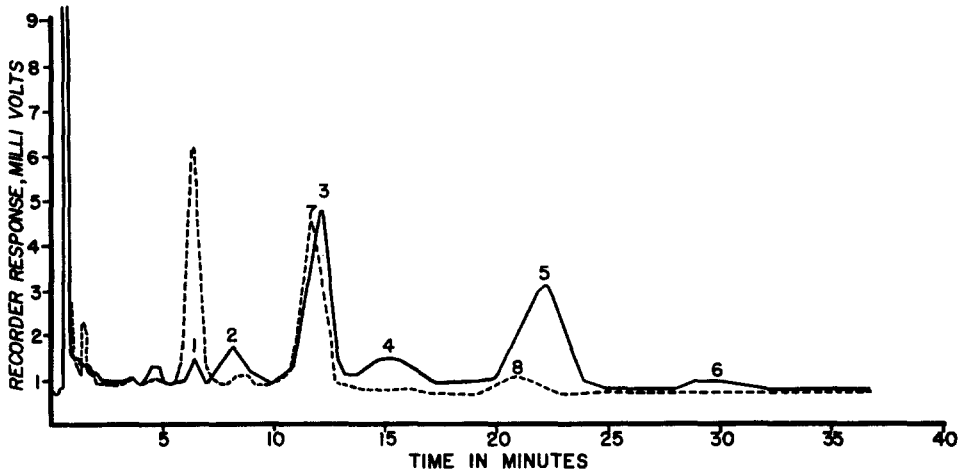


FIG. 2. Gas chromatographic analysis of the aldehydes derived from the periodate oxidation of sphingosine bases of human brain gangliosides. Dotted line represents the analysis after hydrogenation of aldehydes. Peak identification: 1, hexadecanal; 2, unidentified; 3, hexadecenal; 4, unidentified; 5, octadecenal; 6, 2-methoxy heptadecenal; 7, octadecanal; 8, 2-methoxy heptadecanal.

fatty acids. The sphingolipids showed myristic acid as the major product along with smaller amounts of palmitic acid. If the sphingosine bases were hydrogenated prior to oxidation, palmitic acid was found to be the major product. The ganglioside preparations showed myristic and palmitic acids (in approximately equal proportions) as the major products of oxidation of the bases. Palmitic acid is the expected product from both dihydrosphingosine and $C_{20}$-sphingosine. In addition to myristic and palmitic acids, a small amount (approximately 1–2%) of stearic acid, which would be produced from $C_{20}$-dihydrosphingosine, was found. If the sphingosine bases from gangliosides were hydrogenated prior to oxidation, palmitic and stearic acids were obtained.

(c) *Studies on the* erythro- *and* threo*sphingosine fractions:* Approximately 49 mg of the sphingosine bases isolated from human brain gangliosides were separated on a series of TLC plates (23). After exposing the plates to iodine vapor for less than 1 min, the spots corresponding to *erythro* and *threo* bases were scraped off the plates and eluted with diethyl ether, and the solvent was removed to yield 20 and 13 mg respectively. Permanganate oxidation and gas chromatography revealed that both fractions contained sphingosine and $C_{20}$-sphingosine. Both fractions showed infrared absorption peaks in the 10.3 $\mu$ region.

*Distribution of $C_{20}$-Sphingosine in Various Lipids.* The GLC analysis of the permanganate oxidation products of sphingosine bases was extended to lipids from several other sources. Gangliosides and non-ganglioside sphingolipids from a Tay-Sachs brain (formalin-fixed), as well as from dog, rat, and rabbit brains, were analyzed. In all cases, the ganglioside preparations yielded myristic and palmitic acids in approximately equal proportions as the major products, indicating sphingosine and $C_{20}$-sphingosine as major components of all gangliosides examined. Stearic acid was seen as a minor product of all ganglioside preparations except the rabbit brain gangliosides. In no case was evidence obtained for the presence of $C_{20}$-sphingosine or $C_{20}$-dihydrosphingosine in non-ganglioside sphingolipids.

TABLE 2 GLC ANALYSIS OF THE SPHINGOSINE BASES OF GANGLIOSIDES AS ALDEHYDES*

| Ganglioside Source | Unidentified† | Sphingosine‡ | Dihydrosphingosine | $C_{20}$-Sphingosine |
|---|---|---|---|---|
| Human brain (mostly grey matter) | 13 | 41 | 3 | 43 |
| Calf brain | 15 | 48 | 3 | 34 |

* Expressed as percentage of total aldehydes.

† This includes "dehydrosphingosine" and the other unidentified peak.

‡ Figures represent the sum of sphingosine and 3-O-methylsphingosine peaks.

## DISCUSSION

Klenk originally identified stearic acid as the fatty acid component of human gangliosides (1) and more recently has reported (7) the following fatty acid composition by GLC: palmitic 1%, stearic 94%, arachidic 4%, and behenic 1%. Trams, Giuffrida, and Karmen (8) extended these studies to include several other species and reported similar results except for minor amounts of $C_{24}$ acids in non-human preparations. These authors did not report palmitic acid as a component of human gangliosides. In the present investigations, components comprising less than 1% of the total

fatty acids were not considered significant. In general, previous studies were confirmed and extended to include rabbit, dog, and rat gangliosides. These data indicate that palmitic, stearic, and arachidic acids are components of all gangliosides tested, including human. Hydroxy fatty acids were found to comprise approximately 1% of the total fatty acid fractions; however, since the fatty acid yield of cerebrosides is much greater than that of gangliosides, as little as 1% cerebroside impurity in the ganglioside preparations could have contributed the hydroxy esters. Recently Kishimoto and Radin (25) have reported the absence of hydroxy acids in rat gangliosides.

Stanacev and Chargaff (9) detected a small amount of hexadecanal, and nearly equal quantities of hexadecenal and octadecenal, as periodate oxidation products of sphingosine bases derived from ox brain mucolipid. Supporting evidence for the presence of sphingosine and $C_{20}$-sphingosine was provided by the detection of myristic and palmitic acids following oxidation at the double bond. In the present studies, very similar results were obtained with human and calf brain gangliosides. In addition to the aldehydes mentioned above, the products from the 3-O-methyl derivatives of sphingosine and $C_{20}$-sphingosine were observed. Two unidentified peaks were noted, one of which corresponded to the peak described by Sweeley and Moscatelli (21) as probably resulting from dehydrosphingosine. Following permanganate oxidation, a small amount of stearic acid was detected in addition to previously reported products. The detection of stearic acid (before hydrogenation) provides evidence for the occurrence of $C_{20}$-dihydrosphingosine, a base not previously reported.

Analysis by TLC of the sphingosine bases revealed spots corresponding to 3-O-methyl-, *erythro*-, and *threo*-sphingosine. It was demonstrated that $C_{20}$-sphingosine was a component of the *erythro* and *threo* spots and thus is not separated from sphingosine in this TLC system. By analogy with sphingosine, it can be concluded that $C_{20}$-sphingosine probably has an *erythro* configuration and that the *threo* isomer was produced during hydrolysis. Majhofer-Oreščanin and Proštenik (26) prepared the four optically active isomers of the *trans* series of $C_{20}$-sphingosine and compared them with $C_{20}$-sphingosine, which they had found earlier in horse and beef brain lipids (10, 27). On the basis of chemical reactions, physical properties, and infrared spectra, they tentatively assigned a *trans-erythro* configuration to natural $C_{20}$-sphingosine. The infrared spectra and TLC behavior of our samples provide additional supporting evidence for the *trans-erythro* structure.

Because $C_{20}$-sphingosine was first reported as a component of horse and bovine brain lipids (10) and later as a component of bovine gangliosides but not human gangliosides (9, 11), it appeared that its occurrence might be species-specific. However, analyses of gangliosides and non-ganglioside sphingolipids from

TABLE 3 DISTRIBUTION OF $C_{20}$-SPHINGOSINE IN VARIOUS LIPIDS

| Lipid Fraction | Tissue | Species | $C_{20}$-Sphingosine | Reference |
|---|---|---|---|---|
| 1. Gangliosides | Brain | Human* | Present | This work |
| " | " | Human (Tay-Sachs) | " | " " |
| " | " | Calf | " | " " |
| " | " | Rabbit | " | " " |
| " | " | Dog | " | " " |
| " | " | Rat | " | " " |
| "Mucolipid" | Brain | Ox | Present | (9) |
| 2. Sphingolipids (non-ganglioside) | Brain | Human* | Absent | This work |
| " " | " | Human (Tay-Sachs) | " | " " |
| " " | " | Calf | " | " " |
| " " | " | Rabbit | " | " " |
| " " | " | Dog | " | " " |
| " " | " | Rat | " | " " |
| "Sphingolipid" | Lung | Bovine | Absent | (31) |
| 3. Cerebrosides | Brain | Human | Absent | (29) |
| " | Spinal cord | Bovine | " | (21) |
| 4. Cytolipin H | Epidermoid carcinoma | Human | Absent | (30) |
| 5. Phosphatides | Plasma | Human† | Absent | (21) |
| " | Intestinal mucosa | Bovine | " | " |
| " | Brain | Bovine | " | " |
| " | Soy bean | Soy bean | " | " |
| 6. Sphingomyelin | Plasma | Human‡ | Absent | (21) |
| 7. Cerebrin | Yeast | *Torulopsis utilis* | Absent | (32) |

* Normal.
† Four normals.
‡ Normal, atherosclerotic, and Niemann-Pick disease, all female.

several species now indicate that the $C_{20}$-sphingosine in these samples is a specific component of gangliosides and does not occur in detectable amounts in the non-ganglioside sphingolipids. The lipid fractions of horse and beef brain, in which $C_{20}$-sphingosine was first found (10), were obtained by a hot alcohol extraction procedure (28), which would extract gangliosides as well as other sphingolipids. Since beef brain sphingolipids obtained by Sweeley and Moscatelli (21) after washing with Folch solvents did not contain $C_{20}$-sphingosine, it can be reasonably concluded that the $C_{20}$-sphingosine in horse and beef brain lipids is contributed by the gangliosides. Other studies, in which sphingosine bases from non-ganglioside sphingolipids have been examined by GLC (21, 29–31), have not detected $C_{20}$-sphingosine. $C_{20}$-Phytosphingosine has been detected in yeast cerebrin (21, 32). The occurrence of $C_{20}$-sphingosine in various sources as reported by these studies and the present study is listed in Table 3.

It is thus postulated from presently available information that $C_{20}$-sphingosine (D-*erythro*-2-amino-1,3-dihydroxy-4-eicosene) and $C_{20}$-dihydrosphingosine (D-*erythro*-2-amino-1,3-dihydroxyeicosane) are specific components of gangliosides. Although Stanacev and Chargaff have used the term icosisphingosine (9), the common name gangliosphingosine, in analogy with phytosphingosine, is proposed for $C_{20}$-sphingosine in order to indicate its specific occurrence. The interesting observation that gangliosides contain only saturated fatty acids and a specific long-chain base may prove useful in elucidation of the structural and metabolic role of these lipids.

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