

Changes in sphingosine and fatty acid components of the gangliosides in developing rat and human brain

ABRAHAM ROSENBERG* and NORA STERN

Departments of Biochemistry and Medicine, College of Physicians and Surgeons, and the Columbia University Research Service, Goldwater Memorial Hospital, Welfare Island, New York, N. Y.

ABSTRACT Rat brain increases in weight after birth in three stages: (I) rapidly for the first 2 weeks, (II) at a lower rate from 2 to 5 weeks, and (III) at a still lower rate from 5 weeks to 5 months. During the succeeding period, designated IV, it maintains constant weight up to 1 year of age. Brain ganglioside content increased linearly during I and II, more slowly during III, and diminished during IV. The appearance of measurable amounts of brain sphingomyelin and cerebroside succeeded that of ganglioside.

Ceramide with C₁₃-sphingosine and C₁₈ fatty acid was found in a large proportion of all three sphingolipids upon their first appearance in measurable quantity. C₁₈ fatty acid in cerebroside rapidly declined to a negligible level, while in gangliosides and sphingomyelin it declined slowly but remained the major fatty acid component.

Cerebroside and sphingomyelin contained C₁₈-sphingosine almost exclusively at all stages of rat brain growth. Gangliosides contained C₁₈-sphingosine almost exclusively at birth, but subsequently accumulated C₂₀-sphingosine until they had nearly equal quantities of each base type.

Changes in human brain gangliosides resemble those in rat. In Tay-Sachs disease, gangliosides have C₁₈-sphingosine predominantly, and a high content of C₁₈ fatty acid.

KEY WORDS brain · age · gangliosides · cerebroside · sphingomyelin · content · fatty acids · C₂₀-sphingosine · rat · human · Tay-Sachs disease · methanolysis

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Abbreviations: TLC, thin-layer chromatography; GLC, gas-liquid chromatography. Solvent ratios, unless otherwise stated, are volume ratios.

THE SIALIC ACID-CONTAINING glycosphingolipids of neural cells are variously termed gangliosides, strandin, or mucolipids (1). Their metabolic role is largely unknown. Changes have been reported to occur in the sialic acid content of the gangliosides of maturing human brain (2). No study has been made of the lipid portions of the gangliosides of the brain during maturation.

This report presents data which indicate that there is a progressive alteration in the nature of the fatty acid and sphingosine portions of the gangliosides isolated from rat and human brains of increasing age. The results of a study of the lipid portions of the cerebroside and sphingomyelin fractions of developing rat brain are also given to provide points of comparison between changes observed in the gangliosides and those occurring simultaneously in other brain sphingolipids. The sulfatides, which comprise a small portion of the glycosphingolipids of rat brain, were not studied.

The fatty acids of the brain are described by some workers as consisting chiefly of stearic acid (3, 4), and by others as comprising palmitic, stearic, and arachidic acids (5), or stearic, palmitic, and oleic acid plus a small amount of arachidic acid (6).

The brain gangliosides have been shown recently to be an important, if not the exclusive, brain lipid fraction in which C₂₀-sphingosine is found (7). This compound was first mentioned in a study of an unidentified brain lipid fraction (8). The occurrence of C₂₀-sphingosine in preparations of ganglioside from various species has been described (5). Evidence for the existence of C₂₀-sphingosine is drawn largely from the nature of oxidation products and is therefore indirect.

Changes in the long-chain fatty acids of the cerebro-
sides isolated from the brain of rats of increasing age
have been described (9), but animals younger than 23
days and shorter-chain fatty acids were not studied. A
recent report describes changes in the fatty acid composi-
tion of the sphingomyelin fraction isolated from the
brains of humans of increasing age (10). These changes
closely resemble those reported here for rat brain sphin-
gomyelin.

METHODS

Preparation of Tissue

Litters of Wistar strain albino rats from a stock colony
were studied. No differentiation was made on the basis of
sex. Newborn rats were killed by immersion in a mixture
of solid carbon dioxide and acetone. Larger animals were
killed by a blow to the cervical region. Brains were re-
moved, transferred to chopped ice, weighed, pooled, and
processed at once. Human brains were collected a few
hours after death. They were enclosed in polyethylene
bags and kept at -20° until processed.

Extraction of Total Brain Lipid

The total brain lipid was extracted by blending the tissue
with chloroform-methanol 2:1 (v/v) in the cold, and the
extract was dialyzed (11). After dialysis, the upper and
lower phases of the dialysate were separated and col-
lected.

The lower phases of the dialyzed total lipid extracts
were concentrated by evaporation with nitrogen. The
concentrated solutions were transferred to 10-ml volu-
metric flasks and diluted to known volume with chloro-
form-methanol. Suitable aliquots were withdrawn and
evaporated to dryness with nitrogen. The residues were
analyzed for sphingomyelin and cerebroside by TLC.

Isolation and Analysis of Rat Brain Sphingomyelin

Sphingomyelin was separated from the other lower phase
lipids by TLC (12) on plates coated with Silica Gel
G and developed with chloroform-methanol-water
65:25:4. A clean separation of the sphingomyelin frac-
tion from the other lower phase lipids is obtainable in this
system. Pure sphingomyelin (Applied Science Labora-
tories, State College, Pa.) was used as a reference. Spots
were made visible by very brief exposure to iodine vapor.
The sphingomyelin spots were scraped from the plate.
The sphingomyelin was completely recovered by ex-
tracting the collected spot scrapings five times with an
equal volume of chloroform-methanol-water 2:1:0.1.
The quantitative recovery of phospholipids from Silica
Gel G by extraction with chloroform-methanol-water is
described in a recent review (13). The extract was
evaporated with nitrogen.

The quantity of sphingomyelin was estimated by
colorimetric phosphorus analysis after wet ashing (14).
The recovery of known sphingomyelin added to the
plate was $100 \pm 2\%$. This value coincided with the
standard error of the analytical procedure for phos-
phorus when applied to unchromatographed pure
sphingomyelin.

The fatty acids of the sphingomyelin fractions were
released as methyl esters by methanolysis with BF_3 -
methanol (15). Since the presence of silica gel during the
methanolysis caused no measurable change in the subse-
quent GLC patterns of the methyl esters, the methanol-
ysis of the sphingomyelin was usually carried out directly
on the spot scrapings without an intervening extraction
of the lipid from the silica gel (16). The preparation of
the esters for GLC will be described later.

Isolation and Analysis of Rat Brain Cerebrosides

Cerebrosides were separated from the total lower phase
lipid by TLC on Silica Gel G with chloroform-meth-
anol-concd ammonia 80:20:0.4 (17). Pure beef brain
cerebrosides (Applied Science Laboratories, State Col-
lege, Pa.) were used as reference compounds. Cerebro-
sides were located and extracted from the spot scrapings
in the same manner as sphingomyelin. Cerebrosides and
cerebroside-like compounds are extractable from TLC
spots with chloroform-methanol (18) with a recovery of
more than 95% of the applied glycolipids, as estimated
by hexose analysis. The cerebroside-like compounds were hydrolyzed
and their quantity was estimated by analysis of hexose
content with the anthrone reaction as described previ-
ously (19).

The TLC system separates cerebroside-like compounds with normal
fatty acids (R_F 0.51) from cerebroside-like compounds with hydroxy
fatty acids (R_F 0.39). No hydroxy acids were found in
the faster moving spot, but the slower moving spot
carried with it a small percentage of cerebroside-like compounds with
normal fatty acids. These amounted to less than 1%
of the total. The cerebroside-like compounds were methanolized as
described for sphingomyelin. At the end of 2 hr, a com-
plete release of fatty acids was obtained.

Isolation of Ganglioside

The upper, aqueous phase from the dialysis of the total
lipid extract was concentrated to a few milliliters under
vacuum at room temperature. Nine volumes of a sat-
urated solution of KCl were added. The solution was
dialyzed against distilled water overnight in the cold. A
small amount of material became insoluble during the
dialysis. This material contained no sialic acid and was
removed by centrifugation. The clarified solution was
lyophilized to yield the total ganglioside preparation as
the potassium salts.

Purity of Total Ganglioside Preparation

Gangliosides prepared in the above manner are low in contaminants. Phosphorus content ranged from less than 0.02% in most preparations to a maximum of 0.35%. Neutral lipids were not detectable by TLC with Silica Gel G and hexane-ether-acetic acid 90:10:1. Only traces of cerebroside and sulfatide (less than 0.1 and 0.05%, respectively, by anthrone analysis) were detectable by TLC with chloroform-methanol-concd ammonia 80:20:0.4. The total ganglioside preparations thus appeared to be sufficiently free from contamination, and they were used without further manipulation for the study of the sphingosine and fatty acid components of rat brain gangliosides except, as noted below, where pure fractions were isolated from TLC to verify (a) the presence in all ganglioside preparations of small quantities of short-chain fatty acids and (b) the preferential susceptibility to methanolysis of gangliosides with shorter-chain fatty acids.

Analysis of Gangliosides

Gangliosides were estimated by sialic acid analysis based on the colorimetric reaction with *p*-dimethylamino-benzaldehyde after heating in dilute HCl according to the method developed by Werner and Odin (20). As these authors have shown, the direct Ehrlich reaction tends to give more reliable values for sialic acid than colorimetric reaction with orcinol in sulfuric acid, since hexoses and hexose-like compounds are chromogenic with the latter reagent. It should be pointed out, however, that some ganglioside preparations can cause slight turbidity which gives high readings in the direct Ehrlich reaction. A satisfactory correction for turbidity is made by subtracting optical density at 700 $m\mu$, where color makes no contribution to the absorption, from the optical density at the absorption maximum at 565 $m\mu$.

Investigation of the fatty acid composition of the gangliosides was preceded by a search for the optimum conditions of fatty acid release. Samples ranging from 0.05 to 1 mg in weight were heated in boiling water with 1.0 ml of $\text{BF}_3\text{-CH}_3\text{OH}$ under nitrogen in screw-cap tubes whose threads were sealed by lining them with Teflon tape (Hercules Tape Dope, Hercules Chemical Co., New York) and whose caps were lined with a Teflon insert. Various periods of heating were investigated to determine the points of complete release of the individual fatty acid species in the ganglioside preparations. The amount of each fatty acid released was estimated by comparing the approximate peak area on GLC with that produced by a known amount of methyl linoleate added, as an internal reference standard, to the methanolysis reagent. No destruction of the internal standard was observed providing (a) the methanolysis was done under nitrogen and (b) there was no

evaporation of reagent due to tube leakage. Peak areas were calculated by multiplying peak height by peak width at half the peak height. The validity of this procedure was checked with appropriate standard mixtures of methyl esters (NIH, and Applied Science Laboratories, State College, Pa.). Values obtained with the standards were consistently within 2% of the stated values.

After 180 min of heating, all of the fatty acid species released from the gangliosides had reached maximum levels which did not change on further heating.

The precision of the internal standard procedure was checked by gravimetric analysis. The internal standard procedure gave values which were $97 \pm 3\%$ of gravimetric values. The total ganglioside preparations were shown by TLC to be mixtures of mono-, di-, and tri-sialogangliosides in undetermined proportions, and thus it was not possible to calculate accurately the theoretical fatty acid content. Values ranging from 14 to 16%, w/w, were obtained for normal brain gangliosides. As a check for the accuracy of the internal standard method, a ganglioside of established composition was isolated and analyzed. The major fraction of the gangliosides of Tay-Sachs brain, a monosialoganglioside lacking the terminal galactose (2) was separated by TLC. It migrated as a single spot in chloroform-methanol-water, propanol- NH_3 , and phenol-water (21). The fatty acids, according to GLC, contained more than 96% stearic acid. The fatty acid value (mass of fatty acyl radical/mass of total molecule $\times 100$) obtained by the internal standard method was $99 \pm 1.5\%$ of the theoretical value of 19.2%.

Preparation of Methyl Esters for GLC

At the end of each period of methanolysis the tubes were cooled in ice before opening, and an equal volume of water was added to the reaction mixture with cooling. The methyl esters were extracted three times with an equal volume of hexane. The hexane was evaporated with nitrogen to approximately 0.1 ml without warming the tube. By means of a Pasteur pipette, the hexane was transferred, with washing, to a glass tube 4 mm i.d. by 40 mm in length, tapered and sealed at one end. The hexane was evaporated just to dryness with a slow stream of nitrogen without warming (the tube must not be held with the fingers). The inner surface of the tube was carefully washed down with 10 μl of hexane. Approximately 30 sec was allowed for drainage. The tube was shaken to insure uniformity of the hexane solution collected at the bottom. Portions (3 μl) of the solution were withdrawn and injected into the GLC apparatus (stationary phase, ethylene glycol succinate polyester at 173°; mobile phase, argon). Replicate analyses were performed.

Isolation of Sphingosine

To avoid Schiff's base formation and other side-reactions with liberated sphingosine, hexoses were first removed from the ganglioside and cerebroside preparations by hydrolysis in 3 N HCl in sealed tubes under nitrogen at 100° for 90 min. Lipid material was extracted five times with an equal volume of chloroform. The chloroform was evaporated with nitrogen. Sphingomyelin was not subjected to the above treatment.

Sphingosine was liberated either by heating the lipid material in 1 ml of 2 N KOH in methanol-water 4:1 in Teflon-sealed screw-cap tubes at 55° for 5 hr under nitrogen, or by methanolysis in 5% concd. HCl in methanol under nitrogen for 8 hr at 70°. No ceramide or sphingosyl phosphoryl choline was demonstrated by TLC after the heating periods.

Samples subjected to HCl-catalyzed methanolysis in aqueous methanol were extracted five times with 1 ml of hexane to remove the methyl esters of the fatty acids. The combined hexane extracts were washed with 0.5 ml of HCl-CH₃OH and this was added to the original HCl-CH₃OH lower phase which was then rendered strongly basic with 10 N NaOH. The sphingosine was extracted five times with 1 ml of diethyl ether. No ester contaminants were found by TLC.

Alkali-saponified samples were extracted directly with diethyl ether. Six extractions with 1 ml of solvent completely extracted the ninhydrin-reacting material. The combined ether extracts were washed with 0.5 ml of 10% NaOH (w/v) in water to remove the small quantity of fatty acids, demonstrable by TLC, which accompanied the sphingosine during the extraction with ether.

Oxidation of Sphingosine to Fatty Acids

Sphingosine was oxidized at the double bond to fatty acids in an extension of the method of Sweeley and Moscatelli (22). A permanganate-periodate reagent (23) was used for the oxidation. This reagent, when employed as described below, produces minimal amounts of over-oxidation products. Eighteen-carbon sphingosine is oxidized to myristic acid, dihydrosphingosine and 20-carbon sphingosine to palmitic acid. The oxidizing reagent was prepared by mixing one volume of permanganate-periodate solution with four volumes of *tert*-butanol. Sphingosine samples from 0.05 to 1 mg were dissolved in 1 ml of reagent, and oxidation was allowed to proceed at room temperature for 4 hr. At the end of this period, solid sodium hydrosulfite was added, with shaking, until all of the purple permanganate and brown manganese dioxide were reduced. The pale yellow solution was cooled in ice and acidified with concd HCl. The fatty acids derived from the oxidation of sphingosine were extracted five times with 1 ml of hexane. The hexane extract was collected in Teflon-lined screw-cap tubes

and evaporated with nitrogen. One milliliter of BF₃-CH₃OH (24) was added. The tubes were sealed and heated for 5 min at 100°. The tubes were cooled before opening, and 1 ml of water was added. The methyl esters were extracted three times with hexane. They were analyzed by GLC as described above.

A very small amount of overoxidation occurred, as evidenced by the appearance of minor C₁₅ and C₁₃ fatty acid peaks. These were taken into account in calculating the relative quantities of C₁₆ and C₁₄ fatty acid derived from oxidation, respectively, of C₂₀- and C₁₈-sphingosine. The small contribution of dihydrosphingosine was ignored since it was less than 3% of the total.

The oxidation procedure is applicable to intact sphingolipids as well as to liberated sphingosine. Sphingolipids containing dihydrosphingosine do not give fatty acids when oxidized as described. Sphingolipids in which the double bond of sphingosine had been reduced by treatment in methanol with hydrogen (at 42 psi) over a Pt catalyst for 2 hr gave no detectable fatty acids with the oxidation procedure. The relative amounts of C₁₈- and C₂₀-sphingosine in the ganglioside preparations were calculated by comparing the GLC peak areas of the C₁₄ and C₁₆ methyl esters derived from the oxidation of sphingosine. Comparable ratios were obtained from the oxidation of intact gangliosides and from their isolated sphingosine components. Calculated on the basis of fatty acid content, yields were 88–91% when the intact lipid was oxidized, and 60–70% when the sphingosine was split off and isolated before oxidation.

TLC of Isolated Sphingosine

Sphingosine samples were fractionated by TLC (26). In our hands, more distinct separations of sphingosine fractions were obtained by modifying the ratios of the volumes of solvent components (chloroform-methanol-concd NH₃-water) to 80:15:0.5:0.5. Spots were made visible with iodine vapor and by spraying with 1% ninhydrin in methanol-pyridine 95:5. All of the sphingosine samples had a minor component which had the mobility of dihydrosphingosine and gave palmitic acid on oxidation, except for the sphingosine preparations derived from rat brain sphingomyelin. These showed no dihydrosphingosine spot, and the spot corresponding to *threo*-sphingosine was also absent.

RESULTS

Methanolysis of Gangliosides

An example of the release of ganglioside fatty acids as a function of time of heating at 100° in BF₃-CH₃OH is shown in Fig. 1. The initial methanolytic attack was on molecules with fatty acid components shorter than

stearic acid. After an initial release of shorter-chain fatty acids, methanolysis of each fatty acid species proceeded at a rate consistent with its concentration in the ganglioside preparation. Figure 2 demonstrates the effect on the

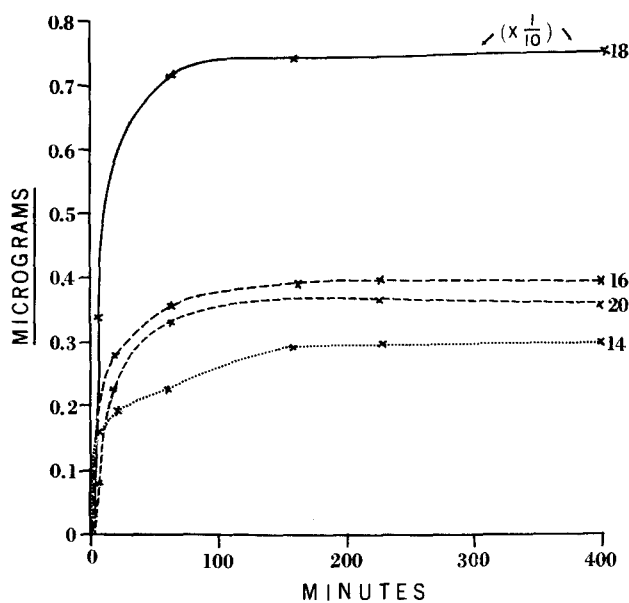


FIG. 1. The release of fatty acids from 0.5 mg of a Tay-Sachs ganglioside preparation heated for time intervals at 100° in boron trifluoride-methanol. The numbers at the end of the curves indicate the fatty acids by carbon number. $C_{18:1}$, not shown, follows almost the same course as C_{18} .

GLC pattern of initial selectivity of methanolysis with respect to gangliosides with shorter-chain acids. The same phenomenon was observed with all of the ganglioside preparations examined regardless of source.

The possibility existed that small amounts of contaminants in the total ganglioside preparations might contribute the short-chain fatty acids (14 carbons and less) found in all ganglioside preparations and might also be responsible for some of the shorter-chain acids which appear during the first part of methanolysis. For examination of this possibility, the pure major fraction in each preparation was isolated by TLC and examined for short-chain acids and for the rate of release of individual fatty acid species.

Isolation of Pure Ganglioside Fractions. Gangliosides were fractionated by TLC with Silica Gel G and chloroform-methanol-water 2:1:0.15. Scrapings from the major spot in each preparation were extracted 10 times with chloroform-methanol-water 2:1:0.25. Yields, by weight, were between 20 and 30% of the total material applied to the plate. The ganglioside subfractions were free from phosphorus (less than 0.02%) and no neutral or glycolipid contaminants were demonstrable by TLC. The fatty acid patterns resulting from a short-term and from a complete methanolysis of pure fractions from rat brain are compared in Table 1. A preferential initial release of shorter-chain fatty acids was noted, just as with the total ganglioside preparations. In addition, all puri-

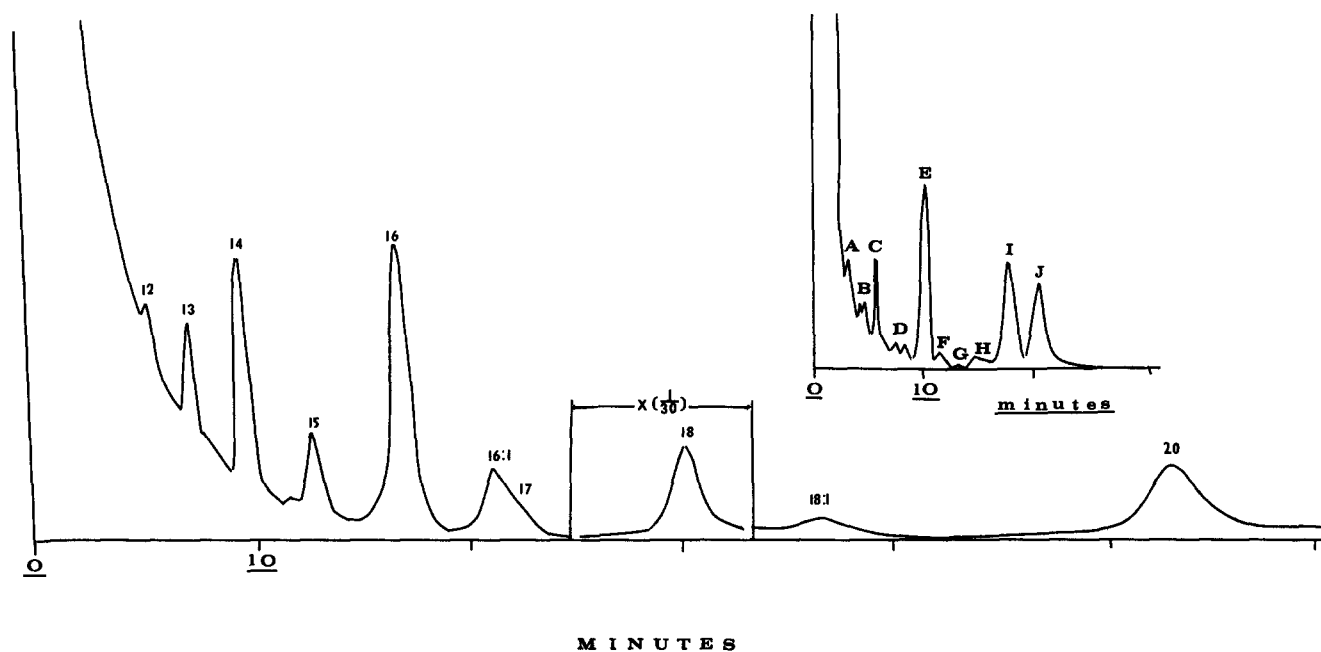


FIG. 2. Preferential release of shorter-chain acids by incomplete methanolysis. The large chromatogram shows a gas-liquid chromatogram of methyl esters of fatty acids from a Tay-Sachs ganglioside preparation after release of all of the fatty acids. The inset shows the chromatogram obtained with the same preparation after a partial release of the fatty acids during a short-term methanolysis for 5 min at 100° . In the large chromatogram, the numbers over the peaks identify the fatty acids by number of carbon atoms and degree of unsaturation. Major peaks in the inset are: C, 14:0; E, 16:0; I, 18:0; J, 18:1.

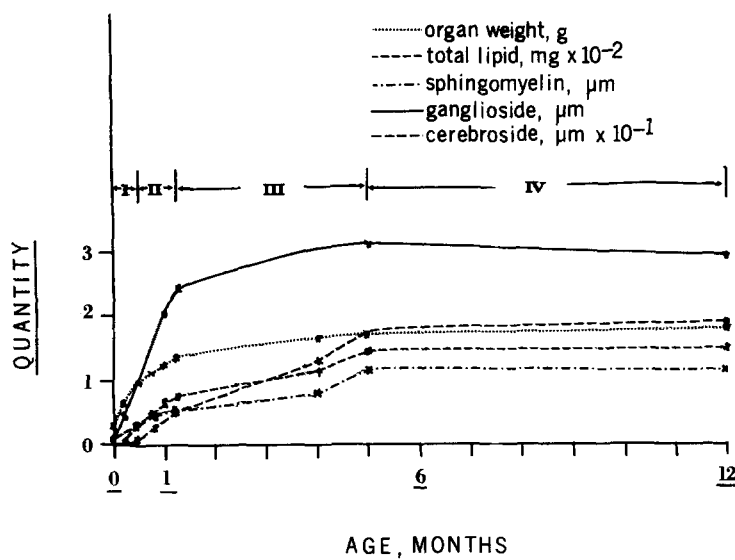


FIG. 3. Changes in the wet weight and the total lipid, ganglioside, sphingomyelin, and cerebroside content of rat brain with increasing age. Values are expressed as the total quantity per brain. Gangliosides are expressed as ganglioside sialic acid. Pooled brains were analyzed in groups of 20–30 for each age except at 5 months and 1 year, where organs were studied in groups of 10. The roman numerals indicate the stages of brain growth. Abbreviations: g (grams); mg (milligrams); μm (micromoles).

fied preparations had small quantities of fatty acids with 14 and fewer carbon atoms.

Growth of Rat Brain

Rat brain weight increased linearly (Fig. 3) from 0.2 g at birth to 1.1 g at the end of 2 weeks (stage I). A new, lower rate of growth to 1.3 g was observed between 2 and

5 weeks (stage II). Between 5 weeks and 5 months, a third, still lower, rate of increase in brain weight to 1.7 g was recorded (stage III). Beyond this period (stage IV) and until the conclusion of the experiment when the brain was 1 year of age, there was only a minor increase in brain weight.

Total Lipid Content of Rat Brain

During stages I and II, the weight of lipid extractable from the fresh brain increased linearly from 5 to 75 mg (Fig. 3). During stage III, there was a further increase to 120 mg, at a greatly reduced rate. During stage IV, a still lower rate of accumulation of lipid in the brain was observed. The lipid content of the brain at 1 yr of age reached 140 mg.

Brain Ganglioside Content

Brain ganglioside content (Fig. 3) was measured in terms of ganglioside-bound sialic acid. During stage I, and continuing until the middle of stage II, ganglioside sialic acid increased linearly, from 0.05 to 1.95 μmoles per brain. At the end of stage II, 2.45 μmoles of ganglioside sialic acid had accumulated per brain. A new, greatly diminished rate of accumulation ensued and continued throughout stage III. At the end of stage III, there were 3.10 μmoles of ganglioside sialic acid per brain. This was the maximum level recorded. During stage IV, there was a slow decrease in brain ganglioside sialic acid content until the end of the experiment when the brain was 1 year of age.

TABLE 1 COMPOSITION OF FATTY ACIDS RELEASED FROM THE PURE MAJOR FRACTION OF RAT BRAIN GANGLIOSIDES ISOLATED BY TLC

Age Group		Fatty Acid Component				
		C ₁₄ and shorter	16:0	18:0	18:1	C ₂₀ and longer
		* % of total fatty acids cleared				
2 wk	A*	1	10	79	10	0
	B†	<1	3	94	1	2
5 wk	A	1	11	67	22	0
	B	<1	6	91	1	2
4 months	A	1	11	62	26	0
	B	<1	8	82	2	8
1 yr	A	2	23	64	13	0
	B	<1	7	84	2	8

The fatty acid compositions of the subfractions shown here differ from those of the total ganglioside preparations (shown in Fig. 5) because distribution of fatty acids among ganglioside subfractions is not uniform, as will be described in a separate report.

* A. After heating the purified ganglioside for 5 min in 14% BF₃ in methanol at 100° in a sealed tube under nitrogen. Approximately 10% of the fatty acids were released.

† B. The same as A, but after 180 min of heating. At this point, all of the fatty acids had been released.

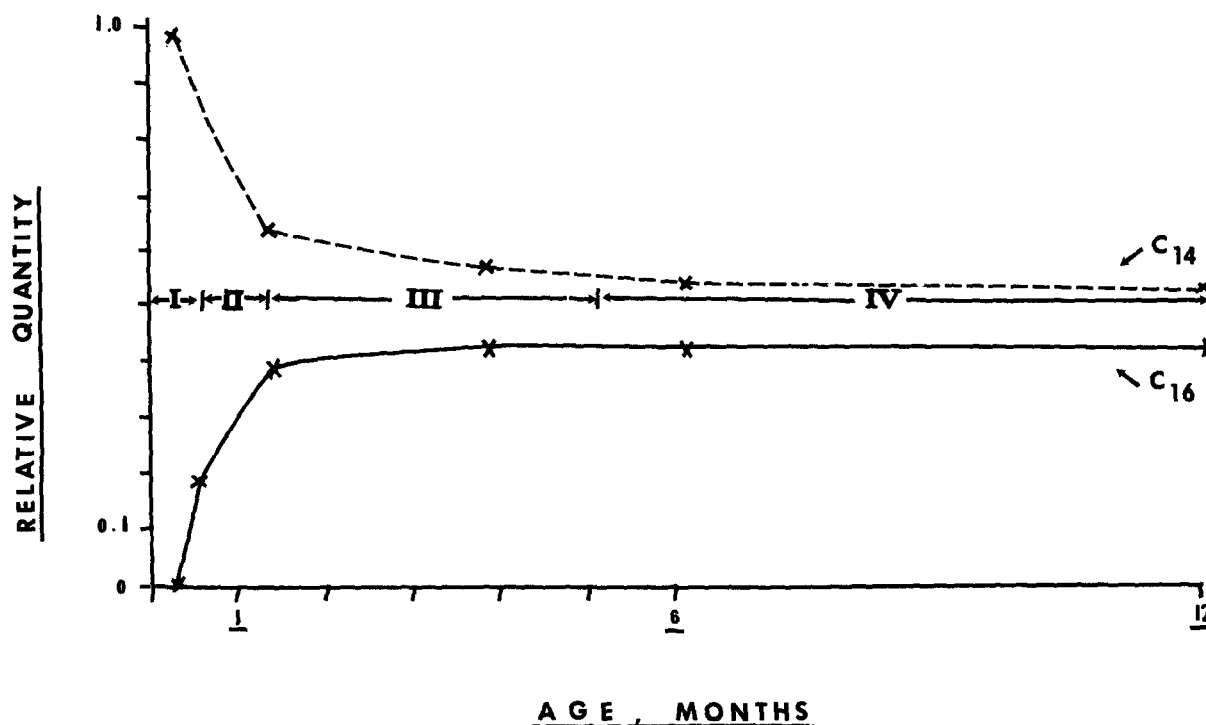


FIG. 4. Effect of age on chain-length of ganglioside sphingosine. Relative proportions of myristic and palmitic acids derived from the oxidation of the double bond of the sphingosine components of total ganglioside preparations from the brains of rats of increasing age. The roman numerals indicate the stages of brain growth.

Rat Brain Cerebrosides and Sphingomyelin

During the early part of stage I, there was insufficient cerebroside and sphingomyelin for measurement. During the latter part of stage I, sphingomyelin and cerebroside began to accumulate. During stage II, the rate of accumulation of sphingomyelin diminished while that of cerebroside did not. At the end of stage II, there were 0.5 μ mole of sphingomyelin and 5.0 μ moles of cerebroside per rat brain. During stage III, cerebroside continued to accumulate more rapidly than sphingomyelin, but at a slower over-all rate than during stage II. At the end of stage III, there were 1.1 μ moles of sphingomyelin and 17.5 μ moles of cerebroside per brain. During stage IV, cerebroside increased only slightly, and sphingomyelin remained relatively constant. Details of these changes are shown in Fig. 3.

Rat Brain Sphingosine

The sphingosine component of rat brain sphingomyelin and cerebroside at all stages of brain growth gave mainly myristic acid on oxidation, indicating that the sphingosine portion of these lipids remains constantly of the 18-carbon variety. The sphingosine portion of rat brain gangliosides gave myristic acid and negligible amounts of palmitic acid at birth (Fig. 4). During the later half of stage I and throughout stage II increasing amounts of palmitic acid were produced on oxidation.

The rate of increase leveled during stage III. At the end of this stage the ratio of C_{16} to C_{14} fatty acid derived from the oxidation of ganglioside sphingosine was 0.76:1. During stage IV, relatively little change was noted in this ratio.

Fatty Acids of Rat Brain Sphingolipids

In the brain of the newborn rat, stearic acid (Fig. 5, top) was the major fatty acid component of sphingomyelin and gangliosides (86 and 73% of the total, respectively), and this acid was present in substantial proportion (23% of the total) in the cerebroside when they first appeared in measurable quantity. With growth of the animal, stearic acid in the cerebroside rapidly declined to a very low level, whereas although the stearic acid content of the sphingomyelin and ganglioside fractions showed decreases with age, it remained high (74 and 55%, respectively, in 1-year old brain).

The C_{18} monoenoic fatty acid fraction (Fig. 5) remained at a relatively constant low level in the cerebroside and sphingomyelin at all stages of rat brain growth. In the gangliosides, there was an increase in $C_{18}:1$ during stage III from 4% to 19%, whereupon the level remained relatively unchanged until the conclusion of the experiment when the brain was 1 year of age. The palmitic acid content of the cerebroside and sphingomyelin of rat brain underwent a continuous decline after birth.

TABLE 2 RELATIVE PROPORTIONS OF THE MOLECULAR SPECIES OF THE FATTY ACID AND SPHINGOSINE COMPONENTS IN TOTAL GANGLIOSIDE PREPARATIONS FROM HUMAN BRAINS AT VARIOUS AGES

The amount of each fatty acid species is expressed relative to the amount of C₁₆. The proportion of C₂₀-sphingosine is calculated from the amount of palmitic acid produced on oxidation. The remainder of the sphingosine was of the C₁₃ variety and gave myristic acid on oxidation.

Specimen	Relative Quantity of Each Fatty Acid										Proportion of C ₂₀ -Sphingosine	
	12:0	13:0	14:0	15:0	16:0	16:1	17:0	18:0	18:1	19:0		20:0
Fetus 1*	—	—	tr.	tr.	1.0	tr.	—	15	tr.	—	0.4	0.00
Fetus 2†	—	—	tr.	tr.	1.0	tr.	—	33	1	—	0.5	0.00
Neonate (3 days)	0.1	—	0.1	—	1.0	tr.	—	22	0.2	—	0.5	0.20
Child (3 yr)	—	—	tr.	tr.	1.0	tr.	—	3.3	0.5	—	tr.	0.33
Adult (30-80 yr)	tr.	tr.	0.2	0.1	1.0	0.2	0.1	3.8	1.1	tr.	0.3	0.44
	tr.	tr.	0.2	0.1	1.0	0.2	tr.	8.6	0.2	tr.	0.9	0.47
	—	tr.	0.1	tr.	1.0	0.2	0.1	4.8	0.7	tr.	0.4	0.53
Tay-Sachs (2-3 yr)	0.1	0.2	0.5	0.2	1.0	0.5	0.1	14	0.1	tr.	0.7	0.12
	—	tr.	0.1	tr.	1.0	0.1	0.1	15	0.1	tr.	0.6	0.12
	—	tr.	0.1	tr.	1.0	tr.	tr.	18	0.2	tr.	0.8	0.09
	—	tr.	0.1	tr.	1.0	tr.	tr.	17	0.2	tr.	2.0	0.14

* 33 weeks gestation.
† 36 weeks gestation.

Palmitic acid in the gangliosides increased during stages I and II, then declined.

These findings indicate that, although details differed, there was a general over-all decline in the stearic acid content of all three sphingolipids with increasing age. An increase in C_{18:1} fatty acid appears to be peculiar to the rat brain gangliosides.

Fatty Acids and Sphingosine in Human Brain Gangliosides

The results obtained with human brain ganglioside preparations are given in Table 2. The trends observed in rat brain with age appear to occur also in human brain. Thus, the gangliosides of fetal human brain are practically devoid of C₂₀-sphingosine, but this compound increases in quantity with increasing age. Likewise the stearic acid content of the human brain gangliosides undergoes a decrease with age. The fatty acid and sphingosine composition of the gangliosides of Tay-Sachs brain resembles that of the normal human fetus rather than that of the age group from which the diseased brain specimens were derived.

DISCUSSION

The results obtained in this study indicate that dependence on arbitrary conditions for their release of fatty acids from gangliosides may give misleading information. Prolonged heating with acidic anhydrous methanol in air can lead to a complete loss of monoenoic fatty acids (5), presumably through oxidation—and perhaps methoxylation (25)—of the double bond. Shorter-chain fatty acids, present in very small amounts in the gangliosides, may be lost through evaporation or distillation. In contrast, incomplete, short-term methanolysis (6) results in a preferential release of shorter-chain fatty acids from the

gangliosides and thus can give a distorted picture of the relative quantity of each fatty acid species. Experimental evidence should be sought to determine whether the preferential release of shorter-chain fatty acids from the gangliosides during methanolysis reflects a preferential susceptibility to hydrolysis and metabolic turnover of the shorter chains in the neural cell.

Ceramide composed of C₁₈-sphingosine and C₁₈ fatty acid is a structural feature of a large proportion of all of the three sphingolipids (gangliosides, sphingomyelin, and cerebroside) studied in the brain of newborn rats. Cerebroside of maturing rat brain diverge from the other two sphingolipids in their percentage of C₁₈ fatty acid, which rapidly diminishes to a negligible level. The C₁₈ fatty acid contents of the gangliosides and sphingomyelin of developing rat brain undergo an over-all decline, but C₁₈ remains the major fatty acid component of these lipids at all stages of brain growth.

The total rat brain ganglioside preparations contained a small amount of phosphorus. It varied from none detectable to a maximum of 0.35% (in one preparation). The decrease in the percentage of C₁₈ fatty acid in rat brain gangliosides with age and the susceptibility of shorter-chain acids to methanolysis bore no quantitative relationship to the amount of phosphorus present. However, one should not overlook the possibility that phospholipid impurities of an unknown nature may have contributed some of the shorter-chain acids. It is also possible that the decrease in the percentage of C₁₈ fatty acid and the increase in C_{18:1} fatty acid with age may have been due in part to the presence of contaminating lipids.

The sphingosine portion of the brain gangliosides changes from almost exclusively C₁₈ at birth to nearly equal quantities of C₁₈ and C₂₀ with organ maturation.

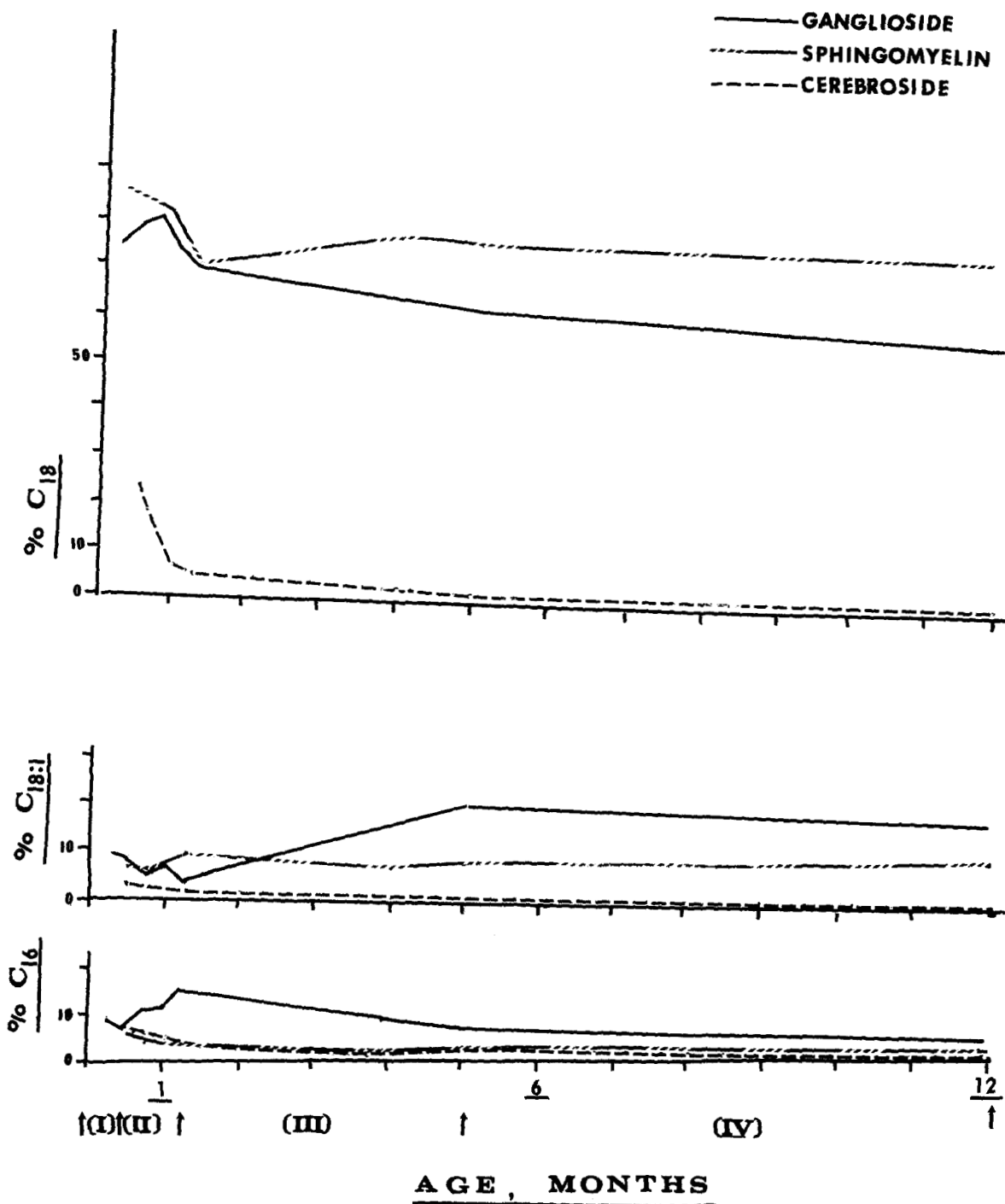


FIG. 5. Changes with increasing age in the relative content of 16:0, 18:0, and 18:1 fatty acid components of the gangliosides, sphingomyelin, and cerebrosides of rat brain. In the cerebrosides, the bulk of the fatty acids was made up by 24h:0, and lesser amounts of 20h:0, 24:1, and 25h:0. The roman numerals indicate the stages of brain growth.

The sphingosine component of the cerebrosides and sphingomyelin remains of the C₁₈ variety at all stages of brain growth. The gangliosides of Tay-Sachs brain have the very high C₁₈ fatty acid and C₁₈-sphingosine content typical of the gangliosides of normal fetal brain. This in-

formation, linked to the previously observed differences between the physical and chemical properties of the gangliosides of the brain of the normal and Tay-Sachs diseased child (27), may help to provide a clue to the pathogenesis of Tay-Sachs disease.

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