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Concentration and fatty acid composition of cerebrosides and sulfatides in mature and immature human brain

JOHN H. MENKES,* MICHEL PHILIPPART, and MARIA CARLA CONCONE

Department of Pediatrics and Division of Neurological Medicine, The Johns Hopkins Hospital, Baltimore, Maryland

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ABSTRACT The fatty acid composition of cerebrosides and sulfatides from frontal lobe gray and white matter was determined for five fresh and four formalinized adult brains and for eight infants. Fatty acid patterns were unaffected by formalinization, but varied considerably from one another in the proportion of saturated to unsaturated fatty acids.

The percentages of 24:0 and 24:1 increased with age. Cerebrosides obtained from areas such as the brainstem and cerebellum, where myelination was more advanced, tended to have a larger proportion of long-chain fatty acids than samples extracted from frontal or parietal lobe white matter. Hydroxy fatty acids showed an adult pattern in all instances in which amounts sufficient for accurate quantification could be isolated.

Lipid hexose, cerebroside + sulfatide hexose, and methanol-eluted hexose were measured in the brains of 12 infants ranging in age from a 4 month fetus to 2 yr. In the most immature, the majority of lipid hexose was in the form of glycolipids more polar than cerebrosides and sulfatides. These have tentatively been identified as hematosides and globosides. With maturation, cerebrosides and sulfatides increased progressively, but the amounts of the more polar glycolipids remained constant in relation to the total lipid content of tissue.

KEY WORDS man · brain · gray matter · white matter · cerebrosides · sulfatides · fatty acids · maturation · individual variation · formalin storage · column chromatography

Abbreviations: Fatty acids are denoted by chain length and number of double bonds. h, hydroxy fatty acid; GLC, gas–liquid chromatography; DEAE, diethylaminoethyl; TLC, thin-layer chromatography; GM, gray matter; WM, white matter; 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 24:0, lignoceric acid; 24:1, nervonic acid; 24h:0, cerebronic acid.

* Joseph P. Kennedy, Jr. Memorial Foundation Senior Scholar in Mental Retardation.

The fatty acid composition of cerebrosides and sulfatides from normal, mature human brain has been determined in a number of laboratories with generally good agreement (1–4). Very long-chain nonhydroxy and hydroxy fatty acids, principally lignoceric, cerebronic, nervonic, and α-hydroxy nervonic acids, predominate in glycolipids isolated from white matter, and lesser amounts of stearic and palmitic acids are also present.

In contrast to those in white matter, cerebrosides isolated from cortical gray matter, cerebellum, and spinal cord contain a smaller percentage of C_{24} and larger amounts of C_{18} and C_{16} fatty acids (2).

In the course of a previously reported study on the composition of cerebral glycolipids in an infant with Maple Syrup Disease, it became evident that both the content and the fatty acid composition of cerebrosides and sulfatides obtained from cerebral white matter of newborn infants differ from that of adult tissue, and undergo considerable modification during the 1st yr of life (5). Analogous changes in fatty acid composition with maturation have already been documented by Kishimoto and Radin (6) in cerebrosides of developing rats, by Ställberg-Stenhagen and Svennerholm in human brain sphingomyelins (7), and by O'Brien and Sampson for all major lipids in one infant and one child (8).

A number of earlier studies have suggested that the total cerebroside and sulfatide concentration within the nervous system remains unaltered by storage in formalin (9–11), but the changes in fatty acid composition have as yet not been investigated. In order to establish an adequate base line for further studies on glycolipid abnormalities, we have undertaken a systematic study

of alterations with maturation and formalin fixation in the distribution and fatty acid composition of lipid-soluble glycolipids. The present report also furnishes data on the natural variability of cerebroside and sulfatide fatty acid composition obtained on a number of normal adult specimens under identical conditions of extraction and purification.

MATERIALS AND METHODS

Gray and white matter taken from macroscopically normal brains of twelve infants and nine adults, who had all died of diseases that were not neurological, were subjected to study. The brains of six infants and five adults were processed fresh, whereas the others had been stored in neutral formalin for a period of 2–4 weeks before processing. Cerebrosides were isolated in a chromatographically pure form from the brains of eight infants and all nine adults. In five specimens cerebrosides and sulfatides were isolated from the same tissue sample. The brains of four infants served only for the determination of the hexose content of various chromatographic fractions.

Analytical Methods

Hexose was measured by duplicate anthrone and orcinol analyses of the lipid fractions, with glucose and galactose standards run concurrently (12, 13).

The cerebral lipids were extracted with chloroform–methanol 2:1 and partitioned against water or, in some of the earlier processed samples, against 0.54% saline. The hexose content of the nonaqueous phase as measured by the anthrone reaction, expressed per unit weight of lipid, was termed *Lipid Hexose*.

Purification of Cerebrosides and Sulfatides

Total lipids were measured gravimetrically and placed on partially deactivated Florisil (14): 100 mg of lipid required 40 g of adsorbent, prepared by the addition of 7 g of water to 100 g of the activated anhydrous adsorbent. Successive elution yielded fraction I with chloroform (0.6 ml/mg of lipid), fraction II with chloroform–methanol 19:1 (0.2 ml/mg of lipid), fraction III with chloroform–methanol 2:1 (1 ml/mg of lipid), fraction IV with methanol (1 ml/mg of lipid), and fraction V with chloroform–methanol 2:1 + 4% water (1 ml/mg of lipid). Glycolipids were detected in all but fraction I, but only fractions III and IV were examined further. The hexose content of fraction III is referred to as *Cerebroside + Sulfatide Hexose* while the hexose content of fraction IV is referred to as *Methanol Eluate Hexose*.

Chromatographically pure cerebrosides and sulfatides were obtained by successive fractionation of the total lipid extract on moistened Florisil, DEAE cellulose, and silicic acid. The exact procedure used in our laboratory for this purpose has been described previously (5, 15).

Cerebrosides and sulfatides were separated from one another on a DEAE cellulose column. Final purification of cerebrosides involved passage through silicic acid to remove traces of impurities present in many of the formalinized specimens, and to separate the "minor cerebrosides" (16, 17). The latter were eluted with chloroform–methanol 98:2 or 97:3, while pure cerebrosides were eluted with chloroform–methanol 95:5. In addition to these, a small amount of ceramide dihexoside was always eluted with chloroform–methanol 9:1. Sulfatides were purified by a final passage through silicic acid and elution with chloroform–methanol 9:1. TLC was employed extensively to verify the identity and purity of all fractions. In very immature tissues, the quantity of hexose-containing material was so small that even slight contamination with adsorbent, particularly silica, affected the apparent weight of the sample and precluded analytically satisfactory hexose values. We were, therefore, forced in some instances to accept the absence of contaminants on TLC or phosphorus analyses as adequate evidence for the purity of our samples.

Determination of Glycolipid Structure

The purified glycolipids were methanolyzed in methanol–concentrated hydrochloric acid 5:1, and the reaction mixture was treated as noted in a previous publication (18).

The identity of the hexose moiety was confirmed by GLC of the trimethylsilyl derivatives of the methyl glycosides (19). The fatty acids were extracted as described by Sweeley and Moscatelli (20). After esterification with diazomethane, hydroxy and nonhydroxy fatty acids were separated on Florisil. Purity of the fatty acid fractions was verified by TLC in hexane–ether 9:1. The normal fatty esters were subjected to GLC at 210°C (diethylene glycol succinate polyester on Chromosorb Z).

Hydroxy fatty esters were methylated with methyl iodide and chromatographed at 216°C on the same column. Each fraction was hydrogenated and rechromatographed. Peaks were identified by comparing their retention times with those of standard fatty acids. Where standards were unavailable, peaks were assigned a tentative identity by their carbon numbers (21). Quantitative results with N.I.H. fatty acid standards agreed with the stated composition data with a relative error of less than 10% for major components and an error of less than 1% in the absolute value of minor peaks.

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TABLE 1 Glycolipid Content of the Mature and Immature Nervous System

Patient	Age	Area	Solids	Total Lipids	Hexose Content of Lipid		
					Total*	C + S†	Methanol Eluate‡
1	4 mos. fetus	G/W	7.9	1.6	0.2	—	—
2	5 mos. fetus	FrG/W	9.2	2.4	0.9	—	—
3	7 mos. fetus	G/W	8.7	2.3	0.31	0.10	0.12
4_F	1 day	GM	9.8	2.6	0.47	0.17	0.21
		WM	8.5	2.6	0.73	0.32	0.29
5_F	1 day	FrG/W	12.0	4.0	0.46	0.21	0.12
		ParG/W	9.6	3.2	0.88	0.51	—
		BG	13.7	4.5	0.61	0.41	—
		BS + Cer	17.3	5.1	1.4	—	—
6_F	1 mos.	FrWM	8.8	2.9	—	0.16	0.25
7_F	$1^1/_2$ mos.	FrWM	9.5	3.6	0.96	0.10	0.29
8	3 mos.	FrWM	12.9	4.2	2.8	0.86	0.37
9	$6^1/_2$ mos.	FrWM	17.4	5.6	1.4	0.70	0.12
10	10 mos.	FrWM	19.4	6.6	2.7	2.1	0.21
11_F	15 mos.	FrGM	13.5	3.7	1.0	0.86	—
		FrWM	19.7	8.7	4.2	1.5	—
12_F	2 yr	FrGM	12.7	3.7	0.59	0.50	—
		FrWM	18.9	8.0	2.2	2.0	—
Adults (Nos. 13–19)	19 to 93 yr	FrGM Fresh (8)	16.7	4.9 (4.2–5.5)	1.2 (0.7–2.1)	1.2 (1.0–1.4)	—
		FrGM Formalin (3)	16.5	5.5 (4.8–6.3)	1.9 (1.6–2.4)	1.9 (1.6–2.4)	—
		FrWM Fresh (4)	25.8	13.9 (11.6–17.0)	4.4 (2.7–6.6)	4.2 (2.7–5.7)	—
		FrWM Formalin (4)	27.2	13.9 (12.6–15.6)	5.4 (2.8–7.1)	4.0 (3.6–4.4)	0.21

F, formalinized; *Fr*, frontal lobe; *Par*, parietal lobe; *BG*, basal ganglia; *BS + Cer*, brainstem and cerebellum; *C*, cerebroside; *S*, sulfatide; *GM*, gray matter; *WM*, white matter; *G/W*, mixed gray and white matter. Figures in parentheses indicate number of specimens analyzed and range of values.

* Hexose value, measured by anthrone reaction in the total lipid extract after equilibration against water.

† Amount of hexose eluted from Florisil by chloroform–methanol 2:1, as measured by anthrone reaction.

‡ Amount of galactose hexose, as measured by anthrone reaction in methanol eluate from Florisil.

RESULTS

Lipid Hexose

Hexose was determined on lipids that did not include the gangliosides that had been removed by the solvent–partition method used in the purification of the total lipid extract. The lipids that were analyzed included, besides cerebrosides and sulfatides, ceramide di- and trihexosides, sialolipids (hematosides), and glycolipids containing hexosamine but free from sialic acid (globosides).

As has already been well demonstrated for the human brain by Cumings, Goodwin, Woodward, and Curzon (22), Brante (23), and several other workers, lipid hexose increased progressively with maturation (Table 1). It should, however, be pointed out that even when analyzing comparable anatomic regions, we noted considerable variation in hexose concentrations among brains of similar chronologic age.

We observed no significant difference in the lipid hexose values between fresh and formalin-fixed adult gray or white matter.

Cerebroside + Sulfatide Hexose

The hexose content of that portion of the washed total lipid extract which is eluted from moistened Florisil by chloroform–methanol 2:1 reflected the cerebroside and sulfatide content of the tissue. Sulfatides, as detected by TLC, accounted for a small proportion of the hexose content of this fraction in the most immature brains. Other glycolipids such as ceramide dihexoside, ceramide trihexosides, and the "minor cerebrosides" (16, 17) were also detected in trace amounts. With maturation, the hexose content of this fraction increased and accounted for a progressively larger percentage of the total lipid hexose value. As estimated by concurrent analyses with anthrone and orcinol (18), this fraction consisted mainly of galactose.

Values for cerebroside + sulfatide hexose did not differ significantly in fresh and formalin-fixed adult

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brains. Cerebroside + sulfatide hexose was 0.7–1.7% of total lipids for fresh, and 1.5–1.7% of total lipids for formalinized GM. It was 4.1–5.7% of total lipids for fresh, and 4.0–6.0% of total lipids in formalinized WM.

Methanol Eluate Hexose

The hexose value of the methanol eluate from Florisil (fraction IV) has up to now not been examined. This fraction contained both hexosamine and neuraminic acid. From preliminary investigation by TLC, it was apparent that it consisted in part of two polar glycolipids, one of which yielded a positive orcinol reaction for neuraminic acid and possessed chromatographic properties similar to those of the hematoside isolated by us from normal and Gaucher spleens (18).

When expressed in terms of dry weight, the hexose content of this fraction remained relatively constant with increasing age. In the more immature brains, therefore, this fraction represented a considerable portion of the total lipid hexose and accounted for a greater part of the difference between total hexose and cerebroside + sulfatide hexose values.

When anthrone and orcinol values obtained on this fraction were expressed in terms of galactose, a considerable discrepancy was noted which indicated the presence of sizeable amounts of a second hexose moiety, most probably glucose.

Cerebroside and Sulfatide Concentration

The cerebroside and sulfatide content of WM increased progressively with maturation. As determined gravimetrically, cerebroside content increased from 0.1% of total lipids in Brain 3, an infant of 7 months' gestation, through 0.28% in parietal mixed GM–WM of Brain 5, a 1-day old full-term infant, and 4.5% of total lipids in the brainstem and cerebellum of the same brain, to 7.5% of total lipids in WM of the 2 yr old child (Brain 12). In adults true cerebroside content of fresh GM was 2.2–3.4% of total lipids, and of formalinized GM 1.3–2.4% of total lipids. The content of pure cerebroside in WM ranged from 9.7 to 15.5% and from 14.8 to 22.7% of total lipids for fresh and formalinized tissues respectively.

Because they were present only in minute amounts, the sulfatides could not be quantified in immature brains.

As estimated from the difference between cerebroside + sulfatide hexose and true cerebroside hexose, sulfatide content of fresh GM ranged from 0.7 to 2.4% of total lipids, and in formalinized GM from 0.4 to 1.9% of total lipids. The estimated sulfatide content in WM was 2.7–4.6% and 2.9–3.5% of total lipids for fresh and formalinized tissues respectively.

WM cerebroside:sulfatide ratios were 3.4–5.4 in fresh and 4.2–6.5 in formalinized tissues. It is possible that the somewhat higher ratio in formalin-stored WM indicates a partial conversion of sulfatides to cerebrosides.

Nonhydroxy and Hydroxy Fatty Acid Distribution in Cerebrosides and Sulfatides

Several changes were noted in the nonhydroxy fatty acid pattern of cerebrosides and sulfatides with maturation (Tables 2 and 3).

(*a*) In the more immature brains, 16:0, 18:0, and 18:1 comprise the major nonhydroxy acids; the quantities of very long-chain fatty acids, particularly 24:0 and 24:1, increase progressively with maturation.

(*b*) In immature brains, the concentration of 24:0 in cerebrosides and sulfatides was generally higher than that of 24:1, a finding never observed by us in normal mature tissues. In the frontal lobe WM the total C_{24} fatty acid concentration was about 80% of adult value at 10 months of age and reached adult values in the 2 yr old. Cerebrosides obtained from the brainstem and cerebellum of a newborn infant contained adult proportions of total C_{24} fatty acids, even though 24:0 rather than 24:1 was the predominant fatty acid.

(*c*) In immature brains, the concentration of 18:1 in both cerebrosides and sulfatides was greater than in mature tissues, and in two specimens—cerebrosides from the basal ganglia of Brain 5 and WM sulfatides from Brain 10—the concentration of 18:1 was greater than that of 18:0. In adult tissues, the concentration of 18:1 in both cerebrosides and sulfatides of GM and WM was always much less than that of 18:0 and never greater than 2.1% of the total fatty acids.

(*d*) In samples in which the fatty acid distribution of cerebrosides and sulfatides was determined concurrently, a fairly good correlation between the fatty acid profile of cerebrosides and sulfatides was generally noted. However, the amount of 16:0 was consistently higher in sulfatides than in cerebrosides, and in two specimens, one derived from the basal ganglia of the newborn infant and the other from frontal WM of a 10 month old, total C_{24} fatty acids were lower in sulfatides than cerebrosides (Table 2).

(*e*) From an examination of the mean values for the percentage of each fatty acid, it is evident that the fatty acid composition in cerebrosides from fresh and formalinized adult brains is essentially identical. In particular, no loss of unsaturated fatty acids could be detected as a consequence of formalin storage.

(*f*) Striking was the great variation noted in the concentrations of some of the major fatty acids in cerebrosides and sulfatides of adult WM and GM (Table 3). For example, in WM cerebrosides the percentage of 24:0 ranged from 4.4 (Brain 15) to 23.6% (Brain 19),

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TABLE 2 Nonhydroxy Fatty Acid Composition of Cerebrosides and Sulfatides from Normal Infant Brains
Fatty Acid Values in Per Cent of Total Nonhydroxy Fatty Acids

Fatty Acid	2	3	4_F 1 day				5_F 1 day					9	10		11_F	12_F 2 yr	
	5 mos. fetus G/W	7 mos. fetus G/W	WM		FrG/W		ParG/W	BG		BS + Cer		6½ mos. FrWM	10 mos. FrWM		15 mos. FrWM	FrGM	FrWM
	C	C	C	S	C	S	C	C	S	C	S	C	C	S	C	G	C
14:0	—	0.3	—	—	—	—	0.2	3.9	—	—	tr.	—	—	—	—	tr.	—
15:0	—	1.1	—	—	—	tr.	—	5.9	tr.	—	tr.	—	—	—	—	tr.	—
16:0	24	14.1	31.5	30.2	14.4	14.2	5.6	8.9	40.3	3.5	6.2	15.1	2.6	12.1	8.1	10.1	1.9
16:1	—	1.6	5.7	1.0	tr.	2.9	0.2	5.9	—	0.2	tr.	—	0.7	1.5	—	—	—
17:0	—	1.2	—	—	—	—	—	9.8	tr.	—	—	—	—	—	—	0.5	tr.
18:0	38	60.0	32.2	35.3	70.3	43.2	37.1	7.6	40.6	16.8	50.3	9.0	13.4	14.8	20.0	74.6	10.3
18:1	—	10.1	14.4	14.7	7.6	31.5	0.9	10.6	9.6	0.3	1.2	5.5	2.2	23.6	—	0.4	0.5
19:0	—	—	—	—	—	—	—	8.1	1.8	—	—	tr.	—	—	—	—	—
20:0	2	2.5	2.6	1.0	—	0.9	1.6	4.2	1.5	2.1	1.4	0.8	4.2	—	2.7	0.6	1.2
22:0	22	—	—	—	2.6	0.9	4.6	—	—	8.4	4.8	6.5	7.0	4.8	8.6	0.8	4.4
23:0	tr.	—	—	1.1	tr.	—	3.7	—	—	2.6	—	2.5	1.5	1.1	4.8	0.7	3.8
24:0	14	1.6	7.8	tr.	3.0	2.5	12.1	35.0	4.7	33.4	21.0	19.1	35.9	20.5	37.2	4.7	12.8
24:1	—	3.6	4.5	3.5	0.7	1.7	15.6	tr.	—	18.5	12.3	15.1	19.7	6.8	7.4	5.8	42.3
25:0	—	—	—	—	—	—	tr.	tr.	—	5.1	2.5	8.0	7.1	3.8	6.3	tr.	6.3
25:1	—	—	—	—	—	—	2.1	tr.	—	1.5	—	11.0	tr.	—	—	1.2	4.0
26:0	—	—	—	—	—	—	tr.	—	—	3.3	—	—	3.1	2.4	4.0	tr.	2.0
26:1	—	—	—	—	—	—	—	—	—	3.8	—	—	7.4	8.6	—	1.1	9.3

For abbreviations see Table 1.

TABLE 3 Nonhydroxy Fatty Acid Composition of Cerebrosides and Sulfatides from Normal Adult Brains
Fatty Acid Values in Per Cent of Total Nonhydroxy Fatty Acids

Fatty Acid	Formalized Adult Brains								Fresh Adult Brains							Mean Values					
	13_F 19 Yr		14_F 42 Yr FrWM		15_F 50 Yr		16_F 52 Yr		17 49 Yr		18 50 Yr	19 55 Yr	20 72 Yr		21 93 Yr	Formalinized		Fresh		Total	
	FrGM	FrWM			FrGM	FrWM	FrGM	FrWM	FrGM	FrWM	FrG/W	FrWM	FrGM	FrWM	FrWM	GM	WM	GM	WM	GM	WM
	C	C	C	C	C	C	C	C	C	C	S	C	C	C	C	C	C	C	C	C	C
16:0	5.9	2.1	0.2	1.3	2.2	1.1	3.7	2.6	4.9	0.6	3.9	4.2	0.9	1.6	0.4	3.9	1.5	2.9	1.7	3.5	1.6
18:0	31.4	7.3	9.3	8.0	20.7	8.1	17.6	9.1	17.2	8.3	5.2	6.2	10.0	8.1	8.3	23.2	8.4	13.6	7.7	19.4	8.1
18:1	1.9	0.8	0.6	—	1.7	0.7	0.6	1.4	tr.	0.1	1.1	2.1	0.4	0.6	0.2	1.4	0.9	0.2	0.7	0.9	0.8
20:0	1.6	0.5	5.1	0.5	1.4	0.8	2.2	0.9	1.7	0.8	0.1	0.4	1.2	0.6	0.8	1.7	1.8	1.4	0.6	1.8	1.2
22:0	3.2	2.1	2.4	3.2	2.5	2.5	4.8	2.9	2.0	3.1	1.8	1.8	3.1	2.0	2.6	3.5	2.5	2.6	2.4	3.1	2.4
23:0	3.3	3.1	3.8	6.4	3.4	3.3	6.3	5.0	2.5	4.3	3.2	3.8	3.8	4.1	3.9	4.3	3.8	3.2	3.9	3.9	3.8
24:0	8.0	10.2	17.5	32.0	8.2	4.4	22.1	6.8	9.3	13.9	27.0	23.6	14.9	14.6	6.9	12.8	9.7	12.1	14.8	12.5	12.2
24:1	31.4	49.3	47.1	32.7	41.2	56.0	23.7	47.6	22.5	45.7	31.0	32.8	38.6	42.4	55.7	32.1	50.0	30.6	44.2	31.5	47.1
25:0	3.7	6.2	6.4	9.8	4.5	9.7	5.7	7.5	8.8	5.5	13.1	8.1	7.9	6.3	3.1	4.6	7.4	8.4	5.8	6.1	6.6
25:1	3.8	8.8	5.0	2.0	7.1	4.0	6.9	7.6	6.0	8.6	6.4	7.8	8.3	9.0	11.9	5.9	6.4	7.2	9.3	6.4	7.8
26:0	0.4	2.0	tr.	—	1.6	5.2	tr.	3.4	1.8	1.8	tr.	tr.	5.1	1.0	2.1	0.7	2.6	3.4	1.2	1.8	1.9
26:1	4.0	5.9	—	3.5	5.1	2.9	5.0	4.9	1.8	6.3	5.6	8.2	3.7	7.2	3.2	4.7	3.4	2.8	6.2	3.9	4.8

For abbreviations see Table 1.

and the percentage of 18:1 varied between 0.1 (Brain 17) and 2.1% (Brain 19). In each case, however, the saturated and unsaturated analogues varied inversely with one another, so that the total percentage of acids of any particular chain length remained remarkably constant from one adult to another in both fresh and formalin-stored specimens.

(*g*) With maturation the amount of hydroxy fatty acids increased (qualitative observation), but no change in the distribution of the various hydroxy fatty acids (Tables 4 and 5) was noted. Thus in the youngest specimen for which accurate quantification was possible (Brain 5) the distribution of very long-chain hydroxy acids was within the limits of variation for adult white matter. In both infants and adults no significant quantities of short chain hydroxy acids could be detected.

Regional Variation in Glycolipid Distribution

There was considerable variation in the glycolipid content of the tissues derived from four areas within the same newborn brain (Brain 5). Cerebrosides + sulfatide hexose was lowest in the frontal lobe and highest in the brainstem and cerebellum. The fatty acid pattern paralleled the cerebroside + sulfatide content, with the greatest proportion of 24:0 + 24:1 nonhydroxy fatty acids and hydroxy fatty acids being isolated from brainstem and cerebellum. These findings agree with the extent of myelin deposition within the central nervous system of

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TABLE 4 Hydroxy Fatty Acid Composition of Cerebrosides and Sulfatides from Normal Infant Brains
Fatty Acid Values in Per Cent of Total Hydroxy Fatty Acids

Fatty Acid	4F 1 Day WM C	4F 1 Day WM S	5F 1 Day Full-term ParG/W C	5F ParG/W S	5F BG C	5F BG S	5F BS + Cer C	5F BS + Cer S	9 6½ Mos. FrWM C	10 10 Mos. FrWM C	11F 15 Mos. FrWM C	12F 2 Yr FrGM C	12F 2 Yr FrWM C
16h:0	1.9	?14.1	2.0		?30.6		0.2	3.4	tr.	0.2	—	tr.	0.5
18h:0	1.1	?32.5	4.7	+	1.7	+	1.9	10.1	0.9	2.6	1.0	0.7	0.9
22h:0	5.7	—	14.2	+	1.8	+	15.2	11.6	6.8	11.8	10.3	8.4	8.7
23h:0	tr.	4.1	4.0	tr.	—	+	6.3	3.3	17.0	9.1	11.2	14.4	12.3
24h:0	25.2	21.6	43.2	+++	12.7	++	61.8	36.4	56.0	49.8	53.4	58.5	47.0
24h:1	21.2	18.6	11.7	—	25.0	+	7.3	15.8	10.7	8.0	4.0	3.0	12.6
25h:0	9.4	—	12.9		—		4.6	3.2	1.0	7.7	12.0	6.8	3.7
25h:1	5.7	—	tr.		—		—	ND*	7.4	—	—	1.7	4.3
26h:0	—	—	—		—		1.4	6.4	—	3.4	2.8	2.9	tr.
26h:1	—	—	tr.		—		—	ND*	—	2.9	3.4	2.8	9.2

For abbreviations see Table 1.
In samples where no adequate quantification was possible, hydroxy acid content is expressed in terms of tr., +, ++, and +++. Hydroxy acids from cerebrosides of Brains 2, 3, 5, FrG/W and from sulfatides of Brain 5 FrG/W and Brain 10 were not detected in quantities sufficient for estimation.
* ND, not determined.

TABLE 5 Hydroxy Fatty Acid Composition of Cerebrosides and Sulfatides from Normal Adult Brain
Fatty Acid Values in Per Cent of Total Hydroxy Fatty Acids

Fatty Acid	Formalized: 13F 19 Yr FrGM C	13F FrWM C	14F 42 Yr FrWM C	14F FrWM S	15F 50 Yr FrGM C	15F FrWM C	16F 52 Yr FrGM C	16F FrWM C	Fresh: 17 49 Yr FrGM C	17 FrWM C	18 50 Yr FrG/W S	19 55 Yr FrWM C	20 72 Yr FrGM C	20 FrWM C	21 93 Yr FrGM C	21 FrWM C	Mean Formalinized GM C	Mean Formalinized WM C	Mean Fresh GM C	Mean Fresh WM C	Mean Total GM C	Mean Total WM C
16h:0	—	0.2	0.1	tr.	tr.	—	0.3	0.4	7.4	0.7	0.2	1.8	1.0	0.1	2.5	0.4	0.1	0.2	3.6	0.7	1.8	0.5
18h:0	0.6	0.6	0.9	0.4	0.5	0.4	0.5	0.4	0.5	0.3	0.2	0.4	0.4	0.5	0.4	0.4	0.5	0.6	0.4	0.4	0.5	0.5
22h:0	9.2	6.1	7.3	12.3	6.5	6.6	9.0	7.3	4.0	5.9	5.3	3.9	5.6	7.0	5.1	5.7	8.2	7.1	4.9	5.6	6.6	6.4
23h:0	17.5	16.0	20.0	12.7	15.4	15.6	20.8	17.3	12.0	14.7	15.4	13.0	14.8	17.2	15.8	16.3	17.9	17.2	14.2	15.3	16.0	16.3
24h:0	31.4	33.5	46.8	38.5	39.0	41.5	44.4	35.6	27.6	37.7	49.1	40.3	39.1	46.0	32.1	28.6	38.3	37.3	32.9	38.2	35.6	38.8
24h:1	23.2	26.6	14.4	7.4	18.8	19.3	7.8	20.0	25.6	21.9	8.9	21.2	16.2	9.3	26.1	31.4	16.6	20.1	22.6	21.0	19.6	20.5
25h:0	8.6	6.5	8.7	8.2	7.2	6.4	6.7	8.7	8.0	9.3	11.3	5.6	10.7	7.6	9.5	6.3	7.5	7.6	9.4	7.2	18.5	7.4
25h:1	2.9	2.7	2.0	tr.	4.4	3.8	5.7	3.2	5.0	2.2	2.3	5.9	3.4	5.3	3.0	5.5	4.3	2.9	3.8	4.7	4.1	3.8
26h:0	3.1	3.2	2.1	2.8	3.2	2.8	2.0	3.3	1.2	2.4	2.1	1.1	4.1	2.7	0.5	1.5	3.4	2.8	1.9	1.9	2.3	2.4
26h:1	2.9	3.3	2.8	3.0	4.0	3.4	2.6	3.6	6.9	4.3	2.4	6.2	4.6	3.8	4.1	3.3	3.2	3.3	5.2	4.4	4.2	3.8

For abbreviations see Table 1.

the newborn infant as demonstrated by histologic techniques (24).

DISCUSSION

Cerebrosides and Sulfatides in Adult Brain

Values for cerebroside and sulfatide content found in the present study agree quite well with those obtained by previous workers who used column chromatography to separate human cerebral lipids, notably Svennerholm (3) and O'Brien and Sampson (8). We too found that cerebrosides and sulfatides from adult GM and WM contain predominantly long-chain fatty acids (C_{22}–C_{26}). The major nonhydroxy acid was 24:1, while 24h:0 was the principal hydroxy acid. No significant amounts of polyunsaturated or branched-chain fatty acids were detected.

Effects of Formalin Fixation

The fatty acid composition of cerebrosides and sulfatides derived from formalinized tissue did not differ significantly from that of fresh brains. Some decrease in the sulfatide content of formalinized WM was probably reflected in the cerebroside:sulfatide ratio, which was significantly higher than that obtained by Austin (25). The method employed in our laboratory for determining the sulfatide content of adult tissues includes ceramide dihexosides with sulfatides and would tend to yield falsely low cerebroside:sulfatide ratios.

Fatty Acid Composition of Cerebrosides and Sulfatides from Adult Brain

Since identical methods were used for the purification, hydrolysis of the glycolipids, and quantification of the fatty acids from each of the nine adult brains examined

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by us, the variations in the relative amounts of saturated and unsaturated fatty acids are probably not artifactual. It is perhaps significant that despite the variation in the ratio of saturated to unsaturated fatty acids in cerebrosides isolated from adult WM, the total percentage of fatty acids of a particular carbon length was almost constant. The coefficient of variation for both hydroxy and nonhydroxy C_{24} fatty acids was less than 5% (Table 6). Quantification of the other fatty acid pairs was less accurate since they accounted for a smaller proportion of the total cerebroside fatty acids, and their coefficient of variation ranged from 9.4 to 12.7%.

Two explanations can be offered for this finding: it may reflect a dynamic equilibrium between saturated and unsaturated fatty acids, which has become fixed at time of death, or in analogy with the observations by Poneleit and Alexander on maize (26), this ratio may be genetically fixed for each fatty acid pair but different in each individual.

Effects of Maturation

The major portion of the small amount of lipid-soluble hexose present in immature brain was found in the form of glycolipids other than cerebrosides and sulfatides. Their exact structure is still uncertain, but according to their chromatographic behavior they are probably hematosides and globosides whose presence in brain has been previously demonstrated. Hematosides or hexosamine-free gangliosides were detected in brain by Klenk and Gielen (27), amongst others, while globosides (trihexosides containing hexosamine but no sialic acid) were isolated from the brains of patients with Tay-Sachs disease by Svennerholm and Raal (28), and Makita and Yamakawa (29).

Svennerholm (30) has observed a considerable amount of glucocerebrosides and other glucose-containing glycolipids in fetal brains. Judging from the relative values of our anthrone and orcinol determinations on the various Florisil eluates, we would suspect that the cerebroside + sulfatide fraction (fraction III) contained mostly galactose, but that the more polar methanol eluate (fraction IV) contains both glucose and galactose.

With maturation the fatty acid distribution in cerebrosides and sulfatides undergoes major changes. As has already been shown for total cerebral lipids (31), the hydroxy fatty acid concentration was low in the immature brain. However, even in the most immature specimens it was evident that C_{24} was the principal hydroxy fatty acid, as in adult brain.

Even though cerebrosides isolated from the most immature brains were almost completely devoid of hydroxy fatty acids, their R_f values on TLC did not differ significantly from those of cerebrosides isolated from adult WM, and a separation into two distinct bands was readily obtained. For example, in Brain 3 the R_f values of the two cerebroside bands in chloroform–methanol 85:15 were 0.30 and 0.24 respectively, as contrasted with 0.31 and 0.25 for cerebrosides purified from adult WM. In another run, cerebrosides from WM of Brain 4 showed R_f values of 0.28 and 0.23 in the same solvent, as compared with a standard from adult WM of 0.32 and 0.24.

TABLE 6 Coefficient of Variation of Major Saturated and Unsaturated Fatty Acid Pairs in White Matter Cerebrosides of Nine Adult Brains

Fatty Acid Values in Per Cent of Total Nonhydroxy or Hydroxy Fatty Acids

Fatty Acid Pair	Mean	Range	Standard Deviation	Coefficient of Variation
18:0 + 18:1	8.9	8.1–10.5	0.84	9.4
24:0 + 24:1	58.9	54.4–62.6	2.8	4.8
25:0 + 25:1	14.4	11.4–15.9	1.4	9.7
24h:0 + 24h:1	59.3	55.3–61.5	2.4	4.0
25h:0 + 25h:1	11.0	8.7–12.9	1.4	12.7

When chromatographing cerebrosides derived from adult WM, we have found that the fast band consisted exclusively of kerasin cerebrosides having both long- and short-chain fatty acids. The slower cerebroside band contained 95% cerebron cerebrosides and 5% kerasin cerebrosides. Stearic acid constituted about 75% of the fatty acids in the latter fraction.[1] We therefore suspect that the chromatographic separation of cerebrosides purified from infant brains may in part be on a basis of fatty acid chain length, a phenomenon previously described for the sphingomyelins (32).

The amount of nonhydroxy fatty acids having a chain length greater than 22 was low in the most immature tissues and increased progressively with age. The concentration of 24:0 rose more rapidly than that of 24:1, which constituted the major nonhydroxy acid component of adult cerebrosides and sulfatides.

The changes in the composition of the fatty acid fraction that accompany the deposition of myelin suggest the presence of two species of cerebrosides and sulfatides within the central nervous system, as follows.

The first, present in brain before the onset of extensive myelination, contains principally 16:0, 18:0, and 18:1, with very long-chain and hydroxy fatty acids as relatively minor components. These glycolipids probably form a portion of the extramyelin cerebrosides and sulfatides.

The second type of cerebroside and sulfatide is one that appears with myelination and consists mainly of the very long-chain and hydroxy fatty acids. In the adult white matter, it constitutes the overwhelmingly greater portion of cerebrosides and sulfatides. In gray matter, mainly as a consequence of the presence of radially

[1] Smith, M., and J. H. Menkes, unpublished data.

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oriented myelinated fibers (33), it also forms the major portion of the cerebroside–sulfatide fraction.

The presence of these two types of cerebrosides and sulfatides corresponds well with our present knowledge of fatty acid biosynthesis. Evidence from a number of sources has indicated that the biosynthesis of very long-chain fatty acids requires two systems. The first of these synthesizes the "primary" fatty acid, mainly 16:0, although data of Hajra and Radin suggest that 18:0 may also serve this function for cerebroside synthesis (34). The second is a chain lengthening process via the successive addition of acetate units to the primary fatty acid. These two systems may occur at different sites within the cell.

It is likely that while the first system is active in the immature brain, the second system does not function until myelin is deposited. The factors responsible for the initiation of chain elongation or its repression are completely unknown.

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References

1. Radin, N. S., and T. Akahori. *J. Lipid Res.* **2:** 35, 1961.
2. O'Brien, J. S., D. L. Fillerup, and J. R. Mead. *J. Lipid Res.* **5:** 109, 1964.
3. Svennerholm, L. In *Brain Lipids and Lipoproteins and the Leucodystrophies*, edited by J. Folch and H. Bauer. Elsevier Publishing Co., Amsterdam, 1963, p. 104.
4. Bernhard, K., and P. Lesch. *Helv. Chim. Acta* **46:** 1798, 1963.
5. Menkes, J. H., M. Philippart, and R. E. Fiol. *J. Pediat.* **66:** 584, 1965.
6. Kishimoto, Y., and N. S. Radin. *J. Lipid Res.* **1:** 79, 1959.
7. Ställberg-Stenhagen, S., and L. Svennerholm. *J. Lipid Res.* **6:** 146, 1965.
8. O'Brien, J. S., and E. L. Sampson. *J. Lipid Res.* **6:** 545, 1965.
9. Brante, G. *Acta Physiol. Scand.* **18 Suppl. 63:** 1949.
10. Rodnight, R. *J. Neurochem.* **1:** 207, 1957.
11. Heslinga, F. J. M., and F. A. Deierkauf. *J. Histochem. Cytochem.* **10:** 704, 1962.
12. Radin, N. S., F. B. Labin, and J. R. Brown. *J. Biol. Chem.* **217:** 789, 1955.
13. Svennerholm, L. *J. Neurochem.* **1:** 42, 1956.
14. Carroll, K. K. *J. Lipid Res.* **2:** 135, 1961.
15. Menkes, J. H., M. Philippart, and D. B. Clark. *Arch. Neurol.* **11:** 198, 1964.
16. Norton, W. T., and M. Brotz. *Biochem. Biophys. Res. Commun.* **12:** 198, 1963.
17. Kochetkov, N. K., I. G. Zhukova, and I. S. Glukhoded. *Biochim. Biophys. Acta* **60:** 431, 1962.
18. Philippart, M., B. Rosenstein, and J. H. Menkes. *J. Neuropathol. Exptl. Neurol.* **24:** 290, 1965.
19. Sweeley, C. C., R. Bentley, M. Makita, and W. W. Wells. *J. Am. Chem. Soc.* **85:** 2497, 1963.
20. Sweeley, C. C., and E. A. Moscatelli. *J. Lipid Res.* **1:** 40, 1959.
21. Woodford, F. P., and C. M. van Gent. *J. Lipid Res.* **1:** 188, 1959.
22. Cumings, J. N., H. Goodwin, E. M. Woodward, and G. Curzon. *J. Neurochem.* **2:** 289, 1958.
23. Brante, J. *Acta Physiol. Scand.* **18 Suppl. 63:** 1949.
24. Dekaban, A. *Neurology of Infancy.* Williams and Wilkins Co., Baltimore, 1959, pp. 2–4.
25. Austin, J. *Arch. Neurol.* **9:** 207, 1963.
26. Poneleit, C. G., and D. E. Alexander. *Science* **147:** 1585, 1965.
27. Klenk, E., and W. Gielen. *Z. Physiol. Chem.* **323:** 126, 1961.
28. Svennerholm, L., and A. Raal. *Biochim. Biophys. Acta* **53:** 422, 1961.
29. Makita, A., and T. Yamakawa. *Japan J. Exptl. Med.* **33:** 361, 1963.
30. Svennerholm, L. *J. Neurochem.* **11:** 839, 1964.
31. Eng, L. G., B. Gerstl, R. B. Hayman, Y. L. Lee, R. W. Tietsort, and J. K. Smith. *J. Lipid Res.* **6:** 135, 1965.
32. Pilz, H., and H. Jatzkewitz. *J. Neurochem.* **11:** 603, 1964.
33. Lewin, E., and H. H. Hess. *J. Neurochem.* **12:** 213, 1965.
34. Hajra, A. E., and N. S. Radin. *J. Lipid Res.* **4:** 270, 1963

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