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THE LIPID COMPOSITION OF TISSUES IN NIEMANN-PICK DISEASE

BY

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ABSTRACT

The lipid composition of liver, spleen, and brain in Niemann-Pick disease was examined. Sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol were determined quantitatively. In liver and spleen, a marked increase in sphingomyelin concentration was found, and cholesterol accumulation in all the tissues.

The fatty acid pattern of these sphingomyelins differed significantly from other phospholipids, and further showed special feature in brain compared with those in liver and spleen. However, as regards other phospholipid components, the fatty acid pattern in liver was distinguished from other tissues.

INTRODUCTION

In 1914, A. Niemann[1] first reported on this disease and L. Pick[2] subsequently added a detailed pathologic description and established a clear differentiation between this disease, now known as the Niemann-Pick disease, and Gaucher's disease. On the chemical disturbance of tissue lipid in this syndrome, E. Klenk[3] demonstrated that the increased tissue phospholipid due to an abnormal accumulation of sphingomyelin, and recently J. A. Balint and his associates[4] showed that the lipid increased in brain as well as in liver and spleen. L. Svennerholm[5,6] reported on the result of fatty acid analysis of sphingomyelin in liver, spleen, and brain from the Niemann-Pick disease patient. Though studies on the lipid composition of these tissues have been reported by many workers, the data are not yet sufficient to summarize precisely the metabolic abnormality of lipids, because of lacking in information on phospholipids other than sphingomyelin. We had an opportunity to study the lipid composition of certain tissues from a patient with the Niemann-Pick disease, and this paper describes the result of detailed analysis of total lipid component and their fatty acid patterns.

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Materials and Method

The samples for this study were supplied by the Toranomon Hospital in Tokyo and the diagnosis was supported by major clinical and pathological examinations. Spleen was obtained by biopsy from a 12-month-old boy, and brain white matter and liver by autopsy from the same patient at 19-months. These tissues were stored in a frozen state until subjected to analysis.

Lipid Extraction Lipid was extracted with chloroform-methanol (2:1, v/v), and the extract was washed by J. Folch's method[7].

Analysis of Neutral Lipid Neutral lipid fraction was isolated from the total lipid extract by silicic acid column chromatography, and then fractionated into each component by rechromatographic separation according to the method of E. J. Barron and D. J. Hanahan[8]. Aliquots of each eluted fractions of neutral lipid were taken and subjected to weight determination, cholesterol assay (by Libermann-Burchard reaction), and thin-layer chromatography on silica gel G. Free fatty acid and triglyceride were separately determined by T. Olivecrona's method[9].

Analysis of Phospholipid Qualitative and quantitative analysis of phospholipid fractions was made by the paper chromatographic examination of water-soluble phosphate esters, obtained by mild alkaline and subsequent acid hydrolysis of the lipids as described by R. M. C. Dawson[10].

Preparation of Phospholipid For analysis of fatty acid pattern of each phospholipid fraction, column chromatographic purification was carried out on silicic acid[11]. The lipid extracted from the tissues was loaded with the aid of chloroform and the column was successively eluted with chloroform, chloroform-methanol 9:1 (v/v), 4:1, 3:2, 1:4, and finally with methanol. The purity of phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine (lecithin), and sphingomyelin fractions was checked by thin-layer chromatography, and the fraction contaminated with other components was further separated by silicic acid rechromatography. Sphingomyelin was purified by mild alkaline hydrolysis to remove glycerophosphatides according to C. C. Sweely's method[12]. Phosphatidylethanolamine and phosphatidylserine were separated by preparative thin-layer chromatography; the spots corresponding to each phospholipid on the chromatogram were separately scraped off and extracted more than twice with chloroform-methanol (2:1).

Gas-Liquid Chromatographic Analysis of Lipids Methyl esters of fatty acids were prepared from each lipid fraction by transesterification with 5% methanolic hydrochloric acid[13] and analyzed by gas-liquid chromatography; a Shimadzu GC-1C instrument equipped with a hydrogen flame ion detector, and the column (0.3×260 cm) containing 60–80 mesh Shimalite W coated with 5% diethyleneglycol succinate polyester, was used at

198° with nitrogen flow rate of 30 ml/min.

Thin-Layer Chromatography Neutral lipid was chromatographed on a thin-layer of silica gel (Weko-Gel B-O with 10% gypsum as a binder) prepared by Desaga's applicator[14]. The solvent system employed was petroleum ether-ether-acetic acid (90:10:1, v/v/v)[15]. The spot was detected by spraying 1% iodine in methanol[14] or 10% phosphomolybdic acid in 95% ethanol[14]. The developing solvents for phospholipid separation consisted of chloroform-methanol-water (65:25:4, v/v/v)[17]. The spot was detected by molybdenum blue reagent[16], ninhydrin solution[17], and Dragendorff reagent[17] sprays.

Results

The lipid content and its distribution in Niemann-Pick disease tissues are given in Table 1. The total lipid content was 15.5% in liver and 13.0% in spleen, and the brain contained lipid in much less amount than in both tissues. The lipid extract was fractionated on silicic acid column into neutral lipids and phospholipids by eluting with chloroform and then with chloroform-methanol (1:1) followed by methanol. Each neutral lipid and phospholipid fraction were evaporated to dryness to weigh the amounts.

Table 1. Lipid distribution in Niemann-Pick disease tissues

	Liver	Spleen	Brain
Total lipid in wet tissue (%)	15.5	13.0	7.6
Phospholipid in total lipid (%)	74.9	62.3	68.8
Neutral lipid in total lipid (%)	19.3	24.2	26.7
Recovery (%)	94.2	86.5	95.5

Table 2. Column chromatographic fractionation of neutral lipid

Fraction	Solvent	Elution volume (ml)	Liver (mg)	Spleen (mg)	Brain (mg)
			239[1]	220[1]	71[1]
I	Hexane	100	2	2	1
II	15% Benzene in hexane	100	9	1	1
III	5% Ether in hexane	200	35	24	11
IV	15% Ether in hexane	200	160	165	48
V		200	14	12	4
VI	30% Ether in hexane	100	2	1	4
VII	Ether	100	3	4	2
Recovery (%)			94	95	100

[1] Amount applied to column chromatography.

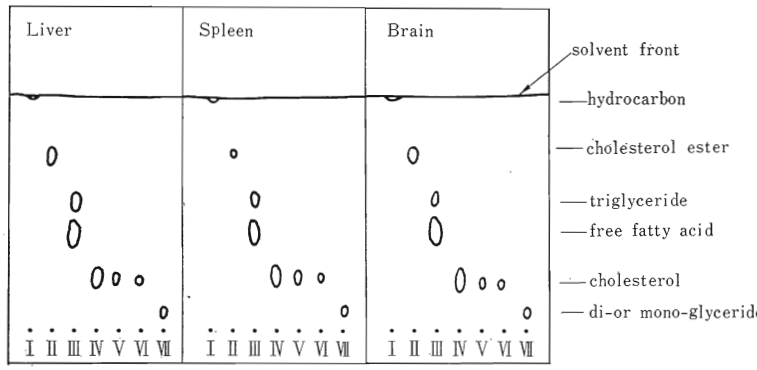

Fig. 1. Thin-layer chromatogram of neutral lipid fraction in Niemann-Pick disease tissues

Neutral Lipid The neutral lipid was further separated into each component by silicic acid column rechromatography. The solvent system for elution, and the result of their analysis are given in Table 2. A typical thin-layer chromatogram of each fraction from three kinds of tissues indicates a satisfactory separation of the lipid classes (Fig. 1).

In this thin-layer chromatogram, the spot of Fraction II showed the Rf value corresponding to that of the authentic cholesteryl palmitate, and Fractions IV, V, and VI all showed Rf values of cholesterol. Fraction III was found to separate into two spots which were identified to be triglyceride and free fatty acid by comparing their Rf values with those of authentic samples. Furthermore, a trace of lipid, probably mono- or di-glyceride, or its mixture, was found in Fraction VII, but it was not analyzed exactly. The amount of neutral lipid composition thus separated is summarized in Table 3. In these tissues, a marked abundance of cholesterol was found, while triglyceride was contained only in a small amount. This result is very different from the neutral lipid pattern in many normal mammalian tissues, in which triglyceride has been found to be the main component of neutral lipid. Cholesterol ester was not large in amount, but free fatty acid was estimated in a considerable amount. The neutral lipid patterns of these three tissues indicated no significant difference.

Phospholipid Table 4 shows the composition of major phospholipid fractions of liver, spleen, and brain, which were examined by mild hydrolysis and paper chromatographic method of R. M. C. Dawson. On the basis of phosphorus analysis giving a satisfactory recovery, it was shown that the sphingomyelin concentration accounted for about 70% of total phospholipid in liver and spleen, and about 50% in brain. This result showed that sphingomyelin abnormally accumulated in these tissues

Table 3. Neutral lipid composition of Niemann-Pick disease tissues

Component	Liver (%)	Spleen (%)	Brain (%)
Hydrocarbon	0.9	1.0	1.4
Cholesterol ester	4.0	0.5	8.5
Cholesterol	78.2	85.2	78.8
Triglyceride	8.0	4.0	2.8
Free fatty acid	7.5	7.5	4.7
Di- or mono-glyceride	1.3	1.9	2.8

Table 4. Phospholipid composition of Niemann-Pick disease tissues

Component	Liver (%)	Spleen (%)	Brain (%)
Phosphatidylethanolamine	9.9	6.3	9.3
Phosphatidylserine	2.0	4.1	5.5
Phosphatidylcholine	8.3	10.6	19.3
Phosphatidylinositol	1.7	1.8	0.3
Sphingomyelin	73.7	67.2	48.2
Plasmalogen	0.7	2.8	8.0
Glycerylether phospholipid	trace	0.6	0.5
Recovery (%)	100.5	94.1	91.5

Table 5. Composition of free fatty acid

Chain length	Liver (%)	Spleen (%)	Brain (%)
12 : 0	0.5		
14 : 0	1.3	1.1	1.2
16 : 0	16.2	16.7	21.3
16 : 1	6.1	3.4	3.3
16 : 2			0.7
18 : 0	6.7	12.9	23.1
18 : 1	53.7	36.2	24.6
18 : 2	6.8	11.7	1.2
18 : 3	0.6	0.3	
20 : 1		0.6	
20 : 2	1.2	2.4	
20 : 3	4.5	10.7	13.5
24 : 0		2.1	4.2
Unknown	2.4	2.5	6.9
Saturated fatty acid (%)	24.7	32.2	49.8

All figures are mole %.

in agreement with the observation of other previous investigation. The phospholipid fraction consists of phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, and phosphatidylinositol in addition to sphingomyelin, but the phosphatidylinositol content was very small in

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Table 4. Phospholipid composition of Niemann-Pick disease tissues

Wrong

Component	Liver (%)	Spleen (%)	Brain (%)
Phosphatidylethanolamine	9.9	6.3	9.3
Phosphatidylserine	2.0	4.1	5.5
Phosphatidylcholine	8.3	10.6	19.3
Phosphatidylinositol	1.7	1.8	0.3
Sphingomyelin	73.7	67.2	48.2
Plasmalogen	0.7	2.8	8.0
Glycerylether phospholipid	trace	0.6	0.5
Recovery (%)	100.5	94.1	91.5

Correct

Component	Liver (%)	Spleen (%)	Brain (%)
Phosphatidylethanolamine	9.9	6.3	9.3
Phosphatidylserine	2.0	4.1	5.5
Phosphatidylcholine	8.3	10.6	19.3
Phosphatidylinositol	1.7	1.8	0.3
Sphingomyelin	73.7	67.2	48.2
Plasmalogen	0.7	2.8	8.0
Glycerylether phospholipid	trace	0.6	0.5
Unknown	4.2	0.7	—
Recovery (%)	100.5	94.1	91.5

Page		Wrong	Correct
215	Table 5	20 : 3	20 : 4
216	Table 6	20 : 3	20 : 4
217	Table 7	20 : 3	20 : 4
218	Table 8	20 : 3	20 : 4

brain. Plasmalogen significantly contained in brain phospholipid was found to be ethanolamine plasmalogen by paper chromatographic separation of mild acid hydrolysate. The glyceryl ether phospholipid accounted for little in all of these tissues.

Free Fatty Acid The composition of free fatty acid in neutral lipid fraction was examined by gas-liquid chromatography as shown in Table 5. The free fatty acid composition in brain had practically the similar pattern as that in the spleen excepting the low content of octadecadienoic acid (18:2), while octadecenoic acid (18:1) showed extremely high concentration in liver and amounted to about eight times that of stearic acid (18:0). The ratio of saturated acid to unsaturated acid in brain was higher than in liver and spleen.

Table 6. Fatty acid composition of sphingomyelin

Chain length	Liver (%)	Spleen (%)	Brain (%)
14 : 0	1.8	3.3	
16 : 0	40.5	50.8	6.1
16 : 1	0.4		
16 : 2	0.3		
18 : 0	15.3	11.7	85.0
18 : 1	3.2	1.8	
18 : 2	0.7		
20 : 0	5.0	4.7	2.5
20 : 1	0.2		
20 : 3	3.8		
21 : 0	0.2		
22 : 0	6.9	7.8	1.9
22 : 1	2.0		2.8
23 : 0	1.9	1.7	
23 : 1	0.3		
24 : 0	2.6	4.7	
24 : 1	13.9	13.5	1.7
Saturated fatty acid (%)	74.2	83.0	95.5

All figures are mole %.

Fatty Acid Composition of Phospholipid Fraction The fatty acid pattern of sphingomyelin (Table 6) in liver and spleen showed a similar feature; namely, the major fatty acid was palmitic acid (16:0), being present in larger amount than stearic and tetracocenoic acid (24:1), while in brain, predominant stearic acid accounted for 85% of the total fatty acid and tetracocenoic acid was contained in a low amount in comparison with liver and spleen. Sphingomyelin fatty acids consisted of a significant amount of saturated fatty acids in all tissues, especially in brain sphingomyelin. The fatty acid composition of phosphatidylcholine fraction is

Table 7. Fatty acid composition of phosphatidylcholine

Chain length	Liver (%)	Spleen (%)	Brain (%)
14 : 0	0.5	0.6	0.4
16 : 0	23.0	34.3	31.4
16 : 1	9.6	2.2	1.8
16 : 2	0.5	0.5	
18 : 0	7.6	15.8	22.7
18 : 1	38.3	17.4	23.6
18 : 2	5.8	5.8	
20 : 0	0.5	0.5	
20 : 1	0.5		
20 : 2	3.8		
20 : 3	11.0	12.7	11.7
21 : 1	1.7	1.4	0.9
21 : 2	0.6	1.3	1.0
21 : 3	0.9	1.5	1.9
24 : 1		2.9	
Unknown	1.7	3.1	4.6
Saturated fatty acid (%)	31.6	51.2	54.5

All figures are mole %.

shown in Table 7. The fatty acid pattern of spleen phosphatidylcholine was similar to that of brain phosphatidylcholine, showing the ratio of octadecenoic acid to stearic acid to be practically equivalent. The fatty acid in liver had lower content of saturated fatty acid than in spleen and brain. The fatty acid pattern of phosphatidylethanolamine in liver, summarized in Table 8, was different from that in spleen and brain. Namely, the ratio of octadecenoic acid to stearic acid in liver was larger than that in spleen and brain, being similar to phosphatidylcholine fatty acid pattern. The fatty acid pattern of phosphatidylserine from spleen and brain, which is given in Table 8, is also similar to the fatty acid composition of phosphatidylcholine and phosphatidylethanolamine, except for the saturated fatty acid ratio being lower than in phosphatidylcholine and higher than in phosphatidylethanolamine.

Discussion and Conclusion

In early studies, the increasing amount of lipid in Niemann-Pick disease tissues has been shown by many workers. A. C. Crocker and S. Farber[18] reported that liver and spleen contained 2 to 6 times more lipid than in normal tissues but brain white matter did not show any increase in lipids, though J. A. Balint and his associates[4] showed the increased amount of lipid in brain. The present result, as listed in Table

Table 8. Fatty acid composition of phosphatidylethanolamine and phosphatidylserine

Chain length	Phosphatidylethanolamine			Phosphatidylserine	
	Liver (%)	Spleen (%)	Brain (%)	Spleen (%)	Brain (%)
14 : 0	0.4	0.4	0.6	0.6	0.7
15 : 1		1.0	4.2		0.5
16 : 0	4.6	9.3	10.4	4.1	3.6
16 : 1	2.7	1.0	2.3	0.8	1.0
16 : 2	0.4	0.5	1.4	0.4	0.3
17 : 1		0.4			0.6
18 : 0	8.4	24.2	27.3	39.7	37.3
18 : 1	70.6	25.0	21.1	29.9	23.4
18 : 2	4.2	5.0		2.6	
18 : 3	0.6				
20 : 0		0.4		0.7	0.9
20 : 1	0.7	0.5	1.2	0.7	1.6
20 : 2	1.2				2.0
20 : 3	5.7	18.0	12.7	6.2	3.1
22 : 1		2.1			6.7
22 : 3		4.6	6.0	4.1	2.7
24 : 1		1.2			2.2
Unknown	0.5	6.4	12.8	10.2	13.4
Saturated fatty acid (%)	13.4	34.3	38.3	45.1	42.5

All figures are mole %.

Table 9. Lipid content of tissues in Niemann-Pick disease

		Liver	Spleen	Brain
Present result	Total lipid	15.5	13.0	7.6
	Neutral lipid	3.0	3.1	2.0
	Phospholipid	11.6	8.1	5.2
	Sphingomyelin	8.5	5.4	2.5
Normal values[1]	Total lipid	3～4	2～3.5	7.8～14.0
	Phospholipid	1.5～2.5	1～2	4.0～6.0
	Sphingomyelin	0.1～0.25	0.1～0.5	1.0～1.8

Values expressed as percent of fresh weight.

[1] From Crocker and Farber[18].

9, indicates that the total lipid content is markedly higher than the normal values in spleen and liver, while remaining normal in brain. These features are also found in phospholipid content. Many of the early studies showed the proportional rate of phospholipid in total lipid to be 40–60% in liver, spleen, and brain in Niemann-Pick patient and J. A. Balint and his associates[4] recently found phospholipid content amounted to 77.3%

of total lipid in liver, 76.7% in spleen, and 54.1% in brain. The results obtained in this experiment somewhat differed from their values, and the cause would be attributed to the diversity of the symptoms and age rather than to analytical method. However, the increase of lipid would due to phospholipid accumulation rather than neutral lipid in liver and spleen, since neutral lipid content amounted only 20% to 30% of total lipid in these tissues. In the analysis of these tissue phospholipids, definite sphingomyelin increase in brain and spleen has been reported by E. Klenk[2]. A. C. Crocker and S. Farber[18] showed that sphingomyelin content accounted for 0.26–6.58% in liver, 1.36–4.57% in spleen, and 0.40–1.59% in brain white matter, compared to the respective figures of 0.1–0.25%, 0.1–0.5%, and 1.0–1.8% in normal cases (Table 9). The present results also exhibited a considerable accumulation of sphingomyelin in liver and spleen but a significant increase was not observed in brain, though the values were slightly higher than those reported by A. C. Crocker and S. Farber. Converting these values into ratio of total phospholipids as shown in Table 4, the result is compared with the figures reported by S. J. Thannhauser[19] of 60.1%, 76.4%, and 28.6% in respective tissue. Data on analysis of the individual phospholipid classes other than sphingomyelin have been scarcely reported in the case of Niemann-Pick disease. E. Klenk[20] reported a slight increase of phosphatidylcholine and E. Chargaff[21] found cephalin content elevated in the spleen, though they lacked in precise analysis. In the present work, phospholipids were fractionated into phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, and phosphatidylinositol, resulting in much smaller content than that of sphingomyelin, as listed in Table 4. From a consideration of these results, it would be reasonable to conclude that the increasing amount of total lipid due to sphingomyelin accumulation in liver and spleen. The plasmalogen content, showing 8.0% of the lipid phosphorus in brain, 0.7% in liver, and 2.8% in spleen, was in close agreement with the findings by G. Schmidt and his associates[22]. Brain plasmalogen was now identified as ethanolamine derivatives in this experiment. The increasing amount of cholesterol in these tissues was found, showing abnormal accumulation amounted to 15.1% of total lipid in liver, 20.6% in spleen, and 21.0% in brain in agreement with the result of A. C. Crocer and S. Farber[18]. As regards neutral lipid component other than cholesterol, the results indicated the abnormally small content of triglycerides and the presence of considerable amount of free fatty acids, but it is not apparent whether these strange evidence is characteristic in Niemann-Pick disease.

E. Klenk[3] found stearic acid was the prominent fatty acid in fatty acid composition of brain sphingomyelin in the Niemann-Pick disease, and L. Svennerholm and his associates[5] recently found low content of nervonic

acid (24:1), which normally makes up 16% of sphingomyelin fatty acid in brain. The present result revealed nearly the same fatty acid pattern of brain sphingomyelin as their findings, while sphingomyelin in liver and spleen had the different pattern of fatty acid, that is, palmitic and nervonic acid were extremely higher but stearic acid showed lower content than that in brain sphingomyelin. Little is known concerning fatty acid distribution of other phospholipid components in Niemann-Pick disease patient. In this study, it was found that fatty acid composition of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine gave the relatively identical pattern for the spleen and brain, differing from that in liver. The molar ratio of octadecenoic acid to stearic acid in these phospholipids was higher in liver than in spleen and brain, and phosphatidylcholine and phosphatidylethanolamine in liver was found to have less of the saturated fatty acid than in spleen and brain. These evidence may suggest that each tissue has characteristic enzyme in phospholipid metabolism. R. O. Brady[23] reported that the activity of sphingomyelin-cleaving enzyme decreased in liver of patient with Niemann-Pick disease comparing to normal tissue, and the deficiency of the activity may be caused the accumulation of the excessive quantities of sphingomyelin. The fatty acid pattern of sphingomyelin accumulated in abundant amount in liver and spleen considerably differs from that in brain in which significant increase of sphingomyelin was not observed. These evidence may be explainable by the substrate specificity of sphingomyelin-cleaving enzyme to fatty acid composition of this phospholipid. On the other hand, it is not obvious whether the difference of fatty acid pattern of phospholipid other than sphingomyelin obtained in the present study is characteristic in Niemann-Pick disease. To clarify this problem, it will be necessary to heap up the detailed data of many cases of this disease.

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