Sapporo Med. J. ***34*** (2/3) 109~120 (1968)

Studies on Liver Phospholipids Metabolism of Sphingomyelin, Lecithin and Cephalin in Subcellular Fractions

Masako AKIYAMA and Toshio SAKAGAMI
Department of Biochemistry (Section 2), Sapporo Medical College
(Chief: Prof. T. Sakagami)

Introduction

It has been reported that sphingomyelin from rat plasma[1], from various rat tissues[2] and also from human plasma[3-5] could be separated into two fractions by thin-layer chromatography (TLC). With large amounts of sphingomyelin from human plasma and erythrocytes[6] it was found that the main difference between two sphingomyelins existed in fatty acid moiety, that is, one of the sphingomyelins, slow-moving sphingomyelin (Sph-S), contained $C_{16:0}$ fatty acid as a main fatty acid and the other, fast-moving sphingomyelin (Sph-F), did C_{24} fatty acids predominantly. Only one report could be found on the composition of the fatty acids of rat liver sphingomyelin[16], but no report has been presented yet on Sph-F and Sph-S. Then it was tried to investigate the nature of these two sphingomyelins. At the same time it was also determined how these two sphingomyelins were distributed in each subcellular fraction of rat liver. Furthermore the metabolism of those two sphingomyelins as well as lecithin and cephalin was studied with ^{32}Pi in each subcellular fraction.

Materials and Methods

Male rats (body weight ca. 200 g) fasted for 12 hrs were used in all experiments. 10 mC of inorganic (^{32}P)-Phospate was injected intraperitoneally.

The animals were sacrificed at 0.5, 1, 3, 6 and 12 hrs by heart puncture. Subcellular fractionation was carried out according to the method of de Duve et al.[7] with a slight modification (conf. Fig. 1). The liver was homogenized with 4 volume of 0.25 M sucrose solution and centrifuged at 900×g for 15 min. The sediment was rehomogenized with the same volume of 0.25 M sucrose solution and centrifuged at the same manner. Each supernatant was combined and centrifuged at 3,500×g (10 min), 18,000×g (20 min), 105,000×g (60 min) to prepare mitochondria, 18,000×g fraction microsomes, and cell sap, respectively.

The lipids of these fractions were extracted with 25 volume of chloroform-methanol (2 : 1 v/v) and washed by Folch's procedure[8]. Then a large part of extract were subjected to mild alkaline and acid hydrolysis with the procedure of Dawson[9]. The alkaline and acid-stable lipid fraction was separated into Sph-F and Sph-S on thin layer plates (Merck, Kieselgel G, 0.5 mm thickness) with chloroform-methanol-water (65 : 25 : 4 by volume) as a developing solvent. These lipids were detected by 2′, 7′-dichlorofluorescein (0.02% of ethanol solution, pH, 7.0). Sph-F and Sph-S on thin layer plates were scraped off and

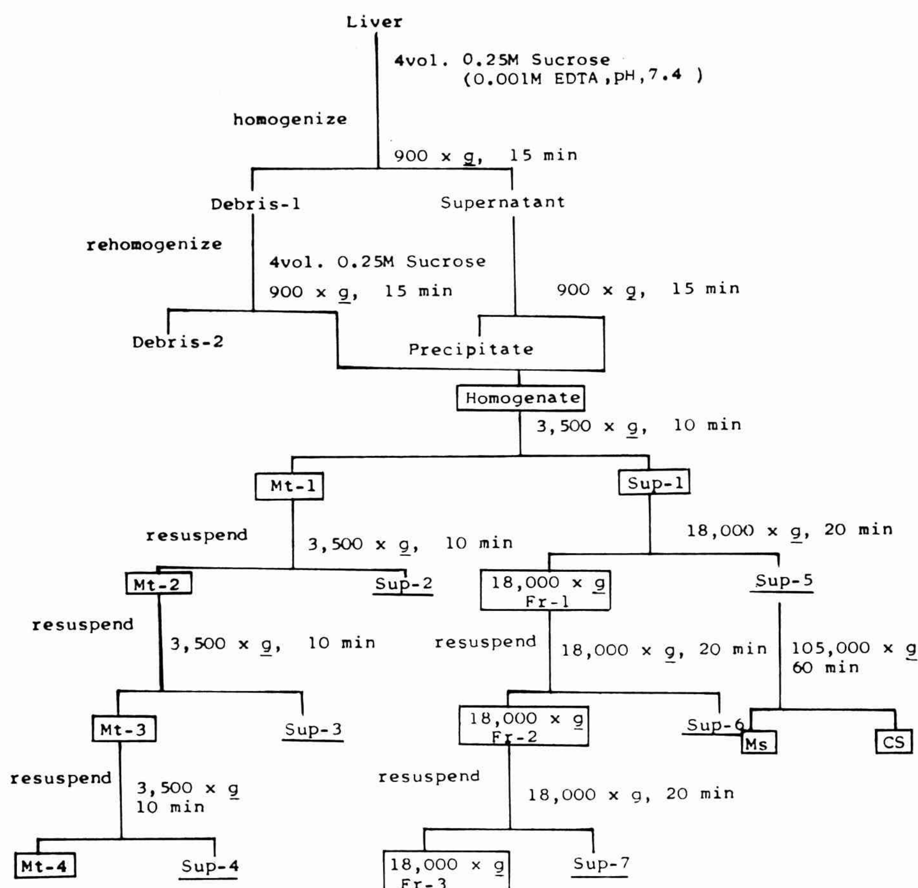

Fig. 1 Subfactionation procedure of rat liver.
Mt: Mitochondria, Ms: Microsomes, CS: Cell supernatant.

extracted with methanol three times by refluxing, respectively. The combined extracts were evaporated to dryness *in vacuo* below 50°C and the residues were redissolved in chloroform-methanol (2:1 v/v) and made up to a known volume. The solution thus obtained was used for gas liquid chromatography of fatty acids from sphingomyelin and for determinations of phosphorus and radioactivity.

The fatty acids from total sphingomyelin, Sph-S and Sph-F were methanolized with 5% H_2SO_4-methanol (v/v) at 70°C for 6 hrs[5]. Gas-liquid chromatography was carried out by Hitachi gas chromatograph KGL-2B. Aliquots of lipid extracts from cell fractions were thin-layer-chromatographed and lecithin and cephalin were scraped off. Lipid-phoshorus was determined by Bartlett's procedure[10] with solutions which were made by exhaustive extraction from Kieselgel-G with methanol or directly by the method of Parker and Peterson[11] with non-extracted one. The results were identical. Therefore the direct method was applied for sphingomyelin-phosphorus determination in some cases.

Protein was analyzed by the method of Lowry et al.[12]. For the measurement of radioactivity Geiger-Müller counter (Nihon Musen Co.) was used. Glucose-6-phosphatase

(G-6-Pase) activity was determined by the method of Langdon and Weakley[13] and acid phosphatase by de Duve et al.[14] with some modification in the presence of 0.1% Triton ×-100 as a releasing agent.

Result

The fatty acid composition of sphingomyelin, Sph-F and Sph-S from rat liver are shown in Table 1. Sphingomyelin contains C_{24} and C_{16} fatty acids as the major fatty acids. The result is very similar to the data obtained by human plasma[6] and human splanchnic tissues[15]. The values shown in parentheses are those presented by Getz et al.[16]. They showed that C_{18} fatty acids were the major fatty acid among which $C_{18:2}$ was as high as 49%. The present data were quite different from those by Getz et al.

Table 1 *Fatty Acid Composition of Sphingomyelin (Sph), Fast-Moving (Sph-F) and Slow-Moving Sphingomyelin (Sph-S)*

	$>C_{15}$	C_{16}	C_{18}	$C_{20}\sim C_{22}$	C_{24}
Sph	—	37.4	5.7	13.7	43.2
	(0.71)	(20.4)	(18:0 11.40, 18:1 11.40, 18:2 49.60)	(3.1)	—
Sph-F	—	14.1	19.6	21.2	45.3
Sph-S	14.4	71.6	13.8	—	—

Values are the percentages of each fatty acid to total fatty acids. Gas chromatographies are carried out with Hitachi gas chromatograph of KGL-2B using Golay column of butanediol-succinate, under the conditions: carrier gas, N_2, operating temperature, 170°C, flow rate, 10.5 mℓ/min, gas pressure, 10.5~0.6 kg/cm^2.

In comparison with Sph-F and Sph-S, the former contains C_{24} fatty acid and the latter does C_{16} fatty acid as a main fatty acid. In human plasma it has been also reported to show the similar patterns[6]. Then the occurrence of the two sphingomyelins in each subcellular fraction was investigated. From Fig. 1 and Table 2 it is known that mitochondria, being washed three times and considered not being contaminated with other subcellular fractions by the measurement of G-6-Pase and acid phosphatase, still contained sphingomyelins, although they corresponded to only about 0.4% of total phospholipids in the fraction. On the other hand, percentages of sphingomyelin in 18,000×g and microsomal fractions were 3.0 and 1.8, respectively. In cell sap fraction it was variable, i. e.

Table 2 *Lecithin, Cephalin and Sphingomyelin of Subcellular Fractions* (% of total phospholipids)

Fraction	Lecithin	Cephalin	Sphingomylelin	Cardiolipin
Mt-4	52.6	33.3	0.44	8.84
18,000×g Fr-3	62.9	20.3	3.00	—
Ms	68.3	26.4	1.80	—
CS	75.0	15.0	6.27	—

3.95 and 8.60. A part of this variation might depend upon the extent of blood contained in the fraction.

Concerning the Sph-F/Sph-S ratios in each fraction it is observed from Table 3 that mitochondrial fraction shows the lowest value and the other three fractions are identical. The amount of lecithin, cephalin and also protein were examined with the same materials as sphingomyelin was determined after subfractionation.

Table 3 *Ratios of Fast-Moving and Slow-Moving Sphingomyelin. Alkali-Acid Stable Phospholipids from Each Subcellular Fraction were Thin-layer-Chromatographed*

Fraction	Sph-F/Sph-S
Mt-4	2.18
18,000 × g Fr-3	2.65
Ms	2.64
CS	2.61

Each value is the average of three experiments.

From Table 4 it is found that in each fraction lecithin and cephalin were not always distributed in the same manner. In mitochondria lecithin/cephalin ratio was 1.5, while it was about 2.6 in microsomes and 3.1 in 18,000×g-Fr-3. Furthermore, Phospholipid/protein ratios were also different in mitochondria and microsomes, that is, it was always

Table 4 *Ratios of Phospholipid/Protein, Lecithin/Cephalin and Lecithin/Sphingomyelin*

	Phospholipid / Protein	Lecithin / Cephalin	Lecithin / Sphingomyelin
Homogenate	0.18	—	—
Mitochondria			
Mt-4	0.15	1.58	120
Sup-2	0.22	2.22	
Sup-3	0.19	1.87	
Sup-4	0.17	1.84	
18,000 × g Fraction			
18,000 × g Fr-3	0.27	3.10	31
Sup-6	0.32	3.00	
Sup-7	0.26	2.79	
Microsomes	0.25	2.60	38
Cell sap	0.05	5.00	12

Each value is average of three experiments.

lower in mitochondria than in microsomes. It is of interest to note that the values of lecithin/cephalin are always about ten times higher than those of phospholipid/protein. Cell sap fraction showed extremely low value. The ratios of lecithin and sphingomyelin, both of which are the choline-containing phospholipid, were significantly different in each fraction. Mitochondria showed the highest value, 120, while 18,000×g fraction and microsomes did about one-fourth of mitochondria. In cell sap the value is close to that of blood plasma.

As it is clarified now that Sph-F and Sph-S are distinctly different from each other on fatty acid moiety and also are present in each subcellular fraction, further, time cource study with ^{32}Pi in vivo was carried out by determining specific activities of Sph-F and Sph-S and their specific activities were compared with those of lecithin and cephalin to investigate their metabolism in living cells.

Table 5 *Time Study of ^{32}Pi-Incorporation into Lecithin, Cephalin and Sphingomyelins*

Time after injection (h)	0.5		1		3		6		12	
Phospholipids \ Subfraction	Mt	Ms	Mt	Ms	Mt	Ms	Mt	Ms	Mt	Ms
Lecithin	4.30	4.86	52.1	80	90.1	137.0	156.0	160.0	154.0	167.0
Cephalin	5.65	22.60	64.5	146	124.0	259.0				
Sph-F	0.204	0.335	—	1.79	(29.6)	31.8	44.0	43.6	104	124
Sph-S	0.307	0.640	—	2.85	25.3	38.0	52.7	61.0	109	124

Values are expressed as cpm per μ mole of phospholipid-P ($\times 10^{-2}$).

The results obtained at 0.5, 1, 3 and 6 hrs showed that specific activities of Sph-S are always higher than those of Sph-S, although the difference between them is much larger at 30 min than at long time period. It is, however, found to give almost the same specific activity at 12 hrs.

In each cell fraction, specific activities of Sph-F, Sph-S and total phospolipids in mitochondria are lower than those in microsomes, respectively. It is however, found that the differences become less at late priod. Comparing speciffc activity of sphingomyelin with those of total phospholipids, there is a great difference between them at eary period and even at 12 hrs the ratio of specific activity of sphingomyelin to that of total phospholipids is 4/5 or less. These data indicate that the turnover of sphingomyelin is very slow among liver phospholipids. The report by Miller and Cornatzer[17] on time course study of ^{32}Pi incorporation into sphingomyelin and total phospholipids in mitochondria showed that the ratio of specific activity of sphingomyelin to that of total phospholipids at 30 min was 1/4. However, the present data show 1/20 or less. As sphingomyelin content in mitochondria is so small that it could be possible to be contaminated with other (^{32}P)-containing substances. Therefore it was tried to check the problem and was

found that sphingomyelin separated with the procedures described above was not contaminated with other (^{32}P)-containing substances at all. So the specific activity of sphingomyelin in the present report is more reliable.

In Table 6 are shown the specific activities of sphinogomyelin in four subcellular fractions at 30 min after (^{32}Pi) injection. It is known that the specific activity of mitochondrial sphingomyelin was lower than that of microsomes at 30 min. Specific activity of sphingomyelin in cell sap can not be presented, because radioactivitis in the fraction was so low as not to be exactly determined. Comparing specific activity of Sph-F with that of Sph-S in each fraction it is demonstrated that the latter was high.

Table 6 *Specific Activities of Sphingomyelin, Lecithin and Cephalin*
(at 30 min after injection)

Subcellular fraction \ Lipid	Total Sphingomyelin	
	Sph-F	Sph-S
Mt-4	238	
	204	307
18,000 × g Fr-3	508	
	468	577
Ms	416	
	335	640
CS	—	
	—	—

Values are expressed as cpm/μ mole lipid-P and the average of two experiments.

On the specific activities of lecithin and cephalin it is found that the latter were always higher than the former in each subcellular fraction. The result on specific activity in mitochondria is not identical with those shown by Miller and Cornatzer[17]. They presented the higher specific activity of lecithin than that of cephalin in mitochondria at 30 min. Although specific activity of lecithin is different from each other in each fraction, the difference is not so clear. On the other hand, specific activity of cephalin in mitochondria does show much lower value than the other two particulate fractions. It is about 1/3 of that in microsomes.

From Fig. 2 and Table 2, it is known that cardiolipin could be demonstrated clearly in mitochondria but negligible in other particulate fractions.

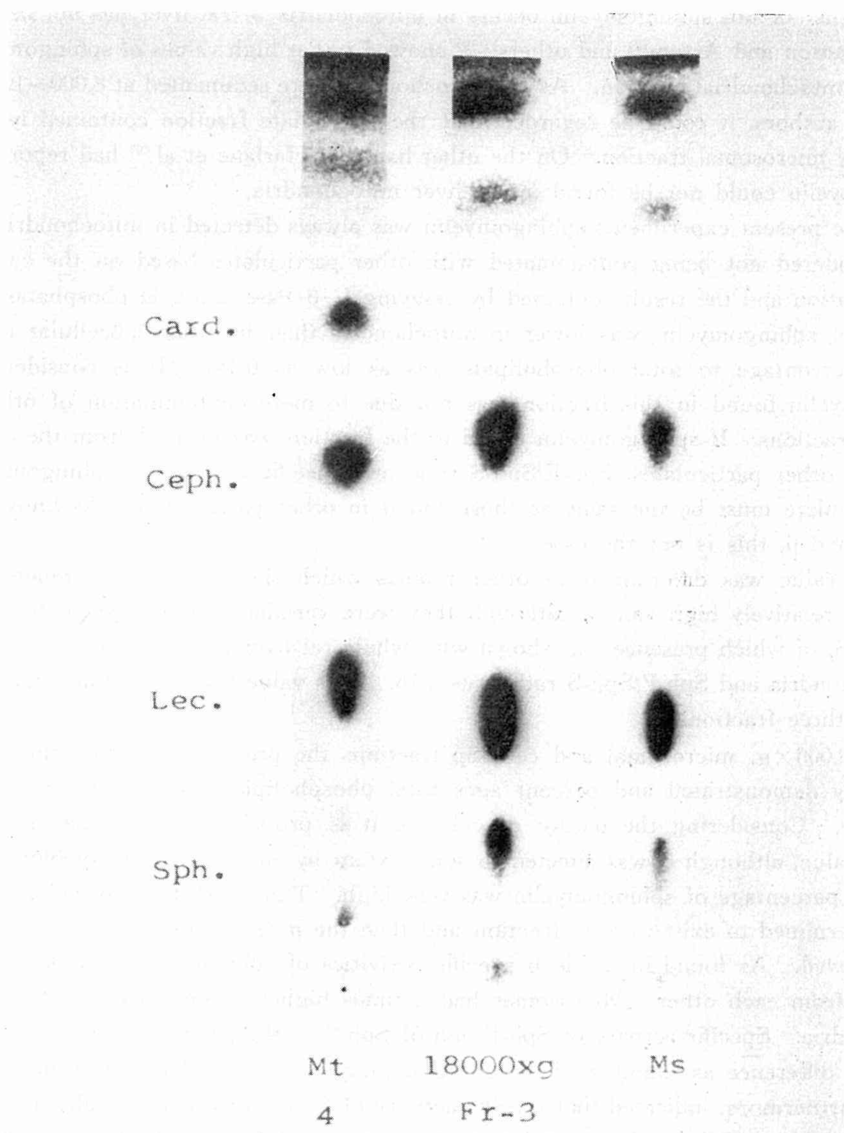

Fig. 2 TLC of phospholipids from rat liver subfractions.
(Silica Gel G, $CHCl_3$-MeOH-H_2O, 65 : 25 : 4, Phosphomolybdic acid spray).

Discussion

The present date show the different pattern on the fatty acid composition from the report by Getz et al.[16] in which they had shown the high $C_{18:2}$ fatty acid content. In our results 80% of the total fatty acid consisted of $C_{16:0}$ and C_{24} fatty acids and could not be found such a high value of $C_{18:2}$ as 49.6% shown by Getz et al. It was also found that in rat liver Sph-F contained C_{24} fatty acid as main one and Sph-S did $C_{18:0}$ as the major fatty acid.

Whether or not sphingomyelin occurs in mitochondria of rat liver has not been fixed yet. Swanson and Artom[18] and others[17-19] showed rather high values of sphingomyelin in rat liver mitochondrial fraction. As the mitochondria were sedimented at 8,000~10,000×g by those authors, it could be regarded that the particulate fraction contained lysosomal and heavy microsomal fraction. On the other hand, Macfarlane et al.[20] had reported that sphingomyelin could not be found in rat liver mitochondria.

In the present experiments sphingomyelin was always detected in mitochondria which was considered not being contaminated with other particulates based on the conditions for separation and the results obtained by assaying G-6-Pase and acid phosphatase. The content of sphingomyelin was lower in mitochondria than in other subcellular fractions and its percentage to total phospholipids was as low as 0.4%. It is considered that sphingomyelin found in this fraction was not due to mere contamination of other subcellular fractions. If sphingomyelin found in the fraction was derived from the contamination of other particulates, Sph-F/Sph-S ratio and specific activity of sphingomyelin in the particulate must be the same as those found in other particulates. As known from Table 5 and 6, this is not the case.

This value was different from other reports which showed the occurrence of this lipid and relatively high values, although they were variable. At the same time Sph-F and Sph-S, of which presence was shown with whole rat liver tissue, were found together in mitochondria and Sph-F/Sph-S ratio was 2.18. This value was lower than those found in other three fractions.

In 18,000×g, microsomal and cell sap fractions the presence of sphingomyelin was apparently demonstrated and percent ages total phospholipids were 3.00, 1.80, 6.27, respectively. Considering the nature of cell sap it is probable that cell sap shows the highest value, although it was affected to some extent by an intermixture of blood plasma in which percentage of sphingomyelin was very high. Thus both types of sphingomyelin were determined to exist in each fraction and then the investigation on their metabolism was followed. As found in Table 6 specific activities of sphingomyelin at 30 min were different from each other. Microsomes had 2 times higher specific activity than that of mitochondria. Specific activity of Sph-F and of Sph-S in the particulate fractions showed the same difference as found in the total sphingomyelin, *i.e.* microsomes>mitochondria. It was, furthermore, indicated that specific activity of Sph-S was metabolically more active than that of Sph-F in each particulate fraction, although among the fractions they are not always metabolized at the same order.

The previous paper presented that sphingomyelin from human blood plasma could be separated into two fractions on a thin layer chromatogram on which Sph-S contained $C_{16:0}$ and Sph-F did $C_{24:0}$ and $C_{24:1}$ as main fatty acids. It was also shown by the authors that Sph-S and Sph-F in rat liver had a close relationship on fatty acid composition to those in blood plasma as described above. Therefore, the results shown above seemed to indicate that sphingomyelin containing $C_{16:0}$ as a major fatty acid is metabolically more active than the other. With chicken liver, Sribney and Kennedy[21] presented that ceramide choline phosphotransferase (EC 2. 7. 8. 3) activity was similar in mitochondria and microsomes in vitro. It was not, however, tried to demonstrate by them the presence

of the enzyme in the particulate fractions of rat liver, although they had shown it in rat liver homogenate. If mitochondria of rat liver would contain the enzyme as mitochondria of chiken liver did, the data shown above might indicate that amount of sphingomyelin synthesized in microsomes is much larger than in mitochondria by the fact that mitochondria contained a very small sphingomyelin and its specific activity is lower than that in microsomes. Until the presence of the enzyme, ceramide choline phosphotransferase, is actually demonstrated in mitochondria of rat liver, it can not be concluded whether sphingomyelin found in mitochondria was biosynthesized *in situ*, or not. Elucidation on the differences in metabolic activities between these two sphingomyelins, consisting of different chain length of fatty acids respectively, would be expected by further studies. Phospholipid/protein and lecithin/cephalin ratios shown in Table 4 also supported that Mt-4 and 18,000$\times$g-Fr-3 were not contaminated with each other. Lecithin/cephalin ratios in mitochondria, 18,000$\times$g fraction and microsomes reported by Strickland and Benson[22] were different from those of the present data in the latter two fractions. According to their findings lecithin/cephalin ratios in mitochondria and microsomes are 1.63, and 1.87 respectively. On the other hand, the present results obtained are 1.58, and 2.60. The values of lecithin/cephalin ratios are always ten times higher than those of phospholipid/protein ratio in each corresponding particulate.

Although the specific activity of lecithin in mitochondria was not very different from those in 18,000$\times$g and microsomal fractions, specific activity of cephalin in the former was significantly lower than those in the latter two particulate fractions. Up to the present the following biosynthetic pathways of lecithin were presented in rat liver: 1. pathway *via* phosphatidic acid, 2. methylation of cephalin, 3. acylation of lysolecithin, 4. 2 lysolecithin$\rightarrow$lecithin$+$glycerylphosphorylcholine. Wilgram and Kennedy[23] and Schneider[24] did find negligible synthesis of lecithin in mitochondrial fraction *in vitro*. The former authors indicated also that choline phosphotransferase (EC 2. 7. 8. 2) was a microsomal enzyme. Bremer and Greenberg[25] and Gibson et al.[26] reported that enzyme system related to the methylation of cephalin existed in microsomal fraction. The original finding of Lands[27] on the acylation of lysolecithin was shown with liver microsomes. It has been, however, found to occur in mitochondria[28]. The pathway 4 was found in a supernatant fraction by Erbland and Marinetti[29]. It has been also shown that lysolecithin was produced by phospholipase A (EC 3. 1. 1. 4) in mitochondria[30,31] and by lecithin cholesterol fatty acyl transferase in cell sap[32]. It is, however, uncertain whether lysolecithin was synthesized in the liver from the structural components or not.

If the facts described above were indeed true, radioactivity found in mitochondrial lecithin and cephalin might depend upon other mechanisms. If it was incorportated in mitochondria *in situ*, these might be two possibilities. First, (^{32}P)-lecithin synthesis might be proceeded by (^{32}P)-lysolecithin synthesis, that is, in mitochondria lysolecithin synthesizing enzyme system might be included with acylating enzyme of lysolecithin and rate of synthesis *via* the pathway 3 might be so rapid as lecithin synthesis in microsomes through other pathways, 1 and 2. On cephalin there might be the same synthetic mechanism and the rate of synthesis might be slow. However it has not yet been shown whether (^{32}P)-lysolecithin was produced with ^{32}Pi, or not.

Second possibility is that lecithin and cephalin might be produced by another pathway which has not been found yet. Furthermore it might be possible to consider that radioactive phospholipids found in mitochondria were not necessarily synthesized in mitochondria themselves and transferred from the site of synthesis to the other organella where the phospholipids were not synthesized *in situ*. If so, difference of specific activity between lecithin and cephalin was ascribed to the difference of rate of exchange or interchange between the phospholipids in membranous lipoprotein of mitochondria and the phospholipids in synthesizing site, for example, endoplasmic reticulum, or of the soluble lipoprotein of the cell cytoplasma. From the preliminary *in vitro* experiments carried out it is suggested now that the last mechanism is quite possible on the phospholipid metabolism. At any rate, the metabolism of each phospholipid in mitochondria has to be elucidated by further investigation.

Summary

It was found that $C_{16:0}$- and C_{24}-fatty acid were predominant in rat liver sphingomyelin and also fast-moving sphinomyelin (Sph-F) contained $C_{16:0}$ as the main fatty acid and slow-moving sphingomyelin (Sph-S) did $C_{20\sim24}$ as the major fatty acids. It was also confirmed by various facts that sphingomyelin existed in the mitochondria as one of the lipid components, although its percentage to total phospholipids was as low as 0.44%. In other subcellular fractions percent of total sphingomyelin in total phospholipids was found to be 1.80 in microsomes and 6.27 in cell sap. Determination of Sph-F/Sph-S ratios showed that the value (2.18) in mitochondria was a little lower than those (2.64, 2.61) in other fractions. The values lecithin/cephalin and phospholipid/protein ratios were always lower in mitochondria than those in microsomes.

Specific activities of sphingomyelin in each fraction were compared with those of lecithin and cephalin at 0.5, 1, 3, 6 and 12 hrs after intraperitoneal injection of ^{32}Pi. Specific activity of total sphingomyelin in mitochondria was clearly lower than those of microsomes until 6 hrs. Specific activities of Sph-S were always higher than those of Sph-F in the same subcellular fraction. Specific activities of lecithin and cephalin were extemely higher than those of sphingomyelin in corresponding subcellular fractions at short time period after injection. Specific activities of cephalin were always higher than those of lecithin. However, the difference in each fraction was divergent, that is, specific activity of microsomal cephalin was 5.6 times greater than that of lecithin. On the other hand, specific activity of cephalin was only 1.3 times greater than that of lecithin in mitochondria.

(Received Sep. 20, 1968)

References

1) Sakagami, T., Minari, O. and Orii, T.: Detection of two sphingophospholipids in rat plasma erythrocytes and liver. J. Biochem. **56**, 294–297 (1964).
2) Minari, O., Tsubono, H., Akiyama, M. and Sakagami, T.: Distribution and matabolism of two sphingomyelins in rat tissues. J. Biochem. **64**, 275–278 (1968).
3) Heverman, E., Bardtlaw, G. and Krusche, B.: Bestimung von Plasmaphospholipoiden nach Dünnschichtchromatographie. Klin. Wshr. **39**, 816–817 (1961).

4) Karlsson, K. A.: Studies on sphingosines. 4. Composition of sphingomyelins from human plasma. Biochem. J. **92**, 39 p–40 p (1964).
5) Wood, P. D. S. and Holton, S.: Human plasma sphingomyelins. Proc. Soc. Expl. Biol. Med. **115**, 990–992 (1964).
6) Minari, O., Tsubono, H., Akiyama, M. and Sakagami, T.: Sphingomyelins in human erythrocytes and plasma. J. Biochem. **62**, 618–620 (1967).
7) De Duve, C., Pressman, B. C., Gianetto, R. G., Wattiaux, R. and Applemans, F.: Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissues. Biochem. J. **60**, 604–617 (1955).
8) Folch, J., Lees, M. and Sloane-Stanley, G. H.: A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226**, 497–509 (1957).
9) Dawson, R. M. C.: A-Hydrolytic procedure for the identification and estimation of individual phospholipids in biological samples. Biochem. J. **75**, 45–53 (1960).
10) Bartlett, G. R.: Phosphorus assay in colnmn chromatography. J. Biol. Chem. **234**, 466–468 (1959).
11) Parker, F. and Peterson, N. F.: Quantitative analysis of phospholipids and phospholipid fatty acids from silica gel thin-layer chromatography. J. Lipid Res. **6**, 455–460 (1966).
12) Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265–275 (1956).
13) Langdon, R. G. and Weakley, D. R.: The influence of hormonal factors and of diet upon hepatic glucose-6-phosphatase activity. J. Biol. Chem. **214**, 167–174 (1955).
14) Wattiaux, R. and De Duve, C.: Tissue fractionation studies. Release of bound hydrolases by means of triton X-100. Biochem. J. **63**, 606–608 (1956).
15) Svennerholm, E., Ställberg-Stenhagen, S. and Svennerholm, L.: Fatty acid composition of sphingomyelins in blood, spleen, placenta, liver and kidney. Biochim. Biophys. Acta **125**, 60–69 (1966).
16) Getz, G. S., Bartley, W., Stirpe, F., Notton, B. M. and Renshaw, A.: The lipid composition of rat liver. Biochem. J. **80**, 176–181 (1961).
17) Miller, J. E. and Cornatzer, W. E.: Time study of incorporation in vivo of inorganic phosphate (^{32}P) into phospholipids of mitochondria. Proc. Soc. Expl. Biol. Med. **118**, 948–951 (1965).
18) Swanson, M. A. and Artom, C.: The lipide composition of the large granules (mitochondria) from rat liver. J. Biol. Chem. **187**, 281–287 (1950).
19) Nelson, D. R. and Cornatzer, W. E.: Role of thyroid in the synthesis of heart, liver and kidney mitochondrial phospholipids. Endocrinology, **77**, 37–44 (1965).
20) MacFarlane, M. G., Gray, G. M. and Wheeldon, L. W.: Fatty acid composition of phospholipids from subcellular particles of rat liver. Biochem. J. **77**, 626–631 (1960).
21) Sribney, M. and Kennedy, E. P.: The enzymatic synthesis of sphingomyelins. J. Biol. Chem. **233**, 1315–1322 (1958).
22) Strickland, E. H. and Benson, A. A.: Neutron activation paper chromatographic analysis of phosphatides in mammalian cell fractions. Arch. Biochem. Biophys. **88**, 344–348 (1960).
23) Wilgram, G. F. and Kennedy, E. P.: Intracellular distribution of some enzymes catalizing reactions in the biosynthesis of complex lipids. J. Biol. Chem. **238**, 2615–2619 (1963).
24) Schneider, W. C.: Intracellular distribution of enzymes. XII. Enzymatic synthesis of deoxycytidine diphosphate choline and lecithin in rat liver. J. Biol. Chem. **238**, 3572–3578 (1963).
25) Bremer, J. and Greenberg, D. M.: Methyl transfering enzyme system of microsomes in the biosynthesis of lecithin (phosphatidylcholin). Biochim. Biophys. Acta **46**, 205–216 (1961).
26) Gibson, K. D., Wilson, J. D. and Udenfriend, S.: The enzymatic conversion of phospholipid ethanolamine to phospholipid choline in rat liver. J. Biol. Chem. **236**, 673–679 (1961).

27) Lands, W. E. M.: Metabolism of glycerolipids. II. The enzymatic acylation of lysolecithin. J. Biol. Chem. **235**, 2233–2237 (1960).

28) Webster, G. R.: On the acylation of lysolecithin by rat-liver and brain mitochondria. Biochim. Biophys. Acta **64**, 573–575 (1962).

29) Erbland, J. and Marinetti, G. V.: In vitro metabolism of lysolecithin. Federation Proc. **21**, 295–295 (1962),

30) Scherphof, G. L. and Van Deenen, L. L. M.: Phospholipase A activity of rat liver mitochondria. Biochim. Biophys. Acta 98, 204–206 (1965).

31) Rossi, C. R., Sartorelli, L., Tato, L., Baretta, L. and Siliprandi, N.: Phospholipase A activity of rat liver mitochondria. Biochim. Biophys. Acta **98**, 207–209 (1965).

32) Akiyama, M., Minari, O. and Sakagami, T.: Cholesterol esterification in rat liver cell sap. Biochim. Biophys. Acta **137**, 525–532 (1967).