STUDIES ON THE LIPIDS IN BRAIN OF BLACK RIGHT WHALE IN THE NORTHERN PACIFIC OCEAN

HIDEO TSUYUKI* AND UHEI NARUSE**

INTRODUCTION

There have been many works on lecithin contained in the brain of human and mammal living on land as well as on lecithin in the egg of hen. The works on lecithin in the brain of human and mammal were reported by Yokoyama & Suzuki (1932 ab) and Levene & Rolf (1921, 1922). Reviewing the works on lecithin in the brain of sea mammal, Igarashi & Zama (1955) studied on lecithin in the brain of sperm whale (*Physeter catodon*) and Alaskan plolack (*Theragra chalcogramma*).

According to the works of Igarashi & Zama (1955), the characteristics of the lecithin in the brain of sperm whale was as follows: Iodine value 71.5, Nitrogen content 1.68%, Phosphorus content 3.61%, Choline content 14.4%. Further the presence of palmitic, stearic, oleic and eicosatetraenoic acids was confirmed and the presence of C_{20}, C_{22}, and C_{24} acids as saturated fatty acids and C_{20} monoenoic acid as unsaturated fatty acids were presumed as the component fatty acids of the lecithin.

In the present work, the writers have studied the properties of the acetone-soluble lipid and phospholipids in the brain of the black right whale in the Northern Pacific Ocean.

It is pleasure that the writers express here their sincere thanks to Dr. Hideo Omura and Dr. Akio Shionoya for their kind advices and thanks to Mr. Seiji Ohsumi who had kindly them the frozen brain of the black right whale.

EXPERIMENTS AND RESULTS

The operation such as the distillation and concentration of solvents were taken in atmosphere of nitrogen gas. Iodine value was measured by Wijs' method, N content by micro-Kjeldahl method, P by method of Beveridge & Johnson (1958), ethanolamine and serine by method of Levine & Chargaff (1951), choline by Glick's method (Glick, 1944) and inositol by method of Böhm & Picharz (1954).

1. Material

Material is the frozen brain of the black right whale, *Eubalaena glacialis* (male, body length: 15.1 m, presumed age: more than 9 years old) which was caught in the southern sea of Kojak Island in the Northern Pacific Ocean (N: 55°53′-54′, W: 154°4′-6′).

* Department of Food Engineering, College of Agriculture & Veterinary Medicine, Nihon University, 49, 3-chome, Shimouma-cho, Setagaya, Tokyo.

** Dept. of Fisheries, Coll. of Agr. & Vet. Med., Nihon Univ.

2. Preparation of Acetone-Soluble Lipid and Phospholipids

The acetone-soluble fraction was 5 times extracted from 500 g of the frozen brain with 5 weights of acetone, and the obtained acetone-soluble fraction was 3 times extracted with 5 weights of ethyl-ether. Then ethyl-ether was distillated off from the ethyl-ether extract and the acetone-soluble lipid was obtained from the 500 g of the frozen brain. The properties of the acetone-soluble lipid are shown in Table 1.

TABLE 1. PROPERTIES OF ACETONE-SOLUBLE LIPID IN BRAIN OF BLACK RIGHT WHALE

Yield		N_D^{20}	Acid value	Sapon. value	Iodine value	Unsapon. matter (%)
g	%*					
6.3	1.26	1.4765	2.6	183.1	142.1	1.37
Phosphorus (%)	Nitrogen (%)	Choline (%)	Ethanolamine (%)	Serine (%)	Insitol (%)	Sterol** (%)
0.03	0.02	0.01	0.01	0.02	trace	18.62

* % of acetone-soluble lipid from the frozen brain.
** % of sterol in unsaponifiable matter by digitonin method.

The residue obtained after extracting the acetone-soluble lipid from the brain and the residue after extracting ethyl-ether-soluble fraction from the acetone-soluble fraction were mixed, and the mixture was 5 times extracted with 5 weights of chloroform-methanol solution (2 : 1). The extract was 3 times treated by the washing of Folch, Ascoli, Leas, Mexth & Lebaron (1951), and the chloroform layer was separated and then chloroform was distillated off from the layer. Thus the phospholipids were obtained. After all 2.1 g of phospholipids was obtained from the material (Fig. 1). The properties of the phospholipids are given in Table 2.

TABLE 2. PROPERTIES OF PHOSPHOLIPIDS IN BRAIN

Yield		Phosphorus (%)	Nitrogen (%)	Choline (%)	Ethanolamine (%)	Serine (%)
g	%*					
2.1	0.42	3.72	1.81	4.68	4.11	3.03
Inositol (%)	N/P**	Choline/P**	Serine/P**	Ethanolamine/P**		
0.82	1.08	0.31	0.24	0.55		

* % of phospholipids from the frozen brain.
** molar ratio.

3. Component Fatty Acids of Acetone-soluble Lipid and Phospholipids

Mixed fatty acids were prepared from the acetone-soluble lipid and phospholipid in the brain of the black right whale by alkali-hydrolysis.

The mixed fatty acids obtained from the acetone-soluble lipid were separated into two portions, solid and liquid fatty acids with lead salt-ethanol fractionation of Hilditch (1956) (Table 3). After synthesizing the acetal-ester of the solid fatty acids with mono-brome-acetone, the acetal-ester of solid fatty acids were dissolved and heated in ethanol, and a little excess volume of 2N-HCl methanol solution contained 2,4-dinitrophenylhydrazine were added to the above solution

Then 2,4-dinitrophenylhydrazone of acetal-ester was educed by cooling this solution at the room temperature, and separated with filtration. Finally 2,4-dinitrophenylhydrazone of the acetal-ester was identified with paper chromatography as its 2% benzene solution by the method of Inoue, Hirayama & Noda (1956 a). The results are shown in Table 4. The other hand the liquid fatty acids were con-

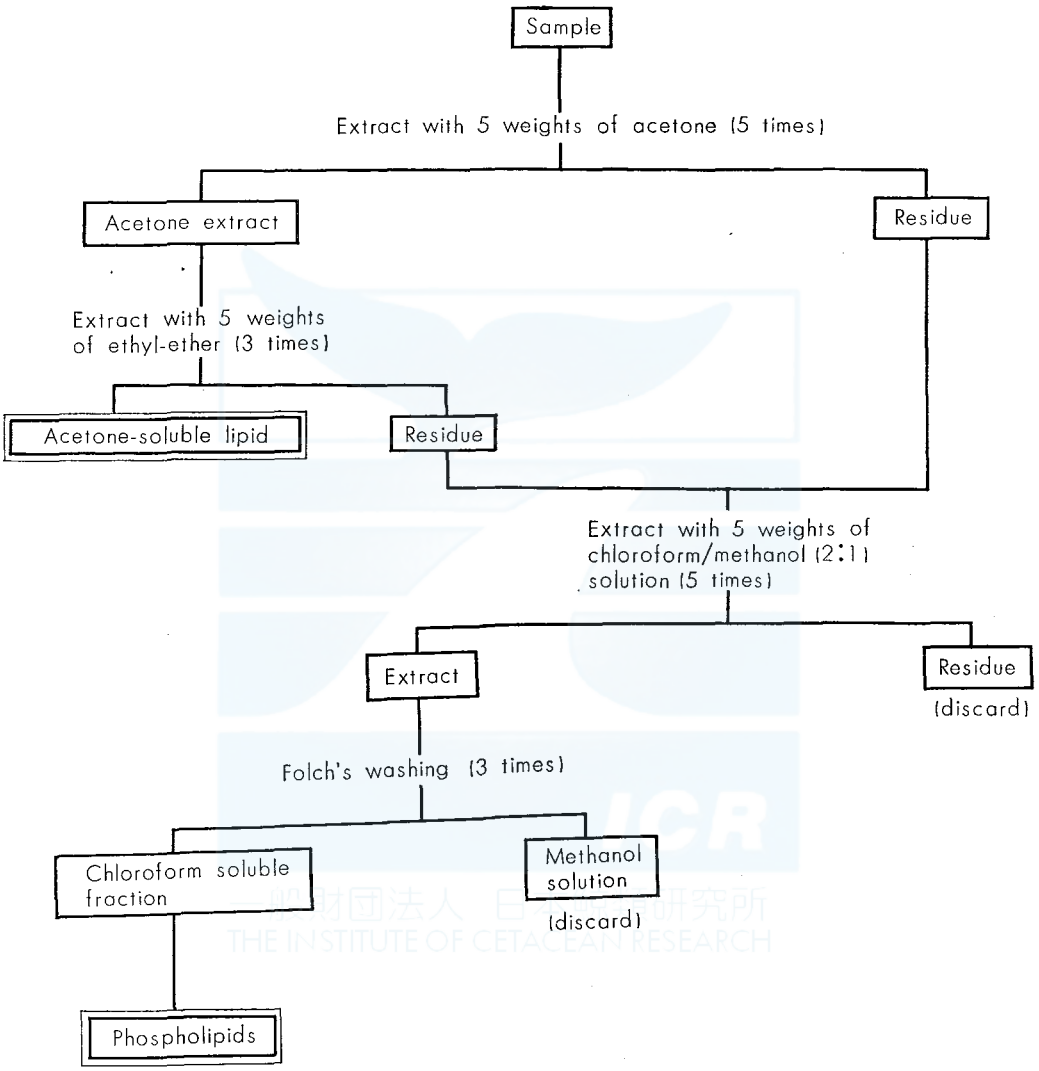

Fig. 1. Extraction and fractionation of lipids from brain of balck right whale.

verted into the methyl ester of the fatty acids by the usual method. Mercuric acetate was added to the methyl ester of the fatty acids in methanol by method of Noda, Hirayama & Inoue (1956), and the liquid fatty acids were indentified with

paper chromatogaphy as their mercuric acetate complexes of methyl ester (Table 5).

TABLE 3. FRACTIONATION OF MIXED FATTY ACIDS OBTAINED FROM ACETONE-SOLUBLE LIPID

Kinds of fatty acids	%	Neutr. value	Iodine value
Mixed fatty acids	—	191.8	151.2
Solid fatty acids	38.6	200.2	10.3
Liquid fatty acids	61.4	183.4	200.3

TABLE 4. R_F VALUE OF 2,4-DINITROPHENYLHIDRAZONES OF ACETAL ESTER OF COMPONENT SATURATED ACIDS OF ACETONE-SOLUBLE LIPID

	Acid				
Sample	Myristic	Palmitic	Stearic	Arachidic	Behenic
Acetone soluble lipid	—	0.29	0.22	0.15	0.10
Standard	0.35	0.29	0.23	0.16	0.11

Paper: Tōyō No. 2. Moving phase: methanol—acetic acid—tetralin (10 : 2 : 1). Stationary phase: Tetralin. Development: Ascending at 30°C. Detection: N/2 KOH in ethanol.

TABLE 5. R_F VALUE OF MERCURIC ACETATE COMPLEXES OF METHYLESTER OF COMPONENT LIQUID ACIDS OF ACETONE-SOLUBLE LIPID

	Acid					
Sample	Zoomaric	Oleic	Eicosenoic	Erucic	Linoleic	Linolenic
Acetonesoluble lipid	0.26	0.14	0.09	0.05	0.51	0.70
Standard	0.25	0.16	0.09	0.05	0.52	0.71

Stationary and moving phase were the same as shown in Table 4. Detection: 0.2% dinitrocarbazone in ethanol.

While para-bromophenacyl bromide were added into the methanol solution contained 20mg of the mixed fatty acids (Table 6) separated from the phospholipids, and the solution was heated for two hours in water bath and then cooled, 2N-HCl methanol solution contained 1% 2,4-dinitrophenylhydrazine was added into the above cooled solution, and the solution was set on for 3 hours at room temperature.

TABLE 6. PROPERTIES OF MIXED FATTY ACIDS OBTAINED FROM PHOSPHOLIPIDS

%	Neutralization value	Iodine value
62.38	155.3	190.1

Thus para-bromophenacyl-ester-2,4-dinitrophenylhydrazine of the fatty acids was synthesized. This ester was extracted with ether. After the extracted solution was washed with water, the water in the solution was removed with anhydrous sodium sulfate and the ether solution was divided into two portions. After each portion of the ether solution was concentrated to 1 ml, anhydrous methanol solution contained mercuric acetate was added to one portion. The solution was heated for 40 minutes at 40°C and cooled. Then 1 ml of benzene, 10ml of water and one

drop of acetic acid were added into the above solution, the benzene-ether solution was produced on the upper layer. This solution was called as Sample B. To another portion, benzene, water and acetic acid were added by the same method mentioned above except mercuric acetate, thus sample A was prepared. The sample A and B of complexes of fatty acids were treated with paper chromatography of Inoue, Hirayama & Noda (1956 b.) *Rf* value of the sample A and B are shown in Table 7 and 8.

TABLE 7. R_F VALUE OF SAMPLE A OF MIXED FATTY ACIDS OBTAINED FROM PHOSPHOLIPIDS BY BROMOZONE METHOD.

	Acid					
Sample	Myristic	Palmitic	Stearic	Arachidic	Behenic	Oleic
Phospholipids	0.34	0.27	0.23	0.18	—	0.25
Standard	0.35	0.29	0.23	0.17	0.12	0.26

Paper: Tōyō No. 2 (40×40 cm). Stationary phase: Petroleum hydrocarbon (b.p. 140–170°C). Moving phase: Methanol-Acetic acid-Petroleum hydrocarbon (5 : 1 : 1). Ascending at 30°C. for 5 hours.

TABLE 8. R_F VALUE OF SAMPLE B OF MIXED FATTY ACIDS OBTAINED FROM PHOSPHOLIPIDS BY BROMOZONE METHOD

	Acid					
Sample	Zoomaric	Oleic	Eicosenoic	Erucic	Linoleic	Linolenic
Phospholipids	0.55	0.51	0.46	0.41	0.73	0.68
Standard	0.57	0.51	0.47	0.39	0.72	0.69

Paper, ascending method, stationary and moving phase were the same as shown in Table 7.

4. Silca Gel Column Chromatography and Silicic Paper Chromatography of Phospholipids

Phospholipids in the brain of black right whale were fractionated with silica gel column by Rhodes' method (Rhodes & Dawson, 1960). At first, 40 g of silica gel (Kanto-kagaku Co.) being suspended in methanol, the silica gel-methanol solution was poured into glass column fulled with glass-fiber for chromatography (30× 1.7 cm), and set until the silica gel was sunk down. Then the solution was flowed down by opening the glass cock of the column. Directly before the surface of the solution reached to the top of silica gel column, the column was washed with 2 volumes of chloroform. When 2–3ml of chloroform was remained on the top of the silica gel column, the cock was closed. Next, the chloroform solution contained 100 mg of phospholipids was added into the column mentioned above. Various phospholipids were eluted with various methanol-chloroform solvents (Table 9). The properties of each phosphlipid are shown in Fig. 2 and Table 10.

Morever, the phospholipids were treated with silicic paper chromatography (Rhodes & Dawson, 1960) to separate each phospholipid. After the filter paper for chromatography (Toyo No. 51) was immersed in sodium silicate solution (sodium silicate : water/1 : 1) for 5 minutes, the paper was suspended in air for 5 minutes and the excess solution was removed from the paper. Further the paper

TABLE 9. SOLVENTS FOR FRACTIONATION OF PHOSPHOLIPIDS

Chloroform : Methanol	Volume (ml)	Kinds of phospholipids
90 : 10	25	Polyglyceroric acid
80 : 20	15	Phosphatidyl serine and Phoshatidyl ethanolamine
75 : 25	50	Phosphatidyl ethanolamine
60 : 40	25	Monophosphoinositide
50 : 50	75	Phosphatidyl choline
10 : 90	25	Sphingomyelin and phosphatidyl choline

TABLE 10. PROPERTIES OF EACH FRACTION OBTAINED FROM PHOSPHOLIPIDS BY SILICA GEL COLUMN CHROMATOGRAPHY

	Fraction					
Component	1	2	3	4	5	6
Phosphorus (%)	3.31	3.72	3.70	3.34	3.36	3.67
Nitrogen (%)	0.23	1.62	1.61	0.39	1.66	3.34
Choline (%)	—	—	—	—	12.40	11.30
Ninhydrin	—	+	+	+	+	+
Inositol	—	—	—	+	+	—
N/P*	0.15	0.95	0.96	0.25	0.99	2.01
Choline/P*	—	—	—	—	0.87	0.83

* Molar ratio

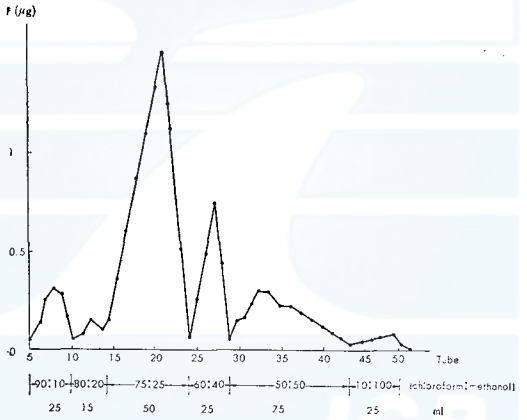

Fig. 2. Silicic column chromatography of phospholipids.

TABLE 11. R_F VALUE OF PHOSPHOLIPIDS BY SILICIC PAPER CHROMATOGRAPHY

Component	Standard	Sample
Phosphatidic acid	0.72	—
Phosphatidyl ethanolamine	0.56	0.54
Phosphatidyl serine	0.52	0.51
Phosphatidyl choline	0.33	0.33
Sphingomyelin	0.23	0.22
Inositol phosphatide	0.20	0.18

Development: di-isobutylketone : acetic acid : water (40 : 25 : 5), ascending at room temperature for 12 hours.

Detection: all lipids—rhodamin 6G, phosphatidyl choline and sphingomyelin—molibdic phosphate, phosphatidyl ethanolamine and phosphatidyl serine—ninhydrin, inositol phosphatide—Elson-Morgan reagent.

was immersed in dilute HCl solution for 30 minutes. Then the paper was 5 times washed with water in order to remove the HCl. After setting the paper in air overnight, the paper was dried for one hour at 110°C. The silicic paper was prepared by the process above mentioned. The result of chromatography which was treated with this silicic paper was shown in Table 11.

DISCUSSION

As has been shown in Table 1, the acetone-soluble lipid in the brain of the black right whale contained a trace of nitrogen, phosphorus, choline, ethanolamine, serine and its acid value and unsaponifiable matter content were lower. Therefore it seems that the main constituent of acetone-soluble lipid is glyceride, and the contents of free fatty acid and unsaponifiable matter are little. The yield of acetone-soluble lipid was 1.26% to the frozen brain of the whale, and was comaratively lower. The content of sterol in unsaponifiable matter was 18.62%. The paper chromatography by the method of Inoue, Hirayama & Noda (1956 a) and Noda, Hirayama & Inoue (1956), indicated that palmitic, stearic, arachidic and behenic acids as saturated fatty acids, and zoomalic, oleic, eicosenoic, erucic, linoleic and linolenic acids as unsaturated fatty acids were contained in the acetone-soluble lipid (Table 4, 5). Myristic acid was unable to be found in the aceone-soluble lipid in the brain.

The yield of phospholipids obtained from the brain of the whale was 0.42%. The properties of the phospholipids were as follows: choline content 4.68%, ethanolamine content 4.11%, serine content 3.03% and inositol content 0.82% (Table 2). According to the bromozone method of Inoue, Hirayama & Noda (1956 b), component fatty acids of the phospholipids seemed to be myristic, palmitic, stearic and arachidic acids as saturated fatty acids, and zoomaric, oleic, eicosenoic, erucic, linoleic and linolenic acids as unsaturated fatty acids. However, the presence ofbehenic acid was not able to be found in the phospholipids.

When the component fatty acids of the acetone-soluble lipid and phospholipids were compared, the presence of palmitic, stearic and arachidic acids were found in both lipids in the case of saturated fatty acids, but myristic acid was not contained in the acetone-soluble lipid and behenic acid was not able to be found in the phospholipids. Considering of the component unaturated fatty acids, their component acids were common to both lipids and the presence of zoomaric, oleic, eicosenoic, erucic, linoleic, linolenic acids were observed in the case of both lipids. Perhaps, various component unsaturated fatty acids seemed to be contained in phospolipids in the whale brain. The results of silica gel column chromatography by Rhodes' method indicated the presence of phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl choline, sphingomyelin and inositol phosphatide was observed in the phospholipids. But phosphatidic acid was not able to found in the phospholipids (Table 11).

SUMMARY

1) The characteristics of the acetone-soluble lipid and phospholipids in the brain of black right whale in the Northern Pacific Ocean were studied.

2) The yield of acetone-soluble lipid obained from the frozen brain was 1.26% and sterol content in unsaponifiable matter was 18.62%. The results of paper chromatography by the methods of Inoue, Hirayama & Noda (1956 a) and Noda, Hirayama & Inoue (1956) indicated that the presence of palmitic, stearic, arachidic, zoomaric, oleic, eicosenoic, erucic, linoleic and linolenic acids in the acetone-soluble lipid.

3) The yield of phospholipids obtained from the frozen brain was 0.42%. The results of the bromozone method indicated the presence of myristic, palmitic, stearic, arachidic, zoomaric, oleic, eicosenoic, erucic, linoleic and linolenic acids in the phospholipids.

The results obtained by the silica gel column chromatography and silicic paper chromatography indicated the presence of phosphatidyl ethanolamine, phosphatidyl choline, inositol phosphatide, polyglyceroric acid, phosphatidyl serine and sphingomyelin in the phospholipids.

REFERENCES

BEVERIDGE, J.M.R. & JOHSON, S.E. (1958). "*Biochemistry of Lipid*", Kyoritsu-Syuppan Co. Tokyo, 311.

BÖHM, P. & G. PICHARZ (1954). Von Quantitative Bestimmung Von Inosit in Phosphstiden, *Z. Phisiol. Chem.*, 298: 110–120.

FOLCH, J., I. ASCOLI, M. LEAS, J.A. MEXTH & F.M. LEBARON (1951). Preparation of Lipide Extracts from Brain Tissue, *J. Biol. Chem.*, 191: 833–841.

GLICK, D. (1944). Concerning the Reineckate Method for the Determination of Choline, *J. Biol. Chem.*, 156: 643–651.

HILDITCH, T.P. (1956). "*The Chemical Constitution of Natural Fats* (3rd. ed.)" Champan & Hall, London, 574.

IGARASHI, H. & ZAMA, K. (1955). Studies on the Phosphatide of Aquatic Animals. Part 2. *Nippon Nogeikagakukaishi*, 29: 958, 961.

INOUE, Y., HIRAYAMA, O. & NODA, M. (1956•a). Paper Chromatography of Fatty Acids as their Acetal Ester Derivatives, *J. Japan Oil Chemists' Soc.*, 5: 16.

—— (1956•b). Separation and Identification of Fatty Acids. Part XX. *Bull. Agr. Chem. Soc. Japan.* 20: 200–205.

LEVENE, P.A. & ROLF, I.P. (1921). Lecithine 4-Lecithine of the Brain, *J. Biochem.* 46: 353–65.

—— (1922). Unsaturated Fatty Acids of the Brain Lecithines, *J. Biochem.* 54: 99–100.

LEVINE, C. & E. CHARGAFF. (1951). Chromatographic Behavior of Analogues of the Nitrogenous Lipide Constitutes, *J. Biol. Chem.*, 192: 481–483.

NODA, M., HIRAYAMA, O. & INOUE, Y. (1956). Separation and Identification of Fatty Acids, Part XIX. *Nippon Nogeikagakukaishi*, 30: 106–111.

RHODES, D.N. & DAWSON, R.M.C. (1960). "*Chromatographic and Electrophoretic Technique 1.*" 363 p. New York.

YOKOYAMA, Y. & SUZUKI, B. (1932•a). Phospholipids in Human Brain-2, *P.I.A. Japan* 8: 375–60.

—— (1932•b). Phospholipids in Human Brain-3, *P.I.A. Japan*, 8: 361–3.