[Chem. Pharm. Bull. 19(2) 385—390 (1971)]

UDC 547.915.5.09 : 615.273.076.9

Abnormal Distribution of Phospholipids in Aorta of the Rabbits with Experimental Atherosclerosis[1]

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(Received August 31, 1970)

Phospholipid class composition was determined on the aorta from normal rabbits and those with experimental atherosclerosis, fed high-cholesterol diet, by means of quantitative thin-layer chromatography with Silica gel G plate. Relative increase in the phosphatidyl choline and relative decrease in phosphatidyl ethanolamine percentage of the total phospholipids were noted on the atherosclerotic aorta in comparison with the normal aorta. Though no change in the percentage of sphingomyelin was observed in the atheromatous aorta, a significant change in the fatty acid composition of this phospholipid was revealed by gas-liquid chromatographic determination of the fatty acids liberated from the purified sphingomyelin preparation, characterized by remarkably high percentage of C_{16} acids and lower percentage of the acids with 20 or more carbon atoms in comparison with the values on the normal aorta.

With the blood plasma of atherosclerotic animals, only a decrease in phosphatidyl ethanolamine, which is not the major component of plasma phospholipids, was detected, with the other phospholipid class composition almost unchanged.

It is a well-known fact that in atherosclerotic arteries considerable accumulation of neutral lipids, mainly cholesterol ester and also of phospholipid occurs,[3] although there is some disagreements as to the mechanism of the accumulation of the latter. Because the arterial tissue can synthesize phospholipids, some authors[4] believe that the synthesis *in situ* could account for the higher amounts of phospholipids present in the atheromatous aorta.

As the changes of individual phospholipid content of arterial walls resulting from atherosclerotic degeneration, an increased sphingomyelin content in human atheromatous aorta was first reported by Weinhouse and Hirsch.[5] Buck and Rossiter[6] and Boettcher and his colleagues[7] made more detailed studies on the lipids in human aorta in several stages of atherosclerosis, and found that sphingomyelin percentage of the total phospholipids increased remarkably whereas the relative percentages of lecithin and cephalin fractions decreased also notably with increasing degree of atherosclerosis.

In the present paper, as a part of the investigations on the mode of abnormal accumulation of phospholipids in atheromatous arteries, the phospholipid class composition of the aorta from the rabbits with experimental atherosclerosis was determined by thin-layer chro-

1) Part of the present report was presented at the 89th Annual Meeting of Pharmaceutical Society of Japan, April 1969, and at the 42 nd Annual Meeting of Japanese Biochemical Society, Hiroshima, October 1969.

2) Location: *Yagoto Urayama 15, Tenpakucho, Showaku, Nagoya.*

3) *a*) E.J. Masoro, "Physiological Chemistry of Lipids in Mammals," Saunders, Philadelphia and London, 1968, p. 267; *b*) R.W. Wissler and D. Vesselinovitch, "Advances in Lipid Research," Vol. 6, ed. by R. Paoletti and D. Kritchevsky, Academic Press, New York and London, 1968, p. 181; *c*) P. Washl and R. Sanwald, "Atherosclerosis," ed. by F.G. Schettler and G.S. Boyd, Elsevier, Amsterdam, 1969, p. 141.

4) *a*) D.B. Zilversmit, E.L. McCandless, P.H. Jordan, Jr., W.S. Henley and R.F. Ackerman, *Circulation*, **23**, 370 (1961); *b*) Y.H. Abdulla and C.W.M. Adams, *J. Atherosclerosis Res.*, **5**, 504 (1965); *c*) H.A.I. Newman, A.J. Day and D.B. Zilversmit, *Circulation Res.*, **19**, 132 (1966).

5) S. Weinhouse and E.F. Hirsch, *A.M.A. Arch. Pathol.*, **29**, 31 (1940).

6) R.C. Buck and R.J. Rossiter, *A.M.A. Arch. Pathol.*, **51**, 224 (1951).

7) C.J.F. Boettcher, "Drugs Affecting Lipid Metabolism," ed. by S. Garattini and R. Paoletti, Elsevier, Amsterdam, 1961, p. 54; C.J.F. Boettcher and C.M. van Gent, *J. Atherosclerosis Res.*, **1**, 36 (1961).

matography (TLC) and compared with the results with the normal adult rabbit. A special attention was paid to the sphingomyelin fraction of the aorta phospholipids of which content in human arteriosclerosis was reported to be significantly altered. Thus, fatty acid composition of this phosphosphingoside in both atherosclerotic and normal aorta of the rabbit was determined by gas-liquid chromatography (as methyl esters).

Material and Method

Materials——Control rabbits were fed a diet of RC 5 of the Oriental Ferment Industrial Co., Japan, for 12 weeks. The experimental animals were kept on the same diet added 1% of cholesterol for the same period of time. At the end of the feeding, all rabbits, weighing 3.20 to 3.82 kg, were sacrificed and the whole aorta were taken away which was then cut into three sections, namely *aorta arcus*, *aorta thoracica* and *aorta abdominalis*. The wet weight of each sample was in a range of 280—684 mg. Each aorta section was homogenized in a small volume of physiological saline by means of Waring blender.

Lipid Analysis——From the homogenate of the aorta tissue, lipids were extracted with 20-fold volumes of chloroform–methanol (1:1, v/v). The extract was filtered, the filtrate was evaporated to dryness *in vacuo* and the residue was reextracted with the same volume of chloroform–methanol (2:1, v/v). The extract was washed with 0.1M potassium chloride[8] and allowed to stand overnight at 4°, and the lower phase was collected, dried under reduced pressure below 40°. The residue was dissolved in chloroform–methanol (1:1, v/v) and this total lipid extract was used for the lipid analysis as described below.

Total phospholipid was estimated by determining the phosphorus in the total lipid extract by the method of Bartlett.[9] Cholesterol determination was performed according to the method of Courchaine, *et al.*[10]

The lipids of plasma from normal and atheromatous rabbits were extracted by the method of Ways and Hanahan.[11]

TLC plate was developed with chloroform–methanol–water (100:40:6, v/v) for phospholipids and with petroleum ether–ether–acetic acid (70:30:1, v/v) for neutral lipids. The lipid bands on the plate were visualized with iodine vapor. Identification of the phospholipid fractions by two-dimensional TLC and quantitative determination of the individual phospholipids on the one-dimensional TLC plate were carried out according to the procedures described in the preceding paper.[12]

Sphingomyelin was isolated and purified from the phospholipid mixture by silicic acid column chromatography and by preparative TLC. The purified preparation gave one spot on two-dimensional TLC. Simultaneous hydrolysis and methylation (*trans*-esterification) of the fatty acids of sphingomyelin were carried out according to the method of Morrison and Smith,[13] with boron trifluoride in methanol as the reagent. The methyl esters thus obtained were analyzed by gas-liquid chromatography through a column of 10% diethylene glycol succinate polyester in Uniport K with Hitachi model K-53 instrument. The experimental conditions are cited on the Table II. The quantitative estimation was made by measuring the peak area (peak height × peak width at half height) and expressed as a percentage of each peak of the total peak area.

Result and Discussion

Phospholipid and Cholesterol Content of the Aorta

The amount of phospholipid and of total cholesterol (free plus ester form) in μg per mg wet aorta tissue, determined on 11 normal rabbits and 13 rabbits with atherosclerosis, is graphically presented in Fig. 1. The degree of the atherosclerosis of the latter group of animals was found to be in the stage III according to the system adopted by the World Health Organization,[6] by histological examination. As is clear from the figure, remarkable accumulation of phospholipid as well as cholesterol in the aorta of such atherosclerosis stage is confirmed.

Accumulation of cholesterol, particularly of the ester type, is macroscopically demonstrated in the TLC for the neutral lipids presented in Fig. 2.

8) J. Folch, M. Lees and G.H. Sloane-Stanley, *J. Biol. Chem.*, **226**, 497 (1957).
9) G.R. Bartlett, *J. Biol. Chem.*, **234**, 466 (1959).
10) A.J. Courchaine, W.H. Miller and D.B. Stein, Jr., *Clin. Chem.*, **5**, 609 (1959).
11) P. Ways and D.J. Hanahan, *J. Lipid. Res.*, **5**, 318 (1964).
12) T. Sato and T. Fujii, *Chem. Pharm. Bull.* (Tokyo), **19**, 377 (1970).
13) W.R. Morrison and L.M. Smith, *J. Lipid Res.*, **5**, 600 (1964).

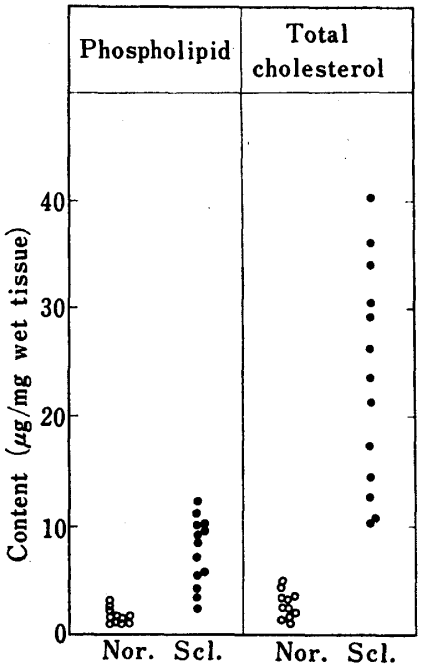

Fig. 1. Phospholipids and Cholesterol Contents of Normal and Atherosclerotic Aorta of Rabbit

Nor.: normal aorta
Scl.: atherosclerotic aorta

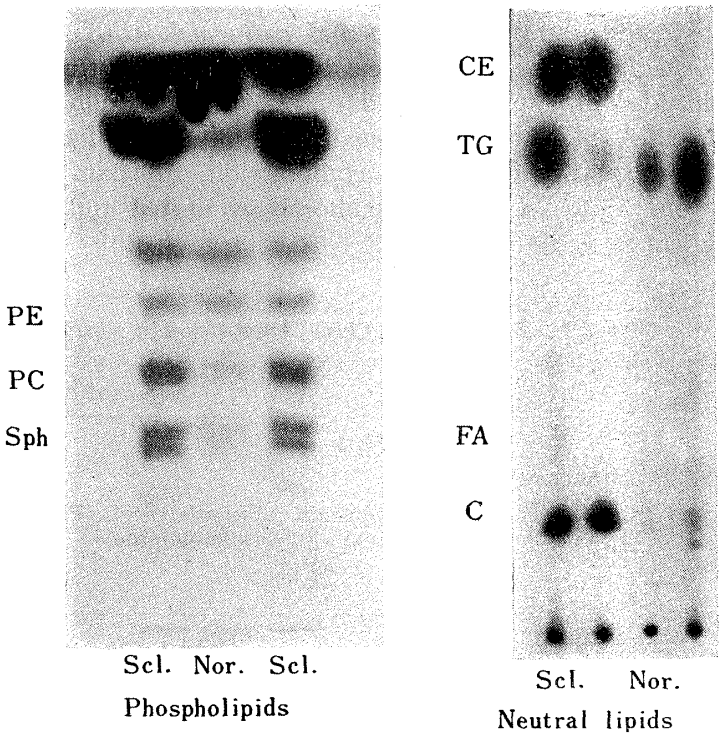

Fig. 2. Thin-Layer Chromatography of Phospholipids and Neutral Lipids Extracted from Normal and Atherosclerotic Aorta of the Rabbit

Nor.: normal aorta
Scl.: atherosclerotic aorta

PE: phosphatidyl ethanolamine
PC: phosphatidyl choline
Sph: sphingomyelin
C: cholesterol
CE: cholesterol ester
TG: triglyceride
FA: fatty acid (free)

The TLC for phospholipids, cited on the left side of the Fig. 2 indicates that three major phospholipids, phosphatidyl ethanolamine, phosphatidyl choline and sphingomyelin, are distinctly separated on the plate. It had been previously shown that with such a solvent system, phosphatidic acid appears above the phosphatidyl ethanolamine band, and phosphatidyl. serine, phosphatidyl inositol and lysophosphatidyl choline are located between the original line and sphingomyelin band.[12] Thus, the aorta phospholipids were fractionated into five and percentage of the phospholipid or phospholipids in each fraction of the total phospholipids was determined on each of three sections of the normal and abnormal aorta, as shown in Fig. 3.

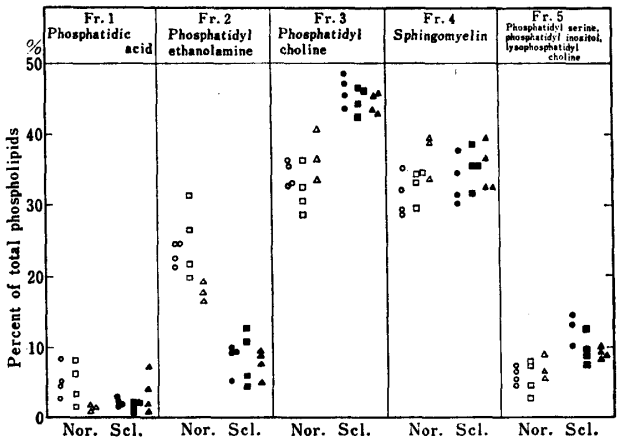

Fig. 3. Phospholipid Distribution in Three Sections of Normal and Atherosclerotic Aorta of the Rabbit

Nor.: normal aorta
Scl.: atherosclerotic aorta

○, ●: *Aorta arcus*
□, ■: *Aorta thoracica*
△, ▲: *Aorta abdominalis*

It was disclosed that as far as the phospholipid class composition be concerned, there seems to be no difference as to the site of the aorta in longitudinal direction, because no differ-

ence in the phospholipid class composition was observed between each of the three sections, *aorta arcus*, *aorta thoracica* and *aorta abdominalis*.

From the Fig. 3, it is easily understood that remarkable alteration in percentage of phosphatidyl ethanolamine and of phosphatidyl choline occurs in atherosclerotic aorta. Considering the above-mentioned fact that each of three longitudinal section of the aorta has similar phospholipid class composition, the data obtained on each three to four specimen of one aorta section were collectively united and were subjected to statistical treatment as shown in Table I.

TABLE I. Phospholipid Class Composition in Normal and Atherosclerotic Aorta of the Rabbit

Groups of animals	Percent of total phospholipids				
	Fr. 1 Phosphatidic acid	Fr. 2 Phosphatidyl ethanolamine	Fr. 3 Phosphatidyl choline	Fr. 4 Sphingomyelin	Fr. 5 Phosphatidyl serine, phosphatidyl inositol, lysophosphatidyl choline
Normal	4.0±2.8	22.2±4.3[a)]	34.1±3.3[a)]	33.5±3.7	6.1±1.8[a)]
Atherosclerotic	2.5±1.8	8.4±2.6	45.3±1.9	34.6±3.1	10.2±2.3

The figures indicate the mean value ± standard deviation.
Numbers of the samples analyzed: Normal aorta 11, atherosclerotic aorta 12.
a) significant difference with $p<0.001$

In normal aorta, the percentage of phosphatidyl ethanolamine and of phosphatidyl choline of the total phospholipids is 22.2±4.3% and 34.1±3.3%, respectively. In the aorta suffering from atherosclerosis, the corresponding value is 8.4±2.6% and 45.3±1.9%, respectively. Such a decrease in the phosphatidyl ethanolamine percentage and increase in the phosphatidyl choline percentage were demonostrated to be statistically significant with a $p<0.001$. Though the percentage of the phospholipids in the Fraction 5 in the atheromatous aorta is significantly raised, no further investigation was performed due to the minute quantities of the individual phospholipids in this fraction.

Table I indicates also that there seems to be no change in the relative content of sphingomyelin in atherosclerotic aorta of the rabbit. This is not in agreement with the experiments with human atherosclerotic aorta as cited already.[5-7)]

It seemed to be worth of note that on the Silica gel G TLC plate, the sphingomyelin fraction obtained from the aorta of the rabbits with atherosclerosis was resolved into two bands, whereas that extracted from normal aorta was not (Fig. 2). Because it was already reported that these two bands correspond to two sphingomyelin fractions with different fatty acid and sphingosine base composition,[14)] the above-mentioned fact suggests possible difference in the fatty acid composition of the sphingomyelin fractions from normal and abnormal aorta tissues. Therefore, this phospholipid was isolated and purified from the total lipid extract of normal and of atherosclerotic aorta separately, by means of silicic acid column chromatography followed by preparative TLC, and the fatty acid composition of each sphingomyelin preparation was analyzed by gas-liquid chromatography. Owing to the fact that complete assignment

14) *a*) P.D.S. Wood and S. Holton, *Proc. Soc. Exptl. Biol. Med.*, **115**, 990 (1964); *b*) H. Jatzkewitz, *Z. Physiol. Chem.*, **336**, 25 (1964); *c*) H. Pilz and H. Jatzkewitz, *J. Neurochem.*, **11**, 603 (1964); *d*) O. Minari, H. Tsubono, M. Akiyama and T. Sakagami, *J. Biochem.* (Tokyo), **62**, 618 (1967); *e*) C. Michalec and Z. Kolman, *J. Chromatog.*, **31**, 636 (1967); *f*) G. Soula, P. Valdiguie and L. Douste-Blazy, *Bull. Soc. Chim. Biol.*, **50**, 887 (1968).

of all the fatty acids detected is not yet attained, the result of the analysis is expressed as the percentage of the sum of the acids with same numbers of carbon atoms, as shown in Table II.

It is clear from the Table that the sphingomyelin from atheromatous aorta contains remarkably higher percentage of C_{16} fatty acids and lower percentage of the acids with 20 or more of carbon atoms than that obtained from the normal aorta (44.5% versus 25.0% for C_{16} acid, and 27.2% versus 50.9% for the acids of C 20). Such a result indicates that though the relative percentage of sphingomyelin of the total phospholipids remains unaltered, the sphingomyelin in atherosclerotic aorta contains different proportions of the molecular species as compared with that in normal aorta.

TABLE II. Fatty Acid Composition of Sphingomyllin Isolated from Normal and Atherosclerotic Aorta of the Rabbit

Fatty acids	Moles per 100 moles fatty acids	
	Normal aorta	Atherosclerotic aorta
C=14	1.4	2.1
C=16	25.0	44.5
C=17	3.8	2.3
C=18	18.9	23.9
C≧20	50.9	27.2

operating conditions of gas-liquid chromatography:
column: 10% diethylene glycol succinate polyester on 60/80 mesh Uniport K (3 mm × 4 m)
oven temperature: 185°
carrier gas: N_2
flow rate: 20 ml/min
detector: hydrogen flame detector

TABLE III. Phospholipid Class Composition in Blood Plasma of Normal and Atherosclerotic Rabbit

Groups of animals	Percent of total phospholipids					
	Fr. 1 Phosphatidic acid	Fr. 2 Phosphatidyl ethanolamine	Fr. 3 Phosphatidyl choline	Fr. 4 Sphingomyelin	Fr. 5 Lysophosphatidyl choline	Fr. 6 Phosphatidyl serine, phosphatidyl inositol
Normal	1.2±0.76	9.2±0.50	55.4±1.2	12.6±1.4	19.5±2.7	2.0±0.26
Atherosclerotic	1.3±0.82	5.2±1.0[a)]	61.2±6.7	13.0±1.3	17.5±3.7	1.8±0.70

The figures indicate the mean value ± standard deviation.
numbers of the samples analyzed: each 3
a) significant difference with $0.001<p<0.01$

The phospholipid distribution of plasma from normal and atheromatous rabbits is shown in Table III. In general, no notable difference in the phospholipid class composition is observed except for phosphatidyl ethanolamine of which percentage is significantly decreased in atherosclerotic animal (from 9.2±0.50% to 5.2±1.0%).

Sphingomyelin from normal and atherosclerotic rabbit plasma also gave two distinct bands on Silica gel TLC, similar to those found on this phospholipid fraction from atheromatous aorta demonstrated in Fig. 2. Therefore, the ratio of the amount of the upper component to the lower one was determined by measuring the amount of phosphorus in each band, and was found to be 0.71 for the plasma from normal rabbits and 0.74 for the plasma from atherosclerotic rabbits (the corresponding ratio for sphingomyelin from atherosclerotic aorta being 1.1).

Thus, it is suggested that there would be practically no difference in the molecular species composition of the plasma sphingomyelin between these two groups of the experimental animals.

From such a finding, it seems to be improbable that the marked alteration in the phospholipid class composition disclosed on atheromatous aorta should be the mere reflection of the altered phospholipid pattern of the plasma. It is suggested that the phospholipids accumulated in atherosclerotic aorta do not entirely originate from infiltration of the plasma lipids into arterial wall in the form of lipoproteins. To what extent the accelerated *in situ* synthesis of phospholipids in atheromatous aorta tissue of the rabbit may contribute to the accumulation there remains to be solved by further investigations.