Phospholipids of Pork Muscle Tissues[1, 2, 3]

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Abstract

The lipids extracted from four locations on a hog carcass were fractionated into nonphospholipids, phosphatidyl ethanolamine, phosphatidyl serine, lecithins, and sphingomyelins. The identities of phospholipid fractions were established and their quantitative contents were determined by three methods: gravimetrically, from analysis of the phosphorus content, and from standard curves of infrared spectra. Variations in content of a phospholipid type were noted in the carcass locations studied. Lecithin and phosphatidyl ethanolamine were found in greatest quantity. These represented ca. 61% and 31%, respectively, of the total phospholipids. The remainder was distributed between phosphatidyl serine and sphingomyelin at 5% and 3%, respectively. The phospholipid content of hog meat was found to be slightly over 0.5%.

Introduction

KNOWLEDGE of the nature of the phospholipids in tissues of meat animals is limited chiefly to tissues of organs, such as brain, liver, etc., which are known to be rich in phospholipids. Knowledge of phospholipids in fresh muscle tissues of meat animals is rather fragmentary. It appears to be important to gain further knowledge of the constituents of meat which contribute to the problems of quality. For this purpose it is important to establish the presence, quantities, and characteristics of phospholipids in meat tissues. The phospholipid composition of muscle tissues in a hog carcass is reported here.

Experimental

Materials. Samples of muscle tissue were taken from four locations on a hog carcass with the following history: sex, male; castrate; age, 5 months; weight, 195 lb; breed, York and Hampshire crossing; feeding, complete.

Silicic acid, cp, precipitated, from Fisher Scientific Co. All solvents were freshly redistilled. Silicic acid was washed with methanol and dried 24 hr at 120C.

Lipid Extraction. Lipids were protected when possible by substitution of nitrogen for air throughout procedures of extraction, separation, and storage. Despite this precaution, the oxidation of isolated fractions could not be entirely eliminated. Extraction of lipids from muscle tissues was made by macerating in a Waring Blendor with chloroform, methanol, and water. The extraction procedure was essentially that of Bligh and Dyer (1), modified to include washing of the extract according to Folch, et al. (2). In order to remove most of the neutral fat fraction from the phospholipids, a rapid method of Choudhury and Arnold (3) was used. No phosphorus was found in the neutral fat fraction, but some of the neutral fats were present in the phospholipid fraction. The content of neutral lipids was determined in suitable aliquots by evaporation of chloroform under a stream of nitrogen, drying overnight in a vacuum desiccator over calcium chloride, and weighing. The phospholipid fraction, after removal of solvents under reduced pressure with a rotary evaporator, and completion of evaporation under a stream of nitrogen, was taken up with a small volume of chloroform and put on a silicic acid column.

Chromatography. Silicic acid columns were prepared in chloroform using at least 50 mg of silicic acid per one mg of phospholipids to be separated. The scheme of chromatographic separation on silicic acid is shown in Table I.

Nonphospholipids were eluted with chloroform as fraction I. The effluent was checked by the Salkowski test for cholesterol and, after obtaining a negative test, the volume of effluent was measured; this was referred to as a volume and is used in the context of the third column in Table I. The volume of chloroform was doubled in order to elute the nonphospholipid fraction.

Acetone, referred to as a scavenger by Nelson and Freeman (4), was used to remove pigmented material. It was usually possible to follow this visually as a band and about ⅓ of a volume of acetone was sufficient.

The third fraction was eluted with one volume of 10% methanol in chloroform, followed with 15% methanol until a ninhydrin positive test was obtained. For this test, equal volumes of sample, ninhydrin, and 2,4-lutidine, were used. A portion giving a ninhydrin positive test was combined with the next fraction.

Cephalins, as fraction IV, were eluted with one volume of 20% methanol followed by 25% methanol in chloroform until disappearance of the ninhydrin test. They were separated into phosphatidyl ethanolamine and phosphatidyl serine on a hydrated silicic acid silicate column as described by Rouser, et al. (5). The elution pattern was followed with the ninhydrin test and phosphatidyl ethanolamine as fraction IVa was eluted with 25% methanol in chloroform followed by phosphatidyl serine as fraction IVb eluted with methanol.

For elution of fraction V, containing lecithins, from a silicic acid column 4 volumes of 35% methanol in chloroform were used, and the sphingomyelins were then eluted as fraction VI with two volumes of methanol.

Solvents were removed from all fractions under reduced pressure in a rotary evaporator and transferred quantitatively to an ultrafine bacteriological filter, and filtered with suction. In this way the escape of silicic acid fines was prevented. Filtrates were transferred to weighed 25 ml glass stoppered Erlenmeyer flasks, and solvents were removed under a

TABLE I

Scheme for Chromatographic Separation on Silicic Acid

Fraction	Eluant	Volume[a]	Components
I	Chloroform	2	Nonphospholipids
II	Acetone	⅓	Principally nonphospholipids
III	10% Methanol[b]	1	Cerebrosides (?)
	15% Methanol	X ml	
IV	20% Methanol	1	Cephalins[c]
	25% Methanol	X ml	
V	35% Methanol	4	Lecithins
VI	Methanol	2	Sphingomyelins

[a] Explained in text.
[b] Listed percentage of methanol is in chloroform (v/v).
[c] Separated into phosphatidyl ethanolamine (IVa) and phosphatidyl serine (IVb) on a hydrated silicic acid silicate column.

[1] Journal Article No. 3077, Michigan Agricultural Experiment Station.
[2] Supported by research grant No. RG-8801, National Institutes of Health, U. S. Public Health Service, Department of Health, Education, and Welfare.
[3] Presented at the AOCS meeting in Toronto, Canada, 1962.

stream of nitrogen. Flask contents were dried overnight in a vacuum desiccator over calcium chloride and weighed. Flask contents were transferred with a suitable spectro grade solvent to volumetric flasks and used for infrared (IR) spectra and other analyses.

Preparation of Standards. Isolated phospholipid standards of desirable purity are usually not available. Synthetic compounds of rigorous purity, if available, have limited solubility for use for IR spectra in solutions, and they may have different extinction coefficient values in IR absorption spectra than naturally occurring phospholipids because of different fatty acid composition and location (6). It was desirable, therefore, to prepare our own standards from hog ham muscle. The phosphorus content, qualitative thin-layer chromatography, and IR spectra were used as criteria of purity of the fractions isolated for standards. Phosphorus was determined by the method of Beveridge and Johnson (7). Thin-layer chromatography was conducted on silica gel G, according to Stahl, with solvent of chloroform-methanol-water, 65/25/4, v/v/v, according to Wagner (8). Chromatograms were developed with 0.2% ninhydrin in *n*-butanol-aqueous acetic acid 10%, 95/5, and aqueous sulfuric acid, 1/1, v/v (9). Both phosphatidyl ethanolamine and phosphatidyl serine were hydrolyzed in 3*N* HCl in sealed tubes at 100C for 16 hr (5). After hydrolysis, the free fatty acids were extracted into petroleum ether, and the water-acid layer was evaporated to dryness in order to remove hydrochloric acid. The residue was dissolved in water and chromatographed with corresponding reference compounds (ethanolamine and serine) on the plate coated with silicic acid (TLC). For developing, a ninhydrin spray was used. The cerebroside fraction after acid hydrolysis was tested with anthrone reagent (10) for possible presence of galactose.

IR measurements were made with a Beckman IR5 double beam recording spectrophotometer, equipped with a sodium chloride prism.

Results and Discussion

Evaluation of Standards. Standards were quite pure on a basis of phosphorus content. The cerebroside fraction had no phosphorus and the phospholipids assayed 97–103% of theoretical phosphorus content.

Thin-layer chromatography of the cerebroside fraction revealed no component which reacted with ninhydrin, but gave a single spot when developed with sulfuric acid, with an R_f value of 0.85. Phosphatidyl ethanolamine and phosphatidyl serine each gave single spots with ninhydrin and sulfuric acid and with R_f values of 0.61 and 0.37, respectively. Acid hydrolysis products of both aminophosphatides gave identical ninhydrin positive spots with their appropriate reference compounds ethanolamine and serine. Lecithins gave a negative ninhydrin reaction only after rechromatographing on silicic acid and gave a spot (developed with sulfuric acid) with an R_f of 0.40. Sphingomyelins gave a ninhydrin negative spot with R_f of 0.31 and another faint spot corresponding to lecithin, even after rechromatographing on silicic acid.

Acid hydrolysis products of the fraction considered to be cerebrosides gave inconclusive results with anthrone reagent. IR spectra of this fraction did not confirm the presence of cerebrosides, and identity was not established. This fraction is not included in further quantitative presentation of phospholipids, because of the small quantities involved. IR spectra of isolated phospholipid standards are shown in Figure 1. Since the absorption cells were not entirely matched, we have discontinued regions on the spectra corresponding to the regions of absorption by solvents. Spectrum A represents phosphatidyl ethanolamine, and spectrum B phosphatidyl serine. Both spectra exhibit band characteristics of cephalins at 9.8 μ, which are poorly developed in isolated cephalins as compared with those of the synthetic, and appear rather as inflections. There is no trace of a lecithin band at 10.3 μ and, common to both cephalins and lecithins, the ester carbonyl band at 5.8 μ is strongly developed. Since there is no ester carbonyl band at 5.8 μ in the sphingomyelin spectrum, and the 10.3 μ band is not present in either isolated cephalin, both bands at 5.8 and 9.8 μ might be used for calibration curves.

Spectrum C represents lecithins with strong bands

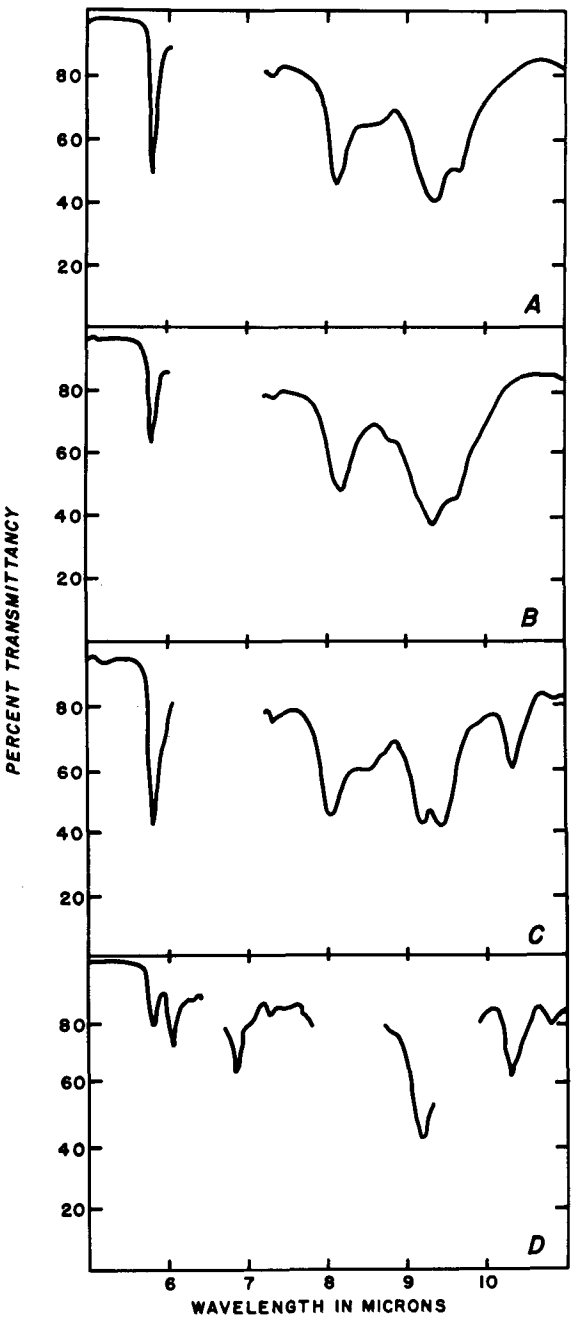

FIG. 1. IR spectra of isolated reference compounds from hog ham muscle. A. Phosphatidyl ethanolamine, 19.86 mg per ml in CS_2; B. Phosphatidyl serine, 18.15 mg per ml in CS_2; C. Lecithins, 20.27 mg per ml in CS_2; D. Sphingomyelin fraction, 18.50 mg per ml in $CHCl_3$. Discontinued regions on the spectra correspond to the regions of absorption by solvents.

TABLE II
Absorption Coefficients

Compound	Wave length μ	$a \times 10^{2}$ [a]
Lecithin, in CS_2	5.8	2.52
	10.3	1.20
Phosphatidyl ethanolamine, in CS_2	5.8	1.53
	9.8	1.63
Phosphatidyl serine, in CS_2	5.8	1.10
	9.8	1.97
Sphingomyelin, in $CHCl_3$	6.1	1.08

[a] a = Absorbancy per mg per ml.

at 10.3 and 5.8 μ. There is no trace of cephalins as indicated by absence of absorption in the 9.8 μ region.

Spectrum D represents the sphingomyelin fraction with a band of 10.3 μ which is common to both lecithins and sphingomyelins, the amide band of sphingomyelins at 6.1 μ and the ester carbonyl band at 5.8 μ which is not present in sphingomyelins. This spectrum actually represents sphingomyelins with some admixture of lecithins. Since only a limited size sample was available for chemical purification of the sphingomyelin fraction, and since a standard curve was already prepared for lecithin, the amount of sphingomyelin in the fraction contaminated with lecithin can be calculated, and a standard curve for sphingomyelin may be constructed. This is possible because the amide band at 6.1 μ, and the ester carbonyl band at 5.8 μ, are independent and are exhibited by two different compounds. This approach was adapted in our quantitative calculations and also permitted proper correction for lecithin content in fraction V of the meat samples by using the sphingomyelin value in fraction VI.

Table II lists absorption coefficients of these bands of isolated standard compounds which were used for calculation or crosschecking of quantitative amounts of phospholipids in a hog carcass. These values for absorption coefficients are for a particular instrument and the set of cells used and, therefore, comparison of them with the published data would be difficult. Phosphatidyl ethanolamine and phosphatidyl serine are believed to exhibit similar (11) or identical (12) IR spectra. On the basis of our results, although both of them exhibit the same bands, absorption coefficients for both bands at 5.8 μ and 9.8 μ in each

TABLE III
Lipid Fractions Isolated from 100 g Fresh Hog Meat

Sample	Method		
	Gravimetric	Phosphorus content	IR spectra [a]
Nonphospholipids			
Belly	7.1322	.....	.....
Ham	2.1636	.....	.....
Loin	2.4076	.....	.....
Ribs	5.5944	.....	.....
Principally Nonphospholipids [b]			
Belly	0.0305	.....	.....
Ham	0.0433	.....	.....
Loin	0.0241	.....	.....
Ribs	0.0245	.....	.....
Phosphatidyl Ethanolamine			
Belly	0.1910	0.1939	0.1826
Ham	0.1616	0.1531	0.1727
Loin	0.1526	0.1515	0.1343
Ribs	0.1631	0.1562	0.1576
Phosphatidyl Serine			
Belly	0.0275	0.0276	0.0281
Ham	0.0370	0.0371	0.0368
Loin	0.0216	0.0214	0.0213
Ribs	0.0142	0.0143	0.0139
Lecithins			
Belly	0.3408	0.3323	0.3280
Ham	0.2587	0.2490	0.2692
Loin	0.2794	0.2801	0.2618
Ribs	0.3623	0.3623	0.3889
Sphingomyelins			
Belly	0.0226	0.0226	0.0167
Ham	0.0155	0.0158	0.0119
Loin	0.0054	0.0056	0.0047
Ribs	0.0353	0.0352	0.0277

[a] Phosphatidyl ethanolamine at 9.8 μ, phosphatidyl serine at 5.8 μ, lecithins at 10.3 μ, and sphingomyelins at 6.1 μ.
[b] Pigmented material containing 0–0.25% phosphorus.

TABLE IV
Percentage of Each Phospholipid Type in the Phospholipids of Hog Muscle Tissue

Phospholipid	Carcass Location			
	Belly	Ham	Loin	Rib
Phosphatidyl ethanolamine	32.8	34.2	33.3	28.4
Phosphatidyl serine	4.7	7.8	4.7	2.5
Lecithin	58.6	54.7	60.8	63.0
Sphingomyelin	3.9	3.3	1.2	6.1

phospholipid class are different. This may be due to different fatty acid groupings within their molecules.

Comparison of Chromatographic Fractions. Table III lists the quantities of isolated lipids in 100 g samples taken from the hog carcass at each of the sites of study. There is wide variation in content of nonphospholipids composed of cholesterol, cholesteryl esters, and glycerides. Removal of depot fat when taking a muscle sample in the ham or loin is rather simple but, on the other hand, because of fine overlapping of fat and muscle tissue in rib or belly tissue, the separation of fat is more difficult and variation in neutral fat content was rather expected.

Fraction II contains undefined pigmented material eluted with acetone. There were traces of phosphorus in this fraction which would indicate that minute amounts of phospholipids could be present in this fraction.

Phospholipid content was determined by three different methods: gravimetric analysis, analysis of the phosphorus content in the various fractions isolated, and from standard curves established from IR spectra. The contents of phosphatidyl ethanolamine, phosphatidyl serine, and lecithins, as determined by these three different methods, are generally in good agreement. On the other hand, sphingomyelin content, determined from IR spectra, is lower by almost one-fourth when compared with the results obtained by gravimetric analysis or phosphorus content. This was due to contamination of the sphingomyelin fraction with lecithin, as discussed above.

The phospholipids in greatest quantity in hog muscle tissue are lecithins and phosphatidyl ethanolamine. In addition to a variation of phospholipid class at a given carcass location, Table IV shows the relative amounts of each phospholipid among the four determined at each carcass location. The variations, while small, show that phosphatidyl ethanolamine tends to be present in greater quantities in the tissues with lesser total lipid content, while the sphingomyelin is relatively somewhat more abundant in the tissues with greater total lipid content. Corresponding distribution patterns are not recognized for lecithins and phosphatidyl serine. The lecithin content agrees quite well (Table IV) with the values reported by Hornstein, et al. (13) for aged pork muscle, but the cephalin content found in the present instance is substantially lower than reported.

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[Received November 19, 1962—Accepted May 16, 1963]