SOME STUDIES ON THE COMPOSITION AND STRUCTURE OF PHOSPHOLIPIDS IN CHICKEN MUSCLE

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ABSTRACT

SOME STUDIES ON THE
COMPOSITION AND STRUCTURE OF PHOSPHOLIPIDS
IN CHICKEN MUSCLE

by Chung-yen Peng

The phospholipids from chicken muscle tissues have been studied from the standpoint of occurrence, nature, composition, and structure. The leg and breast muscles of one-year old chicken on a standard MSU Z-4 diet were chosen for this investigation. Lipids extracted by a chloroform-methanol solvent system have been fractionated into non-phospholipids, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl choline, and sphingomyelins by silicic acid column chromatography. Phospholipid fractions were identified by thin-layer chromatography, and the quantity of each component was determined by gravimetric analysis, phosphorus content determination, and infrared spectral analysis.

The phospholipid content of leg muscle (dark meat) lipids was higher than that in the breast muscle (white meat) lipids. Phosphatidyl choline (lecithin) and phosphatidyl ethanolamine were found in relatively greater amounts than phosphatidyl serine and sphingomyelin. Hydrolysis of each phospholipid was carried out by enzyme, alkali, or acid,

and the liberated fatty acids were converted to methyl esters. Gas-liquid chromatographic analysis of the fatty acids from either position was used in order to establish the positional specificity of the fatty acids in the phosphoglycerides. Stearic acid was the predominant fatty acid in phosphatidyl ethanolamine while palmitic acid was dominant in lecithin. Oleic acid was the main fatty acid in both phosphatidyl serine and sphingomyelin. The polyunsaturated fatty acids were located primarily at the β position and the saturated fatty acids mainly at the α' position. The fatty acid composition of sphingomyelins was such that the dark meat contained a number of the longer-chain fatty acids from C_{21} through C_{25}, whereas white skeletal muscle had none.

The presence of plasmalogens in each phospholipid fraction was detected by mercuric chloride, and by thin-layer and column chromatography. The quantity of plasmalogens present in each phospholipid was determined by specific iodination and gravimetric analysis. Phosphatidal ethanolamine was found to be the predominant plasmalogen in both chicken dark and white muscles. The fatty aldehyde composition of the plasmalogens was analyzed by gas-liquid chromatography of dimethylacetal derivatives of the aldehydes. The resulting analyses showed that palmitaldehyde, stearaldehyde, oleylaldehyde, and capraldehyde were the main aldehydes, and that palmitaldehyde was in the greatest amount in each

plasmalogen component. A definite positional relationship between dimethylacetals and methylesters in a given chromatographic peak was also illustrated.

The phosphoglycerides were the phospholipids present in the greatest quantity in chicken muscle tissues, plasmalogens were next, and sphingolipids were present in the least quantity.

SOME STUDIES ON THE COMPOSITION AND STRUCTURE OF PHOSPHOLIPIDS IN CHICKEN MUSCLE

By

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TABLE OF CONTENTS

TABLE OF CONTENTS - Continued

LIST OF TABLES

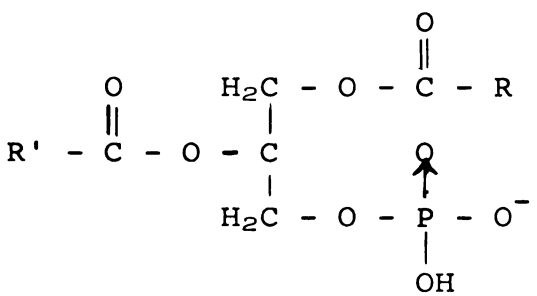
LIST OF TABLES - Continued

LIST OF FIGURES

LIST OF APPENDICES

INTRODUCTION

Phospholipids are regarded as complex lipids. Many of the naturally occurring compounds are derivatives of L-α-glycerophosphoric acid



where R and R' are the hydrocarbon residues of long-chain fatty acids, saturated or unsaturated, attached to the α'- and β-positions of glycerol through an ester linkage, and the phosphoric acid esterified at the α-position is also esterified to a simple nitrogenous base or the cyclic hexose inositol to form a complete phosphatide molecule. Phosphatidyl ethanolamine has an aminoethanol molecule esterified with the phosphoric acid, phosphatidyl serine has a serine molecule, lecithin (phosphatidyl choline) contains a choline base, and phosphatidyl inositol has inositol esterified with the phosphoric acid. Sphingomyelin is a phospholipid which contains sphingosine base in place of glycerol.

The glycerophosphatides are the more abundant of the naturally occurring complex lipids, which in many instances comprise well over 70% of the lipids of a particular tissue.

Phospholipids have been found in brain and other nervous tissues, blood, liver, eggs, muscle, and in the membranes and mitochondria of animals (45,47). In plants, phospholipids are distributed in chloroplasts (81), seeds (147,148), and vegetable tissues (95,260).

Phospholipids and sphingolipids fulfill their functional roles largely by their unusual chemical structure. Each molecule of these complex lipids has both a nonpolar and a polar region from which lipid-protein complexes are formed(86). Beveridge (18), Kanfer and Kennedy (119), and Rossiter (213) have postulated that phospholipids play multi-roles in biologic and metabolic systems. Lipoproteins in plasma function as vehicles to transport cholesterol and exogenous and endogenous triglycerides from the thoracic duct to the blood and to the tissues. In intestinal absorption of triglyceride and cholesterol, phospholipids act to stabilize lipid particles. Phospholipids are considered as principal components of the myelin sheath of a nerve fiber, a highly organized plasma membrane, and closely related to the transport of ions, in particular cations, across the cell membrane. Functionally and structurally, electron transport in mitochondria, protein synthesis in endoplasmic reticulum, and cell division in the nucleus are governed by the phospholipids in the intracellular membranes. Phospholipids are also found as major constituents of atheromatous deposits in the blood vessels, and cephalins are important in blood

coagulation. Phospholipids also play a role as lipid antigens (202), an energy source (94), antioxidant (188), prothrombin activator (191), and enzyme activator (31,240).

The phospholipids of skeletal muscle have not been studied to the same extent as the phospholipids of organ and neural tissues and those of bacteria. Chicken muscle tissue in turn have not been studied as much as those in muscle of pork, beef, lamb, and veal.

Besides the glycerophospholipids and sphingolipids which have been mentioned earlier, a third type of phospholipids known as plasmalogens (62) is frequently encountered. These are also glycerophospholipids, but the α'-carbon of the phosphoglyceride is linked to a long-chain aldehyde through an α,β-unsaturated ether linkage (157,159,160,162). Why nature creates this type of lipid is not known.

Chicken meat contains a favorable protein-fat ratio. The small amount of fat in the meat makes it palatable and satisfying, improves the utilization of proteins and facilitates the utilization of fat-soluble vitamins, thus making chicken meat both satisfying and nutritious. Chicken muscle is especially low in total fat content, but the fat present contains a fair amount of unsaturated fatty acids. It is also a good source of "essential" polyunsaturated fatty acids, e.g., linoleic, linolenic and arachidonic acids which are considered essential for nutrition.

The objectives of this study were: To determine the composition and structure of the glycerophospholipids, sphingolipids, and the plasmalogens of both dark (leg) and white (breast) meat in chicken.

LITERATURE REVIEW

Pioneer Studies of Phospholipids

West and Todd (259) wrote in their biochemistry textbook that Gobley isolated a phospholipid from egg yolk which he named "lecithin" (Greek lekithos, egg yolk). Couerbe, Fremy, and Valencienne found phospholipids in a variety of animal sources, and in 1861 Töpler found them in plant seeds. Thus, by the middle of the nineteenth century the widespread occurrence of phosphorus-containing lipids in both plant and animal tissues and products had been established.

During the latter half of the nineteenth century, Thudichum (246) made an exhaustive and careful study of the chemistry of the brain lipids, much of which dealt with the phospholipids. This was summarized in his classical monograph "A Treatise on the Chemical Constitution of the Brain" (London, 1884).

General Review of Phospholipids

In their monograph "Lecithin and Allied Substances. The Lipins," MacLean and MacLean (150) gave a thorough description of the chemistry of the phospholipids--lecithin, kephalin and sphingomyelin. This detailed the nomenclature and classification, the composition and structure, hydrolysis and properties, the occurrence in animals and plants, methods of

extraction, isolation and purification, the alleged lipins, and the biological functions of phospholipids.

Wittcoff (262) published "The Phosphatides" which covered the chemistry of the phosphatides--lecithin, phosphatidyl serine, phosphatidyl ethanolamine, sphingomyelin, and other minor constituents, the analytical methods, and industrial manufacture and utilization. He also speculated on the role of phosphatides in metabolism, in pathological development, their hydrolysis products in metabolism, and their serological and oxidative functions.

Folch (66) reported the chemistry of phosphatides in the symposium on phosphorus metabolism in the Johns Hopkins University in 1952. He defined phosphatides, classified them and discussed structural determinations of phosphoglycerides, phosphosphingosides, phosphoinositides, and three others. He also mentioned that the first phosphatide established as a chemical entity was lecithin, a diacyl-glyceryl-phosphoryl choline. Then followed the discovery of plasmalogen (acetal phosphatides), phosphatidic acid, phosphatidyl inositol, cephalin which was to be a mixture of three different phosphatides, namely, phosphatidyl serine, phosphatidyl ethanolamine, and diphosphoinositide, and cardiolipin, either isolated or established in this order from 1924 through 1941.

Artom (12) discussed the biological functions of phospholipids in animal tissues and their role in fat absorption, fatty acid transport, and fat metabolism.

The chemistry of phosphatides and cerebrosides was studied by Celmer and Carter (30). They worked on the nomenclature, classification, determination, distribution, histochemical staining, isolation, the mechanism of hydrolysis of phosphate esters of glycerol, and enzyme systems active against phosphatides.

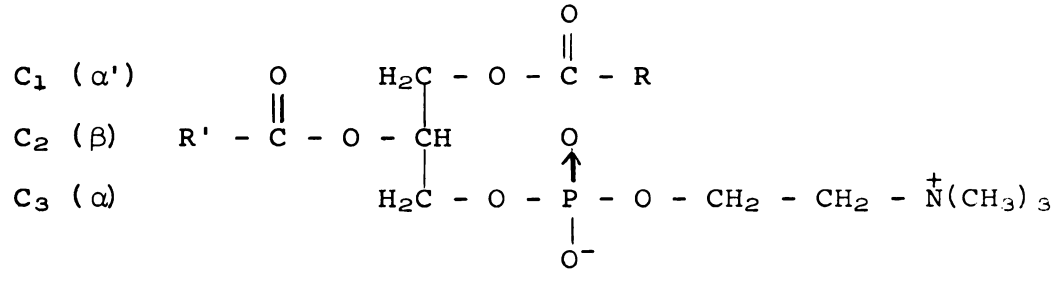
The separation and identification of phosphatides, methods of analysis, naturally occurring phosphatides and their constituents, determination of structure, synthesis and chemical hydrolysis of phosphatides have been thoroughly reviewed by Baer (14).

Recently, Ansell and Hawthorne (10) published "Phospholipids. Chemistry, Metabolism and Function." This could be the first complete report consolidating all elements of the study of phospholipids since Wittcoff (262). The book is concerned with chemical structure of phospholipids, their hydrolysis, analytical methods, preparations, biosynthesis, catabolism, ion transport and other theories of phospholipid function and other related aspects.

Structural Determination of Phospholipids

Lecithin. Since Gobley (74) isolated a phosphatide from egg yolk and gave it the name "lecithin," most studies have been concentrated on this component. Gobley (75) later succeeded in isolating, from a natural source, glycerophosphoric acid which was present in the hydrolysis products.

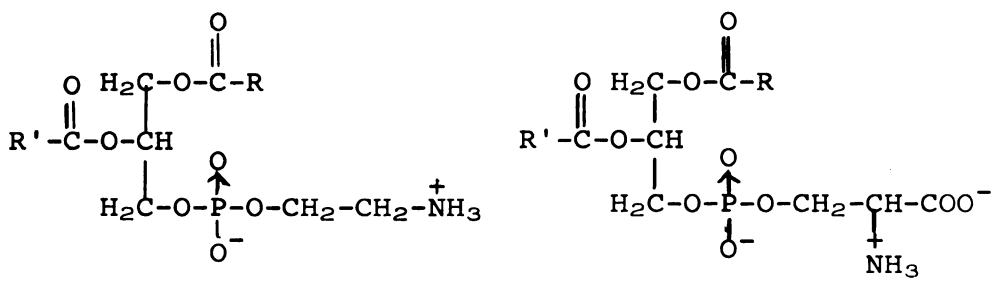
The hydrolysis mixture also provided both a saturated and an unsaturated acid. Eighteen years later, Diakanow (46) and Strecker (238) isolated a nitrogenous base which was named choline (Greek cholē, bile) from bile and found that at least two molecules of fatty acids were present for every molecule of glycerophosphoric acid and of base in the hydrolysis products. In 1901, Thudichum (247) isolated lecithin from brain and confirmed its structure as L-3-phosphatidyl choline. Chemical evidence of its structure was also provided by Levene and Rolf (143).



In the positional distribution of fatty acids on lecithin, Tattrie (241,242), deHaas et al. (43), and Hanahan et al. (93) proved, by the use of pancreatic lipase and phospholipase A, that the saturated acids are on the α' position while the unsaturated acids occupy the β position. Privett et al. (195,196) studied the structural analysis of lecithins and observed that there are four types of egg lecithins, α'- and β-saturated, α'-saturated-β-unsaturated, α'-unsaturated-β-saturated, and diunsaturated units, the second being the

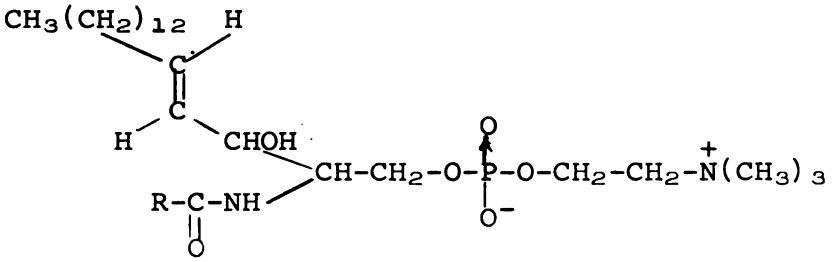
main one. Collins (34) announced that stearoyl arachidonoyl lecithin was the major component of lecithin from rat-liver. Recently, Long et al. (146), Menzel and Olcott (171), and Sastry and Kates (221) confirmed these findings.

Cephalins. The cephalin fraction was first isolated from brain tissue and recognized as a distinct entity by Thudichum (247). Its name is derived from the Greek kephalē, meaning head. The material seemed to be insoluble in alcohol, a characteristic upon which Thudichum and many subsequent workers depended for separating it from lecithin. Trier (250-252) found aminoethyl alcohol in the phosphatides from the bean and egg, and was able to identify the base by the insoluble derivative formed with gold chloride. Folch (64) has shown "cephalin" to be a mixture of three components. The term "cephalin" is ill-defined and certainly is misleading as pointed out by Hanahan (91). He advises the employment of a more definite term, such as phosphatidyl ethanolamine. The structure of phosphatidyl ethanolamine and serine were advanced by Folch (64,65) who postulated the former as an L-α-(diacyl)-glycerophosphoryl ethanolamine, and the latter as L-α-(diacyl)-glycerophosphoryl serine.



The positional distribution of fatty acids in these fractions has not been studied to the same extent as lecithin. Hawke (97) worked on the glycerophospholipids of buttermilk, and Kuchmak and Dugan (137) examined pork muscle tissues. By the use of phospholipase A, it was shown that the same general pattern of distribution of saturated and unsaturated fatty acids occurs in cephalins as in lecithins.

Sphingomyelins. This fraction was first isolated by Thudichum (247) who named it from the Greek sphingein, to bind tight. After hydrolysis with barium hydroxide, he found two bases, sphingosine and choline, a fatty acid, and phosphoric acid. This structure was confirmed and established by Levene (140-142) who reported that the hydrolysis products consisted of phosphoric acid, choline, sphingosine, and fatty acid, chiefly lignoceric acid. He was able to show that the lignoceric acid was linked to the remainder of the molecule by an amide linkage rather than an ester linkage. The phosphoryl choline was attached through ester bonding (29,156,214) to the hydroxyl group at carbon atom 1 of sphingosine. The configuration about the double bond was established as trans (69). On the basis of these data the structure of sphingomyelin was then established as



Sphingomyelin is resistant to basic hydrolysis due to its amide linkage (142,223). The fatty acids of sphingomyelin from a tissue are characteristic of the sphingolipids of that tissue (236), and vary with age and any change during myelin disorders (237). Sphingomyelin from beef brain tissue contained only nonhydroxy fatty acids (184). The fatty acid composition of sphingomyelin from different sources has been investigated by many research workers (26,120,127,225,239,244). The acids range from C_{10} to C_{26} and mainly are saturated and monounsaturated. However, Kishimoto (127) found diunsaturated acids in sphingomyelin from pig brain, and Sweeley (239) found them in sphingomyelin from human plasma. Wood (265) and Eldin (52) observed two spots on thin-layer chromatograms from human plasma and ox-brain respectively, and they suggested this could be attributed to different fatty acid patterns.

Extraction

Because of the complex nature of the phospholipids, the problems associated with their analysis are many. Hoppe-Seyler (102) thoroughly extracted lecithin from Egg yolk with ether and treated the residue with warm alcohol. Thudichum (246) obtained lecithin from brain by a very complicated method using alcohol, cadmium chloride, ether and benzene. MacLean and MacLean (150) believed that drying is essential to isolate phospholipids in an unchanged condition. They extracted them from dried material either with ether or alcohol.

The most applicable and reproducible extraction procedure must be credited to Folch, Lees, and Sloane-Stanley (67). In this method, the tissue is homogenized and extracted with chloroform-methanol (2:1 v/v) and then mixed thoroughly with 0.2 volume of water or an appropriate salt solution to make the final proportion of chloroform-methanol-water 8:4:3 by volume. The resulting mixture separates into two phases, and the lower phase contains the pure total lipid extract.

Bligh and Dyer (21) modified the Folch et al. method and revised the final proportion of chloroform-methanol-water to 2:2:1.8 by volume. Direct use of chloroform-methanol or ethanol-ether mixtures at room temperature is perhaps the most useful method for mammalian tissue extraction (91). Ostrander and Dugan (190) found the proportion of methanol-chloroform-water approximately 2:3:1,v:v:v, to give the best agreement among raw, cooked, and lyophilized samples. Marinetti (164) employed a methanol-ethyl ether or a methanol-chloroform solvent system for serum. For gas-liquid chromatographic analysis, Sheppard (229) found Bloor's reagent (3:1 ethanol and ether) to be much better than the diethyl ether Soxhlet extraction. In extracting milk phospholipids, Duthie and Patton (49) used a solvent partition system with predominantly ethyl and petroleum ethers and 1.5% sodium chloride. Hivon et al. (101) studied seven different solvent systems for extraction of soybean oil. These included

chloroform-methanol; petroleum ether; ethyl ether; chloroform; ethanol-ethers (1:1 ethyl and petroleum); ethanol, 2N HCl, aq. 1:1 ethers (ethyl and petroleum); and ethanol, 8.5N HCl, with the 1:1 ethyl-petroleum ether shown above.

Separation and Purification

In the separation of phospholipids from neutral lipids and other water-soluble contaminants, MacLean and MacLean (150) treated lipid extracts with an excess of acetone, which precipitates the phospholipids, leaving others in solution. Each individual phospholipid was prepared by using a different solvent system based on the solubility of the phospholipids in these systems. Borgström (22), Hanahan (91), and Ansell and Hawthorne (10) introduced the use of acetone-magnesium chloride precipitation to separate phospholipids. Countercurrent distribution has been used by Scholfield (224) to separate phosphoinositides, lecithin and phosphatidyl ethanolamine. However, this method is limited by slow separation of the solvent phase, by emulsion formation, and poor separation due to association of phospholipids.

Although, according to Fruton and Simmonds (69), chromatography was first studied by Schoenbein in 1861, its development as a systematic method came from the work of the Russian botanist Michael Tswett in 1906 in separating the leaf pigments.

Separation of solutes by liquid-solid chromatography depends on (a) the equilibrium established at the interface

between the solid adsorbent and the applied solution, resulting from adsorbent-solvent competition for the dissolved solute; and (b) the relative solubility of the solute in the eluting solvent. The solute is adsorbed from the solution onto the adsorbent, and is later desorbed into the eluting solvent (72). This type of chromatography is usually denoted "adsorption chromatography," and two types are widely used in the lipid field, namely, column and thin-layer chromatography.

Column chromatography. Acid-treated Florisil, a commercially prepared magnesia silica gel, has been employed in column chromatography for the separation of phospholipids and other complex lipids (27). There seemed to be no clean separation of different phospholipid classes.

The first person in the United States to separate phospholipids on a silicic acid column according to Marinetti et al. (158) was D. M. Rathmann who included these observations in her Ph. D. thesis submitted to the University of Rochester in 1944. Borgström (22) investigated chromatographic properties of magnesium oxide, saccharose, and silicic acid adsorbents. He found that silicic acid cleanly separated nonphospholipid from phospholipids and has a higher adsorbing capacity for phospholipids. The silicic acid he used was activated for 24 hours at 120°C and then kept in a tightly stoppered bottle. The neutral lipids are eluted by a less polar solvent (usually ether or chloroform), then the more

strongly adsorbed phospholipids can be eluted with pure methanol or a chloroform-methanol mixture (92).

Phospholipids can be separated from neutral lipids by simply shaking a chloroform solution with silicic acid, which adsorbs the phospholipids, followed by centrifugation or filtration (267). The nature of silicic acid as a chromatographic material in the quantitative estimation of a mixture of phospholipids has also been thoroughly discussed by Wren (267). He was able to list a series of common solvents according to their eluting power, and an order of elution in which higher homologues are eluted more readily than lower homologues.

In 1955 Lea et al. (138) separated egg yolk phosphatidyl ethanolamine from lecithin more easily with silicic acid than with alumina. Twenty percent methanol in chloroform eluted the former much more easily than the latter, and gave a good separation of both components. Hanahan et al. (92) extended these investigations to the more complicated phospholipids in liver and yeast. With an increasing stepwise concentration of methanol in chloroform, 0%, 20%, 40%, and 80%, they separated five phosphorus-containing fractions, an unidentified fraction, phosphatidyl ethanolamine and a serine mixture, phosphoinositides, lecithins, and sphingomyelins in a reproducible manner.

For obtaining best and reproducible results, silicic acid should be pre-treated, and the packed column should be washed with acetone-ether and ether before use (100).

Recent applications of silicic acid chromatography to the separation and estimation of tissue phospholipids are many and excellent, among them Rouser et al. (215,216,219) and Marinetti (164) studied phospholipids in great detail. The former workers even established a monitoring system for column fractions.

Thin-layer chromatography. A method for chromatographic adsorption analysis on a thin layer (less than 1 mm) of suitable adsorbent adhering to a glass plate which has been proved extremely useful for the separation of phospholipids was described as early as 1938 by Izmailov and Shraiber, according to Mangold (152). The method remained in obscurity until 1956 when Stahl described equipment and procedures for the preparation of chromatographic plates, and demonstrated the potential usefulness of thin-layer chromatography (TLC) in the fractionation of substances. The plates are either prepared by a commercially available applicator (152,264), or by a spray technique (17). Commercially available silica gel G is now being widely used by most laboratories for the separation of neutral and acidic lipids (267).

Mangold (152,153) has reviewed the specific application of TLC for the analysis of lipids. He tabulated an eluotropic series of solvents according to their polarity, developing systems for different lipid classes, and many indicators for detection of components on a developed TLC plate. Many workers in the lipid field have adopted TLC for the separation

of phospholipids from neutral lipids (23,48,232,253), determining fatty esters (13), and separating individual phospholipids (20,136,218), and their fatty acid methyl esters and dimethyl acetals (178,183).

Quantitative separation and analysis of phospholipid classes by TLC are much faster than column chromatography on silicic acid (7,20,39,218). TLC is also extremely useful for checking the composition of a fraction eluted from a column (10).

Redman and Keenan (207) employed a method for the unidimensional separation into six fractions of pigeon tissue phospholipids by TLC with a phenol-water solution as developing solvent. Both Skidmore et al. (231) and Abramson et al. (6) developed a quantitative two-dimensional TLC to separate phospholipids from rat liver and thymus into seven and eight components respectively. The former used a basic developing solution, chloroform-methanol-7M ammonium hydroxide; while the latter employed an acid solution, chloroform-methanol-glacial acetic acid-water as the first dimensional developing solution. The second solvent system was also chloroform-methanol-7M ammonium hydroxide, with different proportions of chloroform and methanol. Lepage (139) derived seven major and some minor components from plant phospholipids by using chloroform-methanol-water (65:25:4,v:v:v) and diisobutyl ketone-acetic acid-water (80:50:10,v:v:v) solvent systems. Detecting reagents used by them were ninhydrin for

aminophosphatides, molybdic acid for all phosphatides, ferric chloride-sulfosalicylic acid for phosphate groups, ammoniacal silver nitrate for glycerol and inositol, Dragendorf reagent for choline, fuchsin-sulfurous acid for aldehyde groups, and hydroxylamine-ferric chloride to detect esterified fatty acids.

Identification of Phospholipids

Because of the wide range of components having various functional groups, a solvent eluting organic compounds from an adsorbent column was according to the type and number of functional groups in these compounds (24). These rules apply also in TLC on which different functional groups with different lipids have different rates of migration (152), therefore, on a developed TLC plate, different indicators can be used to identify a specific lipid class from the reactions of indicators with functional groups.

Two types of indicators, as pointed out by Mangold (152), are used for visualization and identification of lipids; destructive and non-destructive. Chromic sulfuric acid solution is an example of the former. This reagent causes charring of the organic materials on the plate. After charring, the lipid spots are measured photodensitometrically. Non-destructive indicators are those used by Skidmore (231). The consecutive use of several properly chosen indicators on one chromatoplate increases the probability that no substance remains undetected or unidentified.

In combination with other analytical methods, Privett and Blank (196) were able to determine the structure of triglycerides and lecithins. Privett, Blank and Schmidt identified four different structures in egg lecithins (195).

Rouser et al. (219) observed that there is a considerable difference in the strength of binding through water for acidic and nonacidic lipids which accounted for the marked difference in the relative migration of the lipids since the acidic lipids are more firmly bound to some adsorbents in the presence of water. The amount of water in a developing solvent must be kept constant for a given system.

In the identification of brain phospholipids, Horrocks (107) compared separated lipid classes with synthetic samples and noted that the R_f values of each component varied with different developing systems.

Rouser et al. (216) identified phosphatidyl ethanolamine and phosphatidyl serine by hydrolyzing them with 3N HCl. The free acids were removed by petroleum ether, and HCl by evaporation, then the water-soluble hydrolysis products were chromatographed by TLC with corresponding reference compounds, ethanolamine and serine.

Infrared spectra. The measurement of infrared absorption spectra of organic compounds is particularly useful as an aid in characterization (32,215). When a substance appears in the appropriate chromatographic fraction and migrates on a TLC plate as a particular lipid class, infrared

is the single most convenient means to confirm this tentative identification, since different absorption bands are shown by different molecules of the substance and by the functional groups within the molecule, stretching or bending at various wavelengths. Schwarz et al. (226-228) used a potassium bromide disc technique for the analysis of lipids from a complex mixture in amounts less than 1 mg. They demonstrated that phosphatidyl ethanolamine and serine possess strong absorption bands at 5.74 μ and 9.8 μ, lecithin at 5.8 μ and 10.3 μ, while sphingomyelins absorbed at 6.04 μ and 6.44 μ. These absorption bands corresponded with those observed later by Rouser et al. (217) in a study of beef brain phospholipids, and Smith et al. (234,235) in an analysis of milk phospholipids.

Freeman (68) assigned the absorption bands to many specific groups and derived a linear simultaneous equation to calculate concentrations. Nelson and Freeman (179) and Kuchmak and Dugan (136) obtained lipid fractions from a silicic acid column and measured the infrared absorption curves in appropriate solvents. They also carried out quantitative measurements by comparison with absorption curves of purified phospholipids and the intensity of appropriate bands. O'Connor (187) reviewed the recent progress in the application of infrared absorption spectroscopy to lipid chemistry, such as determination of cis-trans structure in unsaturation, molecular structure, hydrogenation, oxidation,

autoxidation, and the composition of lipids. He also tabulated the infrared bands used in analysis of phospholipids (185).

Near-infrared absorption spectroscopy can be used as a new tool for lipid analysis as described by Slover and Dugan (233) and O'Connor (186). Duthie and Patton (49) showed consistent infrared results for phosphatidyl ethanolamine after being purified and recovered from thin-layer chromatograms.

Determination of Position of Fatty Acids in Phospholipids

Hydrolysis. As mentioned previously, the most satisfactory methods for the determination of the individual phospholipids depend on the analysis of certain hydrolysis products. The procedure developed by Schmidt et al. (223) in 1946 was to saponify a phospholipid mixture in normal alkali (KOH) at 37°C for 24 hours. The acyl groups were completely removed from glycerophospholipids and subsequent acidification would break down plasmalogens to give water-soluble phosphates. The phospholipids stable to mild alkali and acid in the procedure were considered for some time to be exclusively sphingomyelins (87). Later Hübscher et al. (108) suggested that plasmalogens were also present in this fraction. Dawson (40) modified and extended the hydrolytic technique to saponify a phospholipid mixture with a minimal amount of dilute sodium hydroxide. The alkali-stable lipid fraction

was then treated with trichloroacetic acid, and the alkali- and acid-stable phospholipids were then hydrolyzed with methanolic hydrochloric acid. The solvent system, the time length, and the concentration of alkali used in the basic hydrolysis of phospholipids did not seem to be significant (108).

Many scientists have been successful in using mild-alkali hydrolysis in determining fatty acid composition from tissue phospholipids (38,106,136) and alternate mild basic- and acid-hydrolysis for liberation of sphingomyelin fatty acids (127,241).

Since basic or acid hydrolysis is considered to cleave all fatty acid ester bonds, it is not applicable to the determination of the positional distribution of fatty acids of the phospholipids. A more gentle and specific means for release of fatty acids concerns the use of enzyme systems, in particular phospholipase A. Phospholipases have been found in many tissues (268). The phospholipase A from snake venom (phosphatide acyl-hydrolase, EC 3.1.1.4), which is now considered to be a β-esterase (41,44,111,134,176,210,241) has been extensively used for positional determination.

It has been repeatedly demonstrated that saturated and unsaturated fatty acids tend to occupy stereospecifically distinct locations in the lecithin molecule (89). deHaas (43), Hanahan et al. (93), Tattrie (241), Moore (176) and Menzel (171) proved, by using either synthetic or naturally

occurring lecithins or both, that the saturated acids are mainly on the α'-position, while the unsaturated fatty acids primarily occupy the β-position.

The snake venom phospholipase A not only liberates the β-attached fatty acids on lecithins, but also exhibits a β-specificity to cephalins (44,137).

Cerebrosides, sphingomyelins, and phosphoinositides are not hydrolyzed, while choline and ethanolamine plasmalogens and phosphatidyl serine may be hydrolyzed slowly, therefore, the action of phospholipase A is relatively unaffected by the nature of the phospholipid polar group (10).

Gas-liquid chromatography. Gas-liquid chromatography (GLC) has been the most significant analytical advance in the lipid field in recent years, and has now all but superseded other chromatographic methods for analysis of fatty acid components on account of its rapidity, reproducibility, and resolving power. In gas-liquid chromatography, a sample injected into a heated zone is vaporized and swept by a flow of carrier gas into the column. The column is packed with a liquid phase on a solid support. The vaporized components of the injected sample show different affinities toward the column packing; hence, they are separated into discrete moving bands as the carrier gas continually flushes the column. When each band emerges from the end of the column, it enters the thermal conductivity detector which, in conjunction with a Wheatstone bridge, generates an electrical

signal proportional to the amount of component in the carrier gas. This signal is fed to an electronic recorder which produces the chromatogram (55).

Although the concept was proposed by Martin and Synge (168) in 1941, the finite experimental method of separating C_1 to C_{12} fatty acids was not introduced until 1952 by James and Martin (115,116). They made two assumptions: (a) Partition coefficient is constant, the mobile phase is compressible and thus produces a gradient of gas velocity down the column; (b) the partial pressures of the substances to be separated are negligible in relation to that of the stream of carrying gas. The method was extended by Cropper and Heywood (35) to the separation of C_{12} to C_{22} fatty acids as their methyl esters by using a silicone grease as stationary phase and a katharometer as detector. The separation of methyl esters of C_{12} to C_{26} straight-chain fatty acids by gas chromatography has been reported by Beerthuis and Keppler (16). Kahn and Whitham (118) have not only been able to analyze quantitatively a mixture of C_8 to C_{18} saturated and unsaturated fatty acid esters, but also extended the analytical separation of methyl esters up to and including the C_{34} straight-chain fatty acids. Ray (206) suggested the use of a thermal-conductivity cell for analysis of the effluent gas, a procedure now widely used. He pointed out that a thermal-conductivity cell is very sensitive in measuring thermal-conductivity changes by using an

alternating-current bridge, and amplifying the out-of-balance potential with a conventional a. c. amplifier. Zero drift was eliminated by the use of two thermal-conductivity cells, one in the carrier gas stream before the column, and one at the column exit. Since both cells were contained in the same block of metal, a thermostat was unnecessary. James and Martin (117) introduced the gas density balance as a detection unit. Using Apiezon grease or heavy lubricating oil as stationary phase, they obtained fairly good separations of a series of straight-chain and branched-chain saturated esters and unsaturated esters of C_{10} to C_{18} acids.

Orr and Callen (189) utilized a polar stationary phase of Reoplex 400 Plasticizer (a polyoxyalkalene adipate polyester), since those non-polar liquid phases such as silicone oil or Apiezon grease had difficulty in separating isomers with the same carbon number. This greatly shortened the analysis time and gave fairly complete resolution of esters of the common fatty acids in which monoenes, dienes, trienes, etc., of substances with the same carbon number were eluted in that order after the corresponding saturated ester. Shortly afterwards, the adipate and succinate polyesters of diethylene glycol were introduced by Lipsky and Landowne (144,145). For the first time, it became possible to separate the methyl esters of the C_{18} unsaturated fatty acids completely from each other and from methyl stearate.

The addition of 3% (w/w) phosphoric acid to the liquid phase proved to be useful in retaining defined, symmetrical peaks for long-chain fatty acids (173).

Since Cropper and Heywood (35) converted fatty acids to their methyl esters for GLC analysis, many methods have been worked out and adopted because methyl esters have a lower boiling point than fatty acids, vaporize at a lower temperature, and thus minimize the danger of decomposition (170).

Hornstein et al. (105) have prepared fatty acid methyl esters on a strong anion exchange resin, Amberlite IRA-400. Gehrke and Goerlitz (71) have made them by the reaction of iodomethane with the silver salts of the acids. Transesterification with BF_3-methanol reagent was used by Metcalfe and Schmitz (172) and Hyun et al. (110). Refluxing lipids with HCl-methanol was also widely used (56,121). Rogozinski (211, 212) introduced a methanol-sulfuric acid esterification procedure, and McGinnis and Dugan (169) and Feldman and Rouser (59) have extended this method successfully to make methyl esters from sphingolipids.

The retention value of a substance is a measure of its volatility when dissolved in the stationary phase (116). If the solvent properties of the stationary phase are similar to those of the pure substance, the retention value will be proportional to the saturated vapor pressure at the same temperature. Two important forces between molecules are involved: Van der Waals forces and hydrogen bonding.

Van der Waals Forces between molecules depend on the number and type of atoms in the molecule, and hydrogen bonding involves the sharing of a proton.

Quantitative estimation of the components of a mixture may be carried out by relating the peak areas produced on the recorder trace to the concentration of the individual components. The measurement of peak area has been investigated by Horning et al. (103,104) in several ways. The integrator readout on a strip chart recorder is simplest.

The separation of fatty acid methyl esters is influenced by the flow rate of carrier gas, partition coefficient, and rates of diffusion in the gas phase and in the liquid phase (125); column temperature, chain length, number of double bonds or their position (1,2); and column effects, detection systems, temperature control, and sample size (103,104,197). The use of an internal standard has been recommended for quantitative analysis by GLC (103,104,249,269).

The identification of different peaks on a chromatogram may be accomplished by using retention data alone. It may involve an auxiliary detection system operating concurrently with the conventional system, or it may require fractionation and isolation of components as they emerge from the gas chromatograph and subsequent identification of the components by chemical and physical means (174). More accuracy can be achieved by the use of known mixtures or by comparison with results obtained by other methods (96,98,99,222).

The logarithmic retention time plotted against carbon number was introduced by James (113,114) and Woodford and van Gent (266); James (113) also devised a method to determine the degree of unsaturation of straight-chain and simpler branched-chain components by plotting the logarithm of the retention time measured on one stationary phase against similar values from a different stationary phase. Saturated components lie on one straight line and monoenes, dienes, trienes, etc., lie each on a separate parallel line.

Peterson and Hirsch (192) and Kieselbach (126) studied the air peak (carrier gas front) and found that the retention time of a component should be measured from this air peak to the peak of the component.

A systematic identification method has been proposed by Ackman (3,4,5). Said (220) suggested an optimum point for the measurement of peak areas. Edwards (51) derived a mathematic method of calibrating a GLC column. Thorough reviews of quantitative analysis of fatty acids by GLC have been made by Farquhar et al. (56), Ettre et al. (54), and Horning et al. (103,104).

Plasmalogens

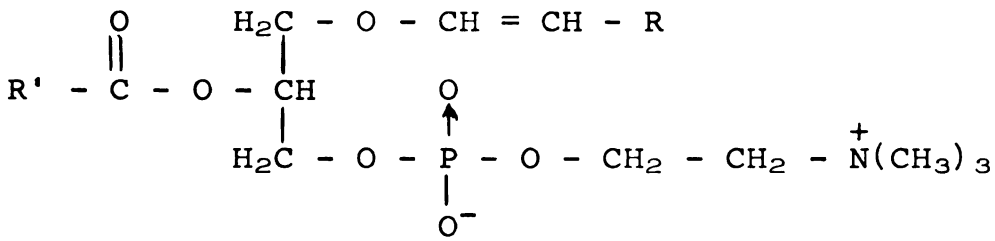
Historically the presence of plasmalogens in tissues was first discovered by Feulgen and Bersin (62) in the isolation of a crystalline acetalphosphatide containing long-chain fatty aldehyde, glycerol, phosphorus, and ethanolamine.

Later Klenk et al. (129,130) were able to isolate long-chain C_{14}, C_{16}, and C_{18} aldehydes from skeletal and heart muscle phospholipids as dimethyl acetal derivatives. Thannhauser et al. (245) isolated a similar lipid from brain. It was a saturated compound containing palmitaldehyde and stearaldehyde residues. The plasmalogens occurring in the tissues also contain esterified fatty acids which would have been removed by alkaline hydrolysis (132).

Thannhauser et al. (245) suggested the chemical structure of plasmalogens to be acetalphosphoglyceride. Klenk and Debuch (131,132) found considerable amounts of mixed chimyl and batyl phosphoric acids after hydrogenation and hydrolysis, therefore, they made the suggestion that the aldehyde might be linked, to one OH of glycerol only, by hemiacetal or α,β-unsaturated ether structure. The linkages of these aldehyde residues in natural plasmalogens exist in the enol form as pointed out by Debuch (42).

The position of the hemiacetal or unsaturated ether was once thought to be at the β-carbon atom of the glycerol moiety (82,90). Marinetti et al. (157,159-163) made correct use of phospholipase A to produce a lysoplasmalogen which on reduction gave an α'-glycerol ether, and arrived at the concept of an α'-structure by a purely chemical method. The plasmalogen preparation was reduced, the acyl group and base removed by alkali and the phosphate by acid to give the same α'-glycerol ether. Marinetti et al. (157,159-163), Rapport

et al. (25,182,199-201,203-204) and Debuch (42) concluded that the α,β-unsaturated ether structure and not the hemiacetal, is responsible for the structure of the original plasmalogens. The configuration of the double bond of the alkenyl ether is cis, not trans (182,257). Gottfried and Rapport (76), and Warner and Lands (255-256) confirmed the structural formula of choline plasmalogen to be α'-(1-alkenyl)-β-acyl glyceryl-phosphorylcholine.



Formation of the cyclic acetal phospholipid during alkaline and enzymic hydrolysis of choline plasmalogen has been reported (193). However, Ferrans et al. (60) demonstrated that the true aldehydogenic group of plasmalogen is an α,β-unsaturated glyceryl ether and that the acetal phosphatide is a degradation product formed during the isolation procedure.

Besides phosphatidal choline and ethanolamine, Klenk and Böhm (128), and Ansell and Norman (9) found serine plasmalogens. Russian chemists (135) also reported that sphingoplasmalogens have been isolated from brain by TLC. Choline plasmalogens have been observed in lipid fractions of soybeans, peanuts and green peas (133).

Some plasmalogen derivatives exhibit hemolytic activity (77), and the concentration of plasmalogens in rat tissues is a function of age (78).

Gottfried and Rapport (76) have produced a method for the isolation of pure choline plasmalogens from heart tissue, since choline plasmalogens of ox-heart are deacylated more slowly than the corresponding phosphatidyl choline by rattlesnake venom. However, this enzyme method can not be adapted to the preparation of all different plasmalogens, since only a few types of phospholipids are deacylated by snake venom. Recently, Renkonen (209) isolated native choline and ethanolamine plasmalogens by mild alkaline treatment of lecithin from ox-heart and phosphatidyl ethanolamine from ox-brain by different rate of destruction, but the isolated plasmalogens still remained contaminated with small amounts of the corresponding phospholipids. To date no generally applicable methods have become available.

Paper chromatographic analysis of plasmalogens has been employed (85,88,164), however, a clean separation from corresponding phospholipids was impossible.

Ansell and Spanner (11) reported that the cleavage of the vinyl ether linkage of brain ethanolamine plasmalogens was magnesium ion dependent.

The qualitative determination of plasmalogens is accomplished mainly by colorimetric methods, either by the reaction with mercuric chloride and diphenyl carbohydrazide

(180), or by Schiff reagent. Marcus et al. (155) derived a formula to calculate the phosphorus present as plasmalogen as a percentage of the total phosphorus through the latter reaction, because the molar ratio of phosphorus to aldehyde is unity.

Alumina columns have been used in studies of nerve tissue plasmalogens by Webster (258). Katz and Keeney (123) have isolated fatty aldehydes from rumen-microbial lipid as DNP-hydrazones which were regenerated by levulinic acid and then analyzed by GLC.

Colorimetric estimation has been widely used for quantitative estimation of plasmalogens. Wittenberg et al. (263) estimated higher fatty aldehydes in tissues as their p-nitrophenylhydrazones. Siggia and Edsberg (230), Rapport et al. (199,201), Norton (181), and Williams et al. (261) reported that vinyl ethers may be determined specifically by measuring the uptake of iodine and demonstrated the linear relationship between iodine uptake and sample size.

Recently, gas-liquid chromatography has been employed extensively in the determination of long-chain fatty aldehydes as their dimethyl acetal derivatives (DMA). Fatty acid methyl esters and DMA are prepared simultaneously either by boron fluoride-methanol (178), or by methanolic hydrochloric acid (57-58,84,183), then they are separated by different solvent systems (53), by saponification (57-58,84,183), or by TLC (178,183).

Both non-polar (Apiezon L or M) and polar (Reoplex 400, polyesters of diethylene glycol adipate (DEGA), or polyesters of diethylene glycol succinate (DEGS)) stationary phases have been tried at different temperatures (57-58,84,183). Farquhar (57-58), found the DMA to be stable on DEGA at 184.8°C.

The identification of aldehydogenic chains may be made either by comparing dimethyl acetals with reference compounds (84), or by comparison of the retention time of alcohols after reduction of unknown aldehydes and reference fatty acids (57-58). They are most frequently identified by plotting logarithmic retention times versus carbon number (266), molecular weight (57), or degree of unsaturation (113).

The distribution of plasmalogens in lipids from skeletal muscle differs with source and probably with carcass location. Some reported values for plasmalogen content of phospholipids from skeletal muscle are as follows: sheep 8.9% (40), hen 30-31% (258), pigeon breast 11.6% (38), rat 13% (204), rabbit 22% (204), and man 31% (204).

EXPERIMENTAL

Materials

Sample Preparation

Two one-year old Cobb strain chickens on a standard MSU Z-4 diet (Appendix A) were used in these studies. They were killed, defeathered, and eviscerated in the usual manner. Muscle samples were taken and freed of skin, adhering fat, and connective tissues. Samples of white meat and dark meat were taken from breast muscle and leg muscle, respectively, then divided into 100 g each, placed into polyethylene bags, and stored in the freezer.

Chemicals and Reagents

Silicic acid, cp, precipitated, (Fisher Scientific Co., Fair Lawn, New Jersey) was washed with distilled water until no turbidity was observed in the supernatant after coarse particles were settled, then washed once with methanol, dried 24 hours at 120°C, and stored in a tightly stoppered bottle. Chromosorb W, acid washed, mesh size 80/100, was obtained from Applied Science Laboratories, Inc., State College, Pennsylvania. DEGS (diethylene glycol succinate, Lac-728) was obtained from F & M Scientific Corporation, Avondale, Pennsylvania. The methyl esters used as chromatographic

standards for gas-liquid chromatography were from California Corporation for Biochemical Research, Los Angeles, California. Crotalus adamanteus venom, obtained from Ross Allen's Reptile Institute, Silver Springs, Florida, was used as a source of phospholipase A. Silica Gel G, used for thin-layer chromatography, was purchased from Brinkmann Instruments, Inc., Chicago, Illinois. Lithium aluminum hydride was made by Metal Hydride Inc., Beverly, Massachusetts. The long chain aldehydes were prepared by K & K Laboratories, Inc., Plainview, New York. All chemicals were analytical reagent grades, and all solvents were freshly redistilled and made suitable for spectrophotometric use.

Methods

Moisture Determination

Fresh samples of white and dark meat were cut into small strips. Duplicate samples were placed in weighed beakers, weighed, and dried at 105°C for 46 hours. The percentage moisture content was calculated from the weight difference.

Total Lipids Extraction

The procedure for extraction of lipids from muscle tissues was essentially based on the method of Bligh and Dyer (21), modified by adopting the washing system from Folch et al. (67). The sample of frozen meat was equilibrated gradually to room temperature in the polyethylene bag in order

to maintain its moisture without any loss. Each 100 g of sample was homogenized in a Waring Blendor for 2 minutes with a mixture of 100 ml of chloroform, 200 ml of methanol, and distilled water to make the total moisture content equivalent to 80% of the sample weight. Then 100 ml of chloroform was added to the mixture. After blending for 30 seconds, 100 ml of distilled water were added and blending was continued for another 30 seconds. The homogenate was filtered through Whatman No. 1 filter paper on a Büchner funnel with slight suction and pressure was applied with the bottom of a beaker to ensure maximum recovery of solvent. For quantitative lipid recovery, the homogenate residue together with filter paper was blended with 200 ml of chloroform for 1 minute and filtered through the same Büchner funnel. The blendor was then rinsed with 50 ml of chloroform and 40 ml of methanol separately. The filtrate and combined washings were transferred quantitatively with the aid of 30 ml of chloroform to a 1,000 ml graduated cylinder. The final proportion of chloroform, methanol, and water was 8:4:3 by volume in the extract. Chloroform to the extent of 7% of the total volume of the extracts was added to the mixture and mixed well to prevent the formation of fluff in the interface. The mixture, protected by a nitrogen atmosphere, was left in a dark place overnight for complete extraction and separation. The upper phase was composed of chloroform-methanol-water in a ratio of 3:48:47 by volume, while the lower phase which contained the

extracted lipids consisted of 86 (chloroform): 14 (methanol): 1 (water). The volume of the chloroform phase was recorded and the alcoholic layer was removed by suction through a tube. The interface and inside wall of the cylinder were washed 3 times with a small amount of pure upper phase during the separation step in such a way as not to disturb the lower phase.

Duplicate 10 ml aliquots were taken from the lipid extracts into weighed Erlenmeyer flasks, the solvents evaporated by a stream of nitrogen while warming on a water bath, and the flasks placed in a vacuum desiccator over calcium chloride for 24 hours and weighed. The percentage of lipid content in the extracts was computed from their weight differences.

Gravimetric Determination of Lipids

The lower phase was transferred quantitatively with a small amount of chloroform to a 1,000 ml round-bottom flask, evaporated by a Rinco rotating high vacuum-type evaporator almost to dryness, and transferred to a weighed Erlenmeyer flask with a disposable capillary pipet. The flask was rinsed with a small amount of chloroform. The lipid was dried in a vacuum desiccator over calcium chloride overnight and weighed. The weighed dried residue was reported as crude total lipids.

Separation of Lipid Class by Column Chromatography

A column of activated silicic acid was prepared by pouring 40 g of silicic acid, slurried in an excess of chloroform

in a beaker with a magnetic stirrer until a homogeneous translucent mixture was obtained, into a column (2.5 x 23 cm) in such a way that no air bubbles would be trapped. The silicic acid was allowed to settle and washed respectively with acetone, methanol, and chloroform to check whether undesirable channeling existed in the column and to remove colored materials. A 1-2 cm layer of powdered anhydrous sodium sulfate was then placed on the top of the packed column.

Lipid extracts, redissolved in a small portion of chloroform, were carefully applied onto the column in a ratio of 1 g per 50 g of silicic acid. Elution was accomplished by various solvent systems and successive 35 ml fractions were collected. The flow rate was adjusted to approximately 3 ml per minute, by applying slight pressure with nitrogen.

Nonphospholipids or neutral lipids were eluted by chloroform and monitored by the Salkowski test (136) until a negative reaction was achieved. This effluent was referred to as fraction 1 in Table 3. Acetone, used as a scavenger for oxidized materials by Nelson and Freeman (179), was used to remove pigmented substances in the column. It was usually possible to follow the pigments visually as a brownish band or to detect them by a negative ninhydrin test. The third fraction was eluted as cephalins with 15% by volume methanol in chloroform. This fraction was followed during the course of development with a rapid ninhydrin test: a colorimetric test consisting of equal volumes of eluate, ninhydrin, and

2,4-lutidine for amino-containing compounds (216). Phosphatidyl ethanolamine was found to come down the column faster than phosphatidyl serine in dark meat phospholipids. Lecithins (phosphatidyl choline) were eluted with 35% by volume methanol in chloroform and their presence was confirmed by negative ninhydrin and positive molybdate tests. Phosphatidyl serine of white meat was found to overlap with lecithins and was separated by absolute ethanol. The former was eluted first, and the latter was recovered by methanol in another column. The fifth fraction was composed of sphingomyelins which were eluted by 100% methanol and checked by ninhydrin reaction. The scheme of chromatographic separation on a silicic acid column is shown in Table 3.

Samples were collected in 35 ml fractions and these were flushed with nitrogen and stored at -20°C in capped vials.

Phosphorus Determination

Determination of the phosphorus content of each fraction was made by a modification of the method of Beveridge and Johnson (19). One ml of sample from each collection was evaporated in a micro-Kjeldahl flask under nitrogen on a water bath and 1 ml of concentrated sulfuric acid was added. After digesting for 10 minutes, 2 drops of 30% hydrogen peroxide were added, the flasks were shaken between each addition and heated again for another 10 minutes, after which the sample mixture should be clear. The digested sample was cooled and transferred quantitatively to a 50 ml volumetric flask with

the aid of 20 ml of distilled water. Twenty ml of freshly prepared molybdate-hydrazine sulfate reagent were added with a rapid delivery pipet, and the solution was made up to volume with distilled water. The solution was mixed well and stoppers were loosened. After heating in boiling water for 10 minutes, samples were cooled in cold water about 5 minutes to bring the solution level to the mark. The absorbance of the blue-colored solution against blank and standard was measured within 24 hours at 830 mμ in a Beckman DU spectrophotometer. All readings were converted from absorbance to concentration in terms of μg of phosphorus per ml by means of a standard curve, and plotted against collection tube numbers to provide a curve showing the fractions containing phosphorus.

Thin-Layer Chromatography (TLC)

Thin-layer adsorption chromatography on silica gel G was employed to check the identities of phospholipids in each tube (136,216) in order to group together the components with the same R_f value. RSCo equipment consisting of 20 x 20 cm glass plates, spotting template, applicator, plastic mounting board, desiccator, and chromatographic developing chamber was used. Twenty-five g of silica gel G containing approximately 13% of calcium sulfate binder was slurried with 50 ml of distilled water and applied to 5 glass plates in an uniform layer about 250 microns in thickness. The plates were air-dried at room temperature for 15 minutes to allow the binder to set and then activated by heating in an oven at 100°C for

one hour. The activated plates were stored in a desiccator to protect them from moisture, dust, and laboratory fumes. Capillary tubes were used to apply the samples in a solution of chloroform-methanol to the chromatoplates. The plates were developed in an equilibrated chromatographic developing chamber containing a solvent system of chloroform-methanol-water (65:25:4) by volume (254). After the solvent had ascended 10 cm on the plates, the plates were removed from the chamber and allowed to dry at room temperature. Lipid component spots were detected by spraying with different indicators: e.g., ninhydrin solution for amino phosphatides (138), molybdic acid for phosphatides and Dragendorf reagent for choline (231). The relative R_f values in this developing system were found to be 0.79 for phosphatidyl ethanolamine, 0.64 for lecithins, 0.54 for phosphatidyl serine, and 0.48 for sphingomyelins.

Gravimetric Determination of Individual Purified Phospholipids

Tubes from column chromatography which contained components with the same TLC R_f value were combined according to TLC pattern and phosphorus analysis. The grouped samples were checked for purity by TLC. Solvents were evaporated under a stream of nitrogen in weighed 25 ml glass stoppered Erlenmeyer flasks on a water bath. Flask contents were then dried overnight in a vacuum desiccator over calcium chloride and weighed. The weighed dried residues were reported as

individual phospholipid components. The contents of these flasks were then transferred quantitatively with a suitable spectrograde solvent to volumetric flasks and used for infrared spectra and other analyses.

Infrared (IR) Spectral Analysis

IR measurements were made with a Beckman IR-5 double beam recording spectrophotometer equipped with a sodium chloride prism, and an absorption cell with an optical path length of 0.0992 mm. The spectra of the purified samples were determined in different solvents depending upon their solubility, hence, phosphatidyl ethanolamine was dissolved in carbon disulfide, while phosphatidyl serine, lecithin, and sphingomyelins were dissolved in chloroform.

Standards for IR spectra of each phospholipid type from chicken muscle were prepared by repeated purification by silicic acid column chromatography. Phosphatidyl ethanolamine and phosphatidyl serine were eluted by 40% methanol in chloroform, while lecithin was eluted by 45% and 60% methanol in chloroform, respectively. All fractions were checked by TLC and acceptable purity for each fraction was shown by only one spot for each fraction on the chromatoplate. Sphingomyelins were purified by subjecting the sample to alkaline hydrolysis with 25 ml of 0.5N methanolic potassium hydroxide to destroy all contaminants, then the petroleum ether extract from the neutralized solution was placed onto the silicic acid column.

Free fatty acids and esters were eluted by chloroform and sphingomyelins were eluted by methanol. The eluates were checked for purity and uniformity by TLC. Three different concentrations of each standard were prepared ranging from 4.14 mg/ml to 22.20 mg/ml for absorbance determination at the appropriate IR frequency.

The IR spectra of all standards and samples were determined with their corresponding solvents as reference. Each component was identified and measured quantitatively from appropriate absorption bands in the IR spectrum. Therefore, cephalins were identified and measured by bands at 5.8 μ because they have been shown to exhibit similar (235) or identical (234) IR spectra, lecithin at 10.3 μ and sphingomyelins at 6.1 μ.

Three methods, standard curve, absorption coefficient (α), and molecular extinction coefficient (ϵ) have been used for calculation of quantities. The standard curve method consisted of simply plotting the absorbance of standards against known concentrations in terms of mg/ml to provide a standard curve. The concentration of each sample was then directly read out from these curves. Absorption coefficients were calculated by the Beer-Lambert law as $A = \alpha cl$ where A is absorbance, α is the absorption coefficient, c is concentration in mg/ml, and l is the thickness of the absorbing material in cm. The method of molecular extinction coefficient was similar. It involved changing concentration from mg/ml to mole/ml by

dividing mg/ml by mg/mole of each phospholipid component. The relative molecular weights of four components were calculated on the basis of assignment of stearic and linoleic acids to the α'- and β-positions, respectively, thus phosphatidyl ethanolamine has a molecular weight of 743, phosphatidyl serine is 787, lecithin 786, and sphingomyelin 728.

Hydrolysis

Enzymatic hydrolysis of dark meat lecithin was accomplished with snake venom phospholipase A in ethyl ether solution and separation of the hydrolysis products was achieved by the procedure of Tattrie (241). A sample containing approximately 150 mg of lecithin was evaporated to dryness on a water bath with a stream of nitrogen, 100 ml of ethyl ether were added and mixed well, 1 ml of snake venom solution (1 mg/ml) was added, the reaction mixture was shaken about 5 minutes, and left in a dark place overnight. The hydrolyzed mixture was transferred in equal volume to four centrifuge tubes and centrifuged at full speed in an International Clinical Centrifuge for 15 minutes. After decanting and saving the supernatant, the precipitate was washed twice with ethyl ether, thorough stirring was necessary, then centrifuged for 27 minutes. All supernatants were combined. These contained the free fatty acids from the β-position, and possibly unhydrolyzed lecithin. Phosphorus determinations indicated that this portion did not contain any trace of unhydrolyzed lecithin. The dark brownish cake-like precipitate was redissolved in

30 ml of chloroform. No centrifugation was necessary if, after thorough stirring, all precipitate had gone into solution. This component was lysolecithin ready for basic hydrolysis.

Enzymatic hydrolysis of cephalins with snake venom was not successful by the procedure used for lecithin. The method described by Magee et al. (151), as adapted by Kuchmak and Dugan (137), was used in conducting the hydrolysis in water solution. Two portions of 45.83 mg of phosphatidyl ethanolamine were evaporated to dryness in a 125 ml Erlenmeyer flask over a water bath with nitrogen. A 20.36 ml portion of sodium deoxycholate solution (5 mg/ml) was added as emulsifying agent to solubilize phosphatidyl ethanolamine at 50°C with occasional shaking. This was followed by adding 24.75 ml of 0.18M glycylglycine buffer, pH 7.3, which was mixed well, and 2 ml and 3 ml of snake venom solution (1 mg/ml) were pipetted into each mixture, respectively. Only gentle shaking was used since vigorous shaking would possibly cause denaturation of the enzyme. The two reaction mixtures were incubated in a 37°C water bath immediately for 19 hours with occasional shaking. The reaction mixture was then transferred into a 1 liter separatory funnel with the aid of 181.75 ml of chloroform-methanol (2:1 by volume). The contents of the funnels were mixed by rotatory shaking and left at room temperature for 4 hours for complete separation. The upper phase was discarded and the lower phase filtered through a

sintered-glass filter. The filtrate was dried and redissolved in 20 ml of chloroform, and transferred quantitatively to the silicic acid column. Fatty acids, a turbid front in the column, were eluted by chloroform. Unhydrolyzed phosphatidyl ethanolamine, if any, was eluted by 25% methanol in chloroform, and the lysocompound eluted by methanol. The elution pattern was followed by a ninhydrin test as previously mentioned. Phosphorus determinations indicated that hydrolysis was not complete since only the chloroform portion was negative to ninhydrin while both the chloroform-methanol and methanol fractions were positive.

Phosphatidyl serine was hydrolyzed in the same fashion with 44.44% sodium deoxycholate, 54% glycylglycine buffer, and 1.56% enzyme solution, by volume, of the sample.

Basic hydrolysis was accomplished in a manner similar to that described by Hornstein et al. (106) for basic hydrolysis of meat tissue lipids. Fractions subjected to complete alkaline hydrolysis were cephalins, lecithin, lysocephalins, lysolecithin residues, and unhydrolyzed cephalins from partial (enzymatic) hydrolysis. All samples were evaporated to dryness under nitrogen. To the dried residue was added 25 ml of 0.5M methanolic potassium hydroxide and the mixture was gently refluxed for 6 hours.

Preparation of Methyl Esters for Gas-Liquid Chromatography

After enzymatic or basic hydrolysis of cephalins, lecithins, and lysocompounds, all liberated free fatty acids

were converted to their corresponding methyl esters by the method of Hornstein et al. (105). Preparation of the ion-exchange resin involved adding 25 ml of 0.1N sodium hydroxide to 10 g of Amberlite IRA-400 and stirring for 5 minutes. The resin was allowed to settle and the supernatant liquid was discarded. The resin was washed on a Buchner funnel with several portions of distilled water to remove free alkali, then with three 25 ml portions of anhydrous ethanol to remove water, and finally with three 25 ml portions of petroleum ether to displace the ethanol.

For esterification, the free fatty acids of each fraction were transferred to a 125 ml separatory funnel with the aid of 50 ml of distilled water. The solution was made acidic to phenolphthalein by dropwise addition of concentrated hydrochloric acid and then 1 ml excess was added. The acidic solution was extracted three times with 25 ml portions of petroleum ether, and the combined extracts were washed with three successive 25 ml portions of cold distilled water. The petroleum ether solution of fatty acids was decanted onto the prepared resin in a 250 ml Erlenmeyer flask. Two 5 ml portions of petroleum ether were used to wash the residual solution into the flask. The solution, plus resin, was stirred with a magnetic stirrer for 5 minutes, allowed to settle, and the supernatant liquid discarded. The residue was then washed by stirring with three successive 25 ml portions of petroleum ether and the washings were discarded.

The adsorbed fatty acids were converted to methyl esters directly on the resin. A 25 ml portion of 6% dry HCl in anhydrous methanol was added to the resin and the mixture was stirred for 25 minutes. The methanolic solution was decanted through a No. 4 Whatman filter paper into a 250 ml separatory funnel. The resin was washed by stirring for 5 minutes with two successive 15 ml portions of anhydrous methanol-hydrochloric acid, again decanting each liquid portion through the filter. To the combined methanol-hydrochloric acid washes was added 10 ml of water, and the solution was extracted with 50 ml of petroleum ether. The aqueous phase was drained into another separatory funnel and extracted twice with 20 ml portions of petroleum ether. The combined extracts were washed with water until free of acid, dried over sodium sulfate, and then concentrated to 0.2 ml by a stream of nitrogen on a water bath. This aliquot was used for gas-liquid chromatographic separation and identification.

Sphingomyelins were esterified by the method described by McGinnis and Dugan (169). The sphingomyelins in ether solution were hydrolyzed by sulfuric acid at the temperature of a dry ice-acetone bath. Fifteen ml of anhydrous methanol were then added to the ether solution and methanolic potassium hydroxide was added to a phenolphthalein end point. The pink milky solution was transferred to a 500 ml separatory funnel with excess of water and extracted with 20 ml and 15 ml

portions of petroleum ether. The extracts were washed free of base by water and adding table salt if fluff was formed at the interface. The solution was concentrated to 0.2 ml for GLC analysis.

Gas-Liquid Chromatography

Gas-liquid partition chromatography was carried out in an F & M Model 500 temperature programmed gas chromatograph equipped with a thermal conductivity detector, Honeywell Electronik recorder, and the Series 200 Disc integrator. A coiled copper column (7.5 x $\frac{1}{4}$ in O. D.) was used for methyl ester separation. It was packed with 20% by weight Lac-728 (DEGS) and 1% by weight phosphoric acid on acid washed chromosorb W, 80/100 mesh, as a support phase. The column was conditioned at 215°C for at least 24 hours before being used. The operating conditions used in this study were: column temperature, 210°C; injection port temperature, 265°C; detector block temperature, 250°C; carrier gas, helium; carrier gas flow rate, 80 ml/min; reference gas flow rate, 120 ml/min; attenuator setting, 2; and bridge current, 150 ma. A proper sample size was essential in order to maintain uniform distribution of sample peaks on the chromatograph without off-scale deviation.

Identification of fatty acid methyl esters on the chromatogram was made by direct comparison of major peaks with those of chromatographic standards (99+% pure) passed through the same column under the same conditions. When

standards were not available, peaks were tentatively identified by semilogarithmic plots of corrected retention times, which were obtained by subtracting retention time of the air peak from those of sample peaks, against carbon number, and the best straight line joining them was constructed. A value corresponding to the retention time of any other peak could then be read off from the graph, and gave the chain length from the same lipid class, e.g., saturated, monoenes, dienes, trienes, etc. The area percentage of each peak was calculated by dividing counts from the disc integrator trace for each individual peak by the total counts for all peaks.

Plasmalogens

Qualitative determination. The colorimetric method used was essentially that used by Norton (180). This method utilizes the reaction of aqueous mercuric chloride with the enol-ether double bond of plasmalogens to form a mercury-organic compound. No. 4 Whatman filter paper was spotted with cephalins, lecithin, and sphingomyelin, and immersed for 30 seconds in 1% (w/v) aqueous mercuric chloride. Excess mercuric chloride was removed with five washes in 1% (w/v) sodium chloride and five washes in distilled water. The presence of mercury in the lipid spot was detected by immersing for 2 minutes in a 0.1% (w/v) solution of diphenyl carbohydrazide in 70% (v/v) ethanol which was 0.1N in potassium hydroxide. After washing thoroughly in water to remove excess diphenyl carbohydrazide reagent, excess water was removed

by blotting. A deep purple spot of mercury salt was observed against an unstained background if plasmalogens were present.

The enol-ethers encountered in biological material are plasmalogens which can be determined quantitatively by using the iodination reaction of Williams et al. (261). It was thought that the iodinated plasmalogens might have a different rate of migration on TLC from that of other phospholipids. Based on this idea, a TLC analysis was performed including unidimensional and two-dimensional development. The experiment was conducted on five different TLC plates: plain silica gel G, silica gel G plate sprayed with one of the following solutions: 1N sodium hydroxide, 20% (w/v) aqueous silver nitrate, 10% (w/v) aqueous mercuric nitrate, or 10% (w/v) ammonium sulfate. Ten different solvent systems were tried (by volume): chloroform (65):methanol (25): water (4); 1, 2, and 3 parts of ammonium hydroxide in a system of chloroform (70):methanol (30):water (4), respectively; 2-butanone (40):acetic acid (20):water (3); n-hexane (75):ethyl ether (25):acetic acid (2); chloroform (97):methanol (3); and a ratio of chloroform to acidic methanol which contained 5% of 0.1N sulfuric acid in 4:1, 3:1, and 2:1 systems. Six different spray reagents were tried: Skidmore's molybdate (231); concentrated and 50% sulfuric acid; dichlorofluorescein; 2,4-dinitrophenylhydrazine; ninhydrin; and fuchsin-sulfurous acid. Ten time intervals for iodination: 0.5, 1, 2, 4, 6, 8, 10, 20, 30, and 40 minutes, respectively. A system of a 10% (w/v) ammonium sulfate-silica gel G plate with chloroform:acidic methanol (2:1, v/v) developing solvent allowed the iodinated plasmalogens to migrate from other phospholipids.

For confirmation of these findings, a silica gel G-ammonium sulfate TLC plate was treated with iodinated samples in a band and developed with chloroform:acidic methanol (2:1,v/v). The plate was sprayed with molybdate in a narrow band at each side of the plate. After unsprayed portions of each bands were scraped out and eluted with pure ether, the supernatant of these two portions were examined chemically by mercuric chloride, ninhydrin, and molybdate to establish the nature of each fraction.

For further confirmation, an experiment with column chromatography was carried out. Ten g of silica gel G were mixed with 10% (w/v) aqueous ammonium sulfate solution, spread thinly on a big watch glass, and dried in an oven at 105^{o}C overnight. The dried silica gel G was packed into a small column (1.2 cm O.D.) and washed with chloroform. An iodinated plasmalogen-phospholipid mixture in chloroform was transferred to the top of the column, eluted by chloroform:acidic methanol (2:1,v/v) solution, and monitored by ninhydrin and molybdate until molybdate negative, then the column was eluted with chloroform. Two fractions were obtained. The solvent from each was evaporated under nitrogen and the residue was redissolved in pure ether. One half of the first fraction was dehalogenated by refluxing with excess of zinc dust in ether solution for at least 2 hours. The dehalogenated fraction gave a positive reaction with mercuric chloride while the iodinated portion was negative to the

mercuric chloride test. Thin-layer chromatography has shown a very promising separation in which the static portion (unknown component) did not develop any color before and after dehalogenation, but the mobile portion had two blue spots before dehalogenation, and one spot afterwards. This was evidence that the spot with the high R_f value was iodinated plasmalogen.

Quantitative Determination of Plasmalogens

Iodination. This was accomplished by a method by Williams et al. (261) which was modified from Siggia and Edsberg (230). This procedure is as follows: 0.01 ml of sample solution was placed in each of three centrifuge tubes and the solvent was evaporated under nitrogen. To each dried sample, 0.9 ml of anhydrous methanol was added and the contents were mixed. The tubes were allowed to stand for 5 minutes to dissolve the lipid residue and mixed again. Then 3.2 ml of 0.094M sodium citrate (pH 5.5) buffer were added and mixing accomplished. To the sample blank tube was added 0.9 ml of 3M potassium iodide and to the other two tubes were added 0.4 ml of 3M potassium iodide and 0.5 ml of 0.0005M iodine in 3M potassium iodide. The tubes were shaken vigorously again for 10 seconds, and centrifuged for 10 minutes. The upper layer was examined in a Beckman DU spectrophotometer at 363 mμ against a sample blank. A standard curve was constructed by using 0.1, 0.2, 0.3, 0.4, and 0.5 ml of 0.0005M iodine in 3M potassium iodide. The results in μmoles were

read out on the standard curve from the difference of absorbance of sample from that of the standard. The percentage of plasmalogens in each phospholipid component was computed by the following equation:

$$\frac{\text{Concentration (mmole/l) x molecular weight (mg/mmole) x } 10^{-3}\text{g/mg}}{\text{Plasmalogen-phospholipid mixture (g/l)}} \times 100$$

A relative molecular weight for each component was assumed on the basis of assigning stearaldehyde and linoleic acid to the α'- and β-position, respectively. Thus, the standardized molecular weight of phosphatidal ethanolamine is 727, and is 771 for phosphatidal serine, and 770 for phosphatidal choline.

Gravimetric Determination of Plasmalogens in Terms of Dimethyl Acetals (DMA)

After methylation, the phospholipid solution was saponified. DMA were alkali stable and therefore remained in the unsaponifiable portion while methyl esters (ME) of fatty acids were saponified. The saponified salts were acidified with 50% sulfuric acid to a phenolphthalein end point, remethylated by refluxing with 5% HCl-methanol, and the resulting ester extracted with petroleum ether. Both ME and DMA extracts were dried, and weighed in the usual manner. The weight percentages of ME and DMA were calculated. In order to relate weight to moles of ME and DMA, GLC results were used to establish a calculated molecular weight.

The number of μmoles was obtained by dividing the weight of each component by the average molecular weight and multiplying by 10^6 μmole per mole, where the average molecular weight was the sum of the molecular weight of each ME or DMA in each component on the gas-liquid chromatogram divided by the total number of ME or DMA in the component. The mole percentage was computed from the number of μmole of ME or DMA of each component divided by the total number of μmoles.

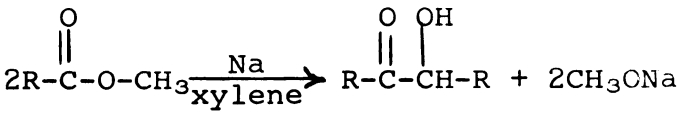
Fatty Aldehyde Chain Determination by GLC

The preparation and isolation of the aldehydes as dimethylacetals were accomplished by refluxing a phospholipid fraction in 20 ml of 5% HCl in anhydrous methanol for 2 hours. After adding 10 ml of distilled water, the resulting fatty acid methyl esters and aldehyde dimethylacetals were extracted with a 25 ml and a 10 ml portion of petroleum ether (b.p. 30-60). The mixture was washed with distilled water until free of acid to methyl red, dried over sodium sulfate, and concentrated to 0.2 ml under nitrogen. This mixture was analyzed by GLC. DMA were isolated from the methanolysate by saponification with 10 ml of 0.5N sodium hydroxide in anhydrous methanol for 2 hours. The methyl esters were converted to the sodium soaps of the respective fatty acids. The unsaponifiable DMA were extracted twice with 15 ml and 10 ml portions of petroleum ether separately, the combined extract was washed with base solution (5 volumes of water to 0.1 volume of 3N sodium hydroxide) three or four times, and then concentrated to 0.2 ml under nitrogen for GLC analysis. The soaps

were acidified. The fatty acids were extracted into petroleum ether, dried, and refluxed with 20 ml of 5% HCl in anhydrous methanol for 2 hours. The methyl esters were extracted, washed, and concentrated to 0.2 ml for GLC. The 5% HCl-methanol solution was also checked by the above procedure for any contamination. All GLC operating conditions were identical with those previously mentioned, except the column temperature was 185°C, and the sample size was 6 μl. This lower temperature was used in conformity with the observation of Farquhar (57) that resolution of DMA is optimum at about 185°C.

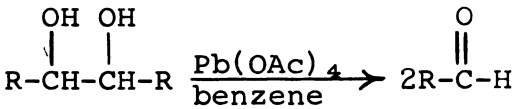
Chromatographic standard preparation: oleylaldehyde was prepared by the method described by Gauglitz and Malins (70):

1. Acyloin condensation:

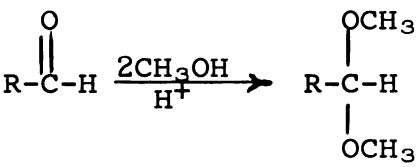


2. Reduction:

3. Oxidative cleavage:



4. Methylation:



A 2.2 g sample of oleic acid was methylated with a 1.4 g yield of methyl oleate which reflected 63.63% recovery.

Twenty ml of freshly distilled xylene and 0.32 g of sodium were placed in a three neck distilling flask equipped with magnetic stirrer, dropping funnel, condenser, and gas-bubbling tube on a Thermix hot plate. The temperature was raised to 115°C, and a fine dispersion of sodium was formed by vigorous stirring of the mixture. After nitrogen had been bubbled through the suspension for 15 minutes (nitrogen was bubbled through during the entire process), 1.4 g of methyl oleate were added over a period of 30 minutes, and an additional 30 minutes given for complete reaction. The reaction mixture was allowed to cool to approximately 85°C, then a slight excess of methanol was added to destroy the residual sodium, and 100 ml of distilled water were added immediately. The mixture was transferred to a separatory funnel and allowed to cool to room temperature. After extracting with 100 ml of ethyl ether, the extract was washed with distilled water until free of soaps, then once with 1N hydrochloric acid, and finally with water to neutrality. The extract was then filtered through anhydrous sodium sulfate to dry it.

The filtrate was added over a period of 15 minutes to a flask containing 0.83 g lithium aluminum hydride in 100 ml of anhydrous ethyl ether and refluxed for 1 hour. The milky mixture was transferred to a separatory funnel, and enough water was added to destroy unreacted lithium aluminum hydride. After washing with water and evaporating under nitrogen, the colorless glycol was redissolved in petroleum ether (b.p.

30-60) and placed onto a column containing 1:1 (w/w) silicic acid and cellulose powder previously saturated with petroleum ether. The glycol was purified by eluting the column with 200 ml of petroleum-ethyl ether (9:1 v/v) solution, the eluate was concentrated to some degree, and stored in the freezer.

Six and one-half g of lead tetraacetate, 25 ml of benzene, and 3 g of the glycol were refluxed for 3 hours. After the excess reagent was destroyed with ethylene glycol, which formed a sticky fluid with lead tetraacetate and stayed on the bottom of the flask, the solution was poured into a separatory funnel with the aid of 125 ml of 20% acetic acid and extracted with peroxide free ethyl ether. The ether solution was washed three times with water, then with 1% (w/v) sodium carbonate, and finally with water until the upper phase became clear (just about neutral). The crude oleylaldehyde solution was drained through anhydrous sodium sulfate, and concentrated under nitrogen.

The dimethylacetal of oleylaldehyde was prepared by methylation, and all contaminants were removed by saponification.

The saturated aldehydes were prepared by reducing fatty acids to their corresponding alcohols, and oxidizing alcohols to their aldehydes. One and one-half g each of decanoic, lauric, myristic, and palmitic acids and 1.3 g of stearic acid were dissolved in ethyl ether, and transferred over a

period of 15 minutes through a dropping funnel to a flask containing 0.83 g of lithium aluminum hydride in 100 ml of anhydrous ethyl ether. The reaction mixture was refluxed for an hour. When lithium aluminum hydride was destroyed by adding water, a very dense soap was formed which was insoluble in water and soluble in ether. After standing for an hour, the upper phase was siphoned off and concentrated under nitrogen.

To a 500 ml two neck round-bottom flask equipped with a dropping funnel and a condenser, and containing 125 ml of distilled water, 40 ml of concentrated sulfuric acid were added slowly. A 250 ml suction flask was attached to the end of the condenser for a receiver and completely covered with ice in an ice pan. Several glass beads were added to the acid solution which was heated to boiling and kept boiling by means of a small flame throughout the reaction. Fifty g of sodium dichromate ($Na_2Cr_2O_7 \cdot 2H_2O$) were dissolved in 50 ml of water, and the alcohols were added to the clear solution. After placing this solution into the dropping funnel, the oxidizing agent and alcohols were added to the boiling acid solution at a rapid drop-by-drop rate over a period of an hour. The alcohols were quickly oxidized to their corresponding aldehydes which were distllled into the receiver along with unchanged alcohols, acetals, and water. When the alcohols all had been added, the distillation was continued

for 5 more minutes (8). The aldehyde mixture was extracted by ethyl ether, methylated, and purified by saponification and extraction.

Three commercially prepared aldehydes, myristic aldehyde, plamitic aldehyde sodium bisulfite, and stearic aldehyde sodium bisulfite were used for chromatographic standards. The same procedures mentioned-above for methylation, purification, and contamination checking were applied. The myristic aldehyde was contaminated mainly by decanoic and lauric and lesser quantities of 12 other carboxylic acids.

The procedure for identifying fatty aldehyde dimethylacetals on the chromatograms was as follows: direct comparison of corrected retention times of main peaks with peaks from the reference compounds; when standards were not available, peaks were then tentatively identified by plotting corrected logarithmic retention times against both the carbon number and the molecular weight of the corresponding DMA. A straight line was constructed by joining points referring to the same type of compounds. The area percentage was calculated as mentioned before.

The presence of plasmalogens in chicken muscle tissues was also checked by the comparison of gas-liquid chromatograms of ME-DMA mixtures with those of residual ME.

Column Chromatography

A Celite column impregnated with 2,4-dinitrophenylhydrazine and phosphoric acid has been used by Mookherjee and

Chang (175) to separate 21 carbonyl compounds which varied from C_2 through C_{13}. The column was eluted by carbonyl-free hexane and monitored spectrophotometrically at 335, 340, 355, and 370 mμ. Chicken white meat lecithins have been applied to this column and eluted in a similar manner. The aldehydes from the 2,4-dinitrophenylhydrazones in the eluate were converted to DMA derivatives by refluxing with 5% HCl in methanol or by sulfuric acid catalysis, and analyzed by GLC at 185°C after extraction and concentration.

RESULTS AND DISCUSSION

Sample Chosen

It has long been known that age and dietary fats affect the fatty acid composition of total lipids and phospholipids of chicken muscle (73,149,165-166). It is therefore desirable to study an experimental animal with a standard diet from which representative results can be obtained. The fat reserves of the chicken are much more uniform with respect to the total body fat composition than is the case in pigs and cattle (36,208). Thus, the one-year old chicken on a standard MSU Z-4 diet was chosen.

Moisture Content

The moisture content of dark and white meat was determined immediately after killing and cleaning. Values are shown in Table 1.

Table 1. Moisture content of chicken muscle tissues.

Muscle	Average moisture content (%)
Dark meat	76.20
White meat	75.80

Knowledge of the moisture content is important for the extraction process in which lipids are concentrated in the chloroform phase of a three component system with a ratio of 8:4:3 chloroform, methanol, and water.

Lipid Extraction

Since a major portion of the tissue lipids is bound to protein as lipoprotein through water molecular bonding (91), it is necessary to employ such dehydrating agents as methanol, ethanol, or acetone to break the linkages in order to achieve extraction of total lipids. They are often used in conjunction with non-polar solvents, e.g., chloroform or ether for lipids not soluble in the polar solvents, to ensure complete extraction. Extraction at room temperature yields solutions containing fewer oxidation products than those obtained at elevated temperature and the reproducibility is good.

The Folch et al. (67) washing procedure was used to remove water-soluble contaminants from the lipid extract. Care must be taken to avoid loss of lipids soluble in both water and organic solvents. A particular problem was the tendency of phospholipids to solubilize non-lipid material in organic solvents (15).

Table 2 shows some discrepancies in values between two methods used to determine crude total lipids in muscle.

Table 2. Crude total lipids in dark and white meat.

Method	Weight of lipids in grams	
	Dark meat	White meat
Aliquot determination	1.52	1.18
Gravimetric determination	1.55	1.05

The 1.93% difference in dark meat could be attributed to some insoluble crystals present in the sample aliquot which made one sample heavier than the other. The white meat had 11.01% difference which could be due to incomplete mixing or loss in transferring. This makes the results from the aliquot method appear greater than the results from the gravimetric determination.

Separation of Lipid Classes

Because of its high capacity (22), silicic acid is the most commonly used adsorbent for column chromatography. Pretreatment was essential in order to obtain the best and most reproducible results.

Phospholipids from each chicken muscle tissue were separated into four main components on a silicic acid column by elution with concentration gradients of methanol in chloroform. Chloroform was used to clear from the column all neutral lipids adsorbed by the silicic acid. The oxidized lipids or colored materials were scavenged by acetone.

Removal of these components could be followed by observation of a brownish ring which moved down the column during the elution process.

The scheme for the separation process is given in Table 3. A 15% mixture of methanol in chloroform (v/v) was used to elute cephalins as a third fraction. Lecithins were eluted by 35% methanol in chloroform. The fifth fraction, sphingomyelins, was eluted with 100% methanol.

Table 3. Scheme for chromatographic separation of lipids on silicic acid.

Fraction	Solvent	Component	Detection
I	Chloroform	Nonphospholipids	Salkowski test
II	Acetone	Principally non-phospholipids	Ninhydrin test and visual observation
III	15% methanol[1]	PE[2] and PS[3]	Ninhydrin test
IV	35% methanol[1]	PC[4]	Ninhydrin and molybdate tests
V	100% methanol	SP[5]	Ninhydrin test

[1]Listed percentage of methanol in chloroform by volume
[2]Phosphatidyl ethanolamine
[3]Phosphatidyl serine
[4]Phosphatidyl choline (lecithins)
[5]Sphingomyelins

The overlap of acidic lipids with nonacidic lipid fractions has been observed, phosphatidyl serine was eluted along with phosphatidyl ethanolamine from dark meat, and with

lecithins from white meat. They were separated in a separate silicic acid column by eluting the mixture with absolute ethanol. This behavior could be explained either by alcohol breaking the electrostatic bonding or by the presence of one compound altering the solubility of the other, or the combination of both.

The amount of phospholipids loaded onto the column is important because this may alter the chromatographic results to a marked extent. In this study, a ratio of 0.02 g of phospholipid to 1 g of silicic acid was found to work best.

The recovery of total dark meat lipids from column chromatography was 91.70%, while 94.92% recovery was achieved for the white meat lipids.

Phosphorus Determination

The phosphorus content of each 35-ml fraction collected was determined by the method of Beveridge and Johnson (19). Although time consuming, the method was accurate, it had excellent color stability (24 hours), and allowed determinations up to a concentration range of 65 μg of phosphorus. A little higher phosphorus content was observed after the column was kept in cold storage overnight. This could be due to a difference in equilibrium between solvent and adsorbent during static and dynamic or mobile conditions. Figure 1 is an example of the use of the phosphorus determination to delineate concentration differences in fractions eluted from

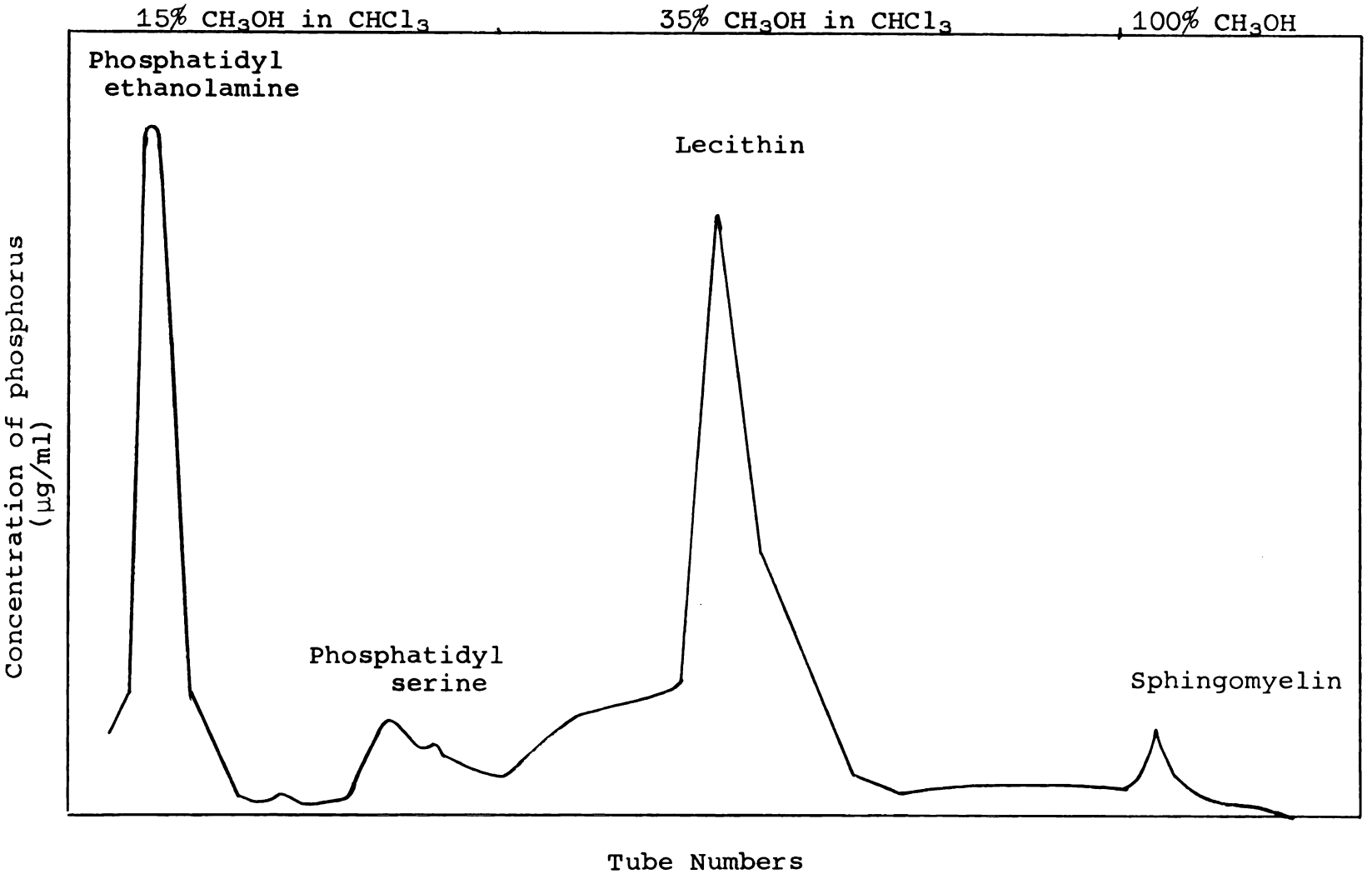


Figure 1. Fractionation of phospholipids on silicic acid as related to phosphorus content of the component.

a silicic acid column. By plotting phosphorus concentration against tube numbers, it was possible to identify the tubes containing a phospholipid component.

Thin-layer Chromatography (TLC)

The identity of each phospholipid component was checked by thin-layer adsorption chromatography on silica gel G plates using a developing solvent system of chloroform-methanol-water (65:25:4) by volume. The lipid spots were detected with ninhydrin solution and molybdic acid. The pattern of the relative R_f values in this system was found to be 0.97 for phosphatidyl ethanolamine, 0.64 for lecithins, 0.54 for phosphatidyl serine, and 0.48 for sphingomyelins.

TLC is simple, fast, effective, sensitive, and has a moderate capacity. The method of development and adsorbent activity, which is controlled by ambient relative humidity, are two major factors affecting the reproducibility (37). Therefore, different developing systems produce different R_f values (107), and different migration patterns occur with different water content of the adsorbent (219). The R_f values found in chicken meat phospholipids agreed with those obtained by Horrocks (107) using similar stationary and mobile phases on brain phospholipids.

Gravimetric Determination of Individual Phospholipid Components

Chicken dark and white meat lipids were determined by the conventional method of weight differences. Table 4 shows

that the quantities of total lipids and phospholipids in dark meat were approximately double those found in the white meat which were contrary to the results obtained by Katz (122). However, the percentage distribution of phospholipids in the total lipids in both dark and white meat was about the same at 40%.

Table 4. Gravimetric determination of individual phospholipid component.

Component	Dark meat		White meat	
	g/100g sample	%	g/100g sample	%
Neutral	1.09	57.56	0.44	48.82
Acetone elutable	0.06	3.37	0.10	10.89
PE	0.18	9.67	0.06	6.23
PS	0.05	2.74	0.03	3.65
PE & PS	0.05	2.47	----	-----
PC	0.43	22.69	0.22	24.81
PC & SP	----	-----	0.04	4.46
SP	0.03	1.50	0.01	1.14
Total	1.89	100.00	0.90	100.00

The proportion of phospholipids to neutral lipids in the dark and white meat was much higher than that observed in broiler tissues by Marion and Woodroof (166-167). This difference may be due to differences in sample sources and diet, or in age.

Infrared (IR) Spectra

Standards for infrared analysis were purified either by column chromatography or by base hydrolysis. They were checked for purity by TLC. An example of this is shown in Figure 2 for a sample containing sphingomyelins, which was submitted to basic hydrolysis. The extracted sphingomyelins, which were not hydrolyzed by base traveled more slowly on the TLC plate than the free fatty acids from the acidified portion containing no sphingomyelin.

Although phosphatidyl ethanolamine and serine exhibited characteristic bands at 5.8 μ and 9.8 μ, the latter was poorly developed in isolated cephalins and appeared rather as an inflection. A lecithin band occurred at 10.3 μ due to P-O-C stretching. The ester carbonyl band at 5.8 μ was strongly developed in the lecithin spectrum as also was the P-O-C vibration at 9.2 μ. The presence of a band at 10.3 μ in the spectrum of sphingomyelins was associated with the existence of a trans configuration of the double bond in sphingomyelin (32) and was also common to both lecithins and sphingomyelins. The main absorption band due to amide stretching was at 6.1 μ.

The absorption coefficients, designated α, absorbance per mg per ml, and molecular extinction coefficients, designated ϵ, of white chicken meat phospholipids as given in Table 5 were calculated by the Beer-Lambert law at their principal absorption bands. Since each substance may have different absorption coefficients due to different fatty acid

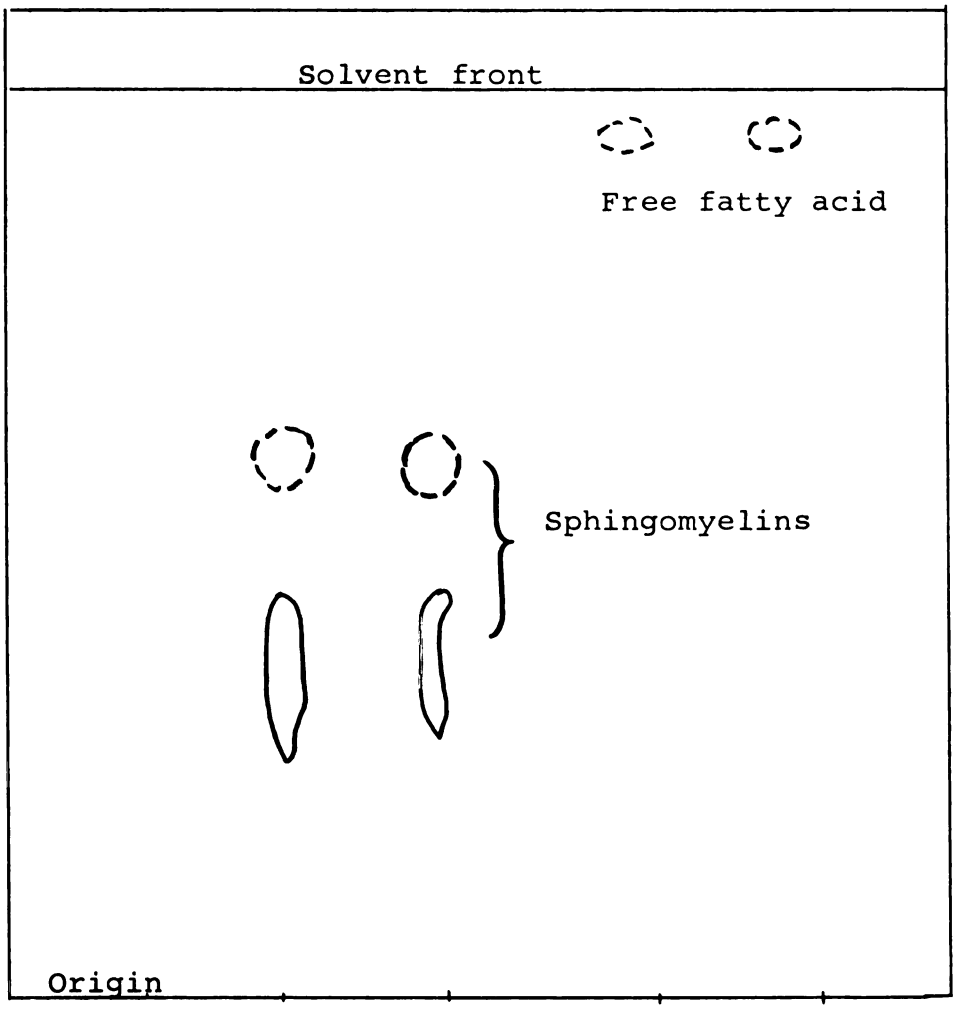


Figure 2. TLC chromatogram of sphingomyelin after basic hydrolysis.

composition and location, it was deemed preferable to prepare standards from the same materials to be determined by IR spectral analysis.

Table 5. Absorption coefficients for IR spectral bands of phospholipids.

Component	Solvent	Wavelength(μ)	$\alpha \times 10^2$	ϵ
PE	CS_2	5.8	0.91	67656
PS	$CHCl_3$	5.8	0.67	53175
PC	$CHCl_3$	10.3	0.45	36008
	$CHCl_3$	5.8	0.77	61666
SP	$CHCl_3$	6.1	0.60	44019

[α]Absorbance per mg per ml
[ϵ]Molecular extinction coefficient

Each component of the phospholipids in chicken white muscle, which was determined by infrared spectral analysis, was calculated by three methods, standard curve, absorption coefficient, and molecular extinction coefficient. The results compared in Table 6 were in agreement.

The calculated absorption coefficients reveal that at a given wavelength and a given absorbance, the higher the concentration of the sample in the solution, the smaller the absorption coefficients; and the larger the molecular weight of the sample, the smaller the molecular extinction coefficients.

Table 6. Infrared spectral analysis of white meat phospholipids (g/100g sample).

Component	Method of calculation		
	Standard curve	α	ϵ
PE	0.06	0.06	0.06
PS	0.03	0.03	0.03
PC	0.27	0.31	0.30
PC & SP	----	0.07	0.07
SP	0.01	0.01	0.01

Solvents selected for this study were based principally on the solubility of the various lipid classes in them. Hence, phosphatidyl ethanolamine was measured in carbon disulfide, while the other three components were measured in chloroform.

The possible sources of error in IR spectral analysis could be from mixtures of components due to the overlapping of fractions in chromatographic separation, deterioration of the sample by oxidation, variations in molecular weight due to variable fatty acid composition, and contamination of the phospholipids with substances containing similar groups, e.g., ester carboxyl or amidë carboxyl.

Comparison of Various Methods in the Determination of Lipid Classes

Each component in dark meat phospholipids was quantitatively determined by two methods: gravimetric analysis

and phosphorus determination. Three methods: gravimetric analysis, phosphorus determination, and infrared spectral analysis were employed to determine white meat phospholipid components. The overall results are presented in Table 7.

Table 7. Lipid fractions determined from 100 g of sample

Component	Dark meat (g)		White meat (g)		
	Gravimetry	P-det'n	Gravimetry	P-det'n	IR spectra
PE	0.18	0.23	0.06	0.07	0.06
PS	0.05	0.07	0.03	0.04	0.03
PE & PS	0.05	0.04	----	----	----
PC	0.43	0.40	0.22	0.25	0.30
PC & SP	----	----	0.04	0.06	0.07
SP	0.03	0.03	0.01	0.01	0.01

PE - Phosphatidyl ethanolamine
PS - Phosphatidyl serine
PC - Lecithins
SP - Sphingomyelins

There was higher concentration of each phospholipid component in the dark meat than that of its counterpart in the white meat. This was probably due to more exercise by leg muscle than the breast muscle in these birds which had not been flying birds. Lecithins and phosphatidyl ethanolamine were present in relatively greater amounts than the phosphatidyl serine and sphingomyelins.

Hydrolysis and GLC Analysis

The specificity of snake venom phospholipase A toward a specific phospholipid is markedly dependent on the reaction medium (79). The relative reaction rate of this enzyme on phosphatidyl ethanolamine is slower in ether than it is on lecithins in ether. However, the reaction rate with phosphatidyl ethanolamine is accelerated in water or buffer systems, so that it was necessary to use a system of this type for hydrolysis of the cephalins.

Enzymatic hydrolysis of lecithins was considered complete because no phosphorus was present in the supernatant after centrifugation. Lysolecithins were hydrolyzed by alkali.

All liberated fatty acids were converted to their methyl esters for GLC analysis. Two methods, those of Hornstein et al. (105) and McGinnis and Dugan (169) have been used to methylate hydrolysis products from lecithins, and the fatty acid content of the lecithin fractions as determined by GLC were in agreement qualitatively and quantitatively.

Table 8 shows the fatty acid composition at each location in lecithin molecules from dark meat in terms of their fatty acid methyl esters. It is apparent that 87.97% of the fatty acids at the α' position were saturated, mainly stearic, palmitic, tricosanoic and lignoceric acids, while 74.37% of the fatty acids in the β position were unsaturated and linoleic,

oleic and arachidonic acids predominated. Palmitic acid was the predominant fatty acid in lecithins, followed by stearic, oleic and linoleic acids.

Table 8. Fatty acid composition and location in dark meat lecithins (area %).

Fatty acid	α' Position	β Position	Intact[1]
9:0[2]	6.68	1.91	1.51
10.0	5.17	2.19	7.52
11:0	4.11	1.06	----
12:0	3.15	2.95	4.08
13:0	----	1.20	1.99
14:0	----	1.15	1.56
14:1	----	----	1.27
15:0	----	1.74	1.01
16:0	19.49	9.23	21.18
17:1	----	0.62	1.89
18:0	21.08	4.20	18.67
18:1	7.78	29.60	14.36
18:2	3.85	35.60	14.25
20:1	0.40	0.38	2.69
21:0	----	----	1.36
20:4	----	8.17	3.46
22:1	----	----	1.12
23:0	14.12	----	1.05
24:0	14.17	----	1.03
Saturated	87.97	25.63	60.96
Unsaturated	12.03	74.37	39.04
Total	100.00	100.00	100.00

[1]Lecithins not subjected to enzymic hydrolysis
[2]Carbon chain length : number of double bond (56)

Two different amounts of enzyme solution, 2 ml and 3 ml, with the same concentration (1 mg/ml) were used to hydrolyze dark meat phosphatidyl ethanolamine. After the

hydrolysis products were separated from a silicic acid column, some residual phosphatidyl ethanolamine was found as indicated by ninhydrin test and phosphorus determination. This demonstrated that the enzymatic hydrolysis of phosphatidyl ethanolamine, even in water solution, was not complete. The lysocompound and residual phosphatidyl ethanolamine were then hydrolyzed by alkali.

The results in Table 9 show the fatty acid composition of the fractions in terms of fatty acid methyl esters from GLC analysis. Results obtained from the use of 2 ml of enzyme solution show that 76.33% of fatty acids in the α' position were saturated and 66.44% of those in the β position were unsaturated. When 3 ml of enzyme were used, the fatty acids in the α' position contained 58.02% of saturated acids, while those in the β position consisted of 71.13% unsaturated acids. The predominant fatty acid in the intact phosphatidyl ethanolamine was stearic acid, while next in order were arachidonic, docosaenoic and linoleic acids. At the α' position, the chief component, stearic acid was accompanied by lesser amounts of oleic, linoleic and palmitic acids when a 2 ml portion of enzyme was used, and linoleic, oleic, arachidonic and palmitic acids when a 3 ml portion of enzyme was used. Arachidonic acid was the main component in the β position, with linoleic, oleic, stearic and palmitic acids following in both the 2 ml and 3 ml portions of enzyme were used. The reasons for the difference of hydrolysis products resulting from the use of

Table 9. Fatty acid composition and location in dark meat phosphatidyl ethanolamine (area %).

Fatty acid	2 ml of enzyme			3 ml of enzyme			Intact[2]
	α' Position	β Position	Unhydro-lyzed[1]	α' Position	β Position	Unhydro-lyzed[1]	
6:0[3]	3.79	----	----	----	----	----	----
8:0	4.73	----	----	----	----	2.09	----
11:0	0.63	----	----	----	----	----	5.03
12:0	4.23	4.88	9.11	3.78	2.37	1.91	5.03
?	----	----	----	----	0.51	----	----
13:0	0.95	0.72	1.54	1.05	----	0.97	1.80
?	2.11	1.97	4.23	1.08	----	----	----
14:0	1.67	2.02	2.36	2.24	1.25	2.16	2.41
14:1	----	----	----	----	----	----	3.93
?	3.16	2.80	4.50	1.74	0.77	1.32	----
15:0	2.84	1.56	2.41	3.00	0.80	2.36	2.32
?	----	----	----	----	----	----	2.71
16:0	8.84	6.84	9.11	6.84	5.16	6.61	5.33
16:1	----	2.08	----	2.62	1.68	2.78	----
17:0	2.21	1.65	2.19	2.12	0.79	1.63	----
17:1	----	----	----	----	----	----	3.62
?	----	----	----	1.95	----	----	----
18:0	44.66	10.39	51.70	35.79	17.22	58.61	18.91
18:1	8.38	14.54	6.81	10.68	13.51	10.08	4.94
18:2	7.89	24.03	6.04	12.54	22.25	9.48	7.84
18:3	----	1.72	----	0.90	2.06	----	----
?	----	0.73	----	----	----	----	----
20:0	1.78	----	----	----	----	----	----
20:1	----	----	----	----	----	----	4.54
21:0	----	----	----	3.20	----	----	----
20:4	1.70	24.07	----	10.47	31.63	----	16.50
22:1	----	----	----	----	----	----	10.86
?	0.43	----	----	----	----	----	----
25:0	----	----	----	----	----	----	4.23
Saturated	76.33	28.06	78.42	58.02	28.10	76.34	45.06
Unsaturated	17.97	66.44	12.85	37.21	71.13	22.34	52.23
Unidentified	5.70	5.50	8.73	4.77	0.77	1.32	2.71
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00

[1]Sample residue after enzymatic hydrolysis
[2]Sample not subjected to enzymic hydrolysis
[3]Carbon chain length : number of double bond (56)

two different quantities of enzyme in the hydrolysis of phosphatidyl ethanolamine was not clear, because the quantities of substrate, emulsifying agent, and buffer, the procedure, and the time and temperature of incubation were all the same. Kinetic studies might provide clues to better evaluate the observed results. It was noted that phosphatidyl ethanolamine appeared to be the most readily oxidized component, as evidenced by brown color formation when exposed to air as compared to others. This may perhaps be attributed to the high level in phosphatidyl ethanolamine of arachidonic acid with its four double bonds.

Table 10. Fatty acid composition and location in dark meat phosphatidyl serine (area %).

Fatty acid	α' Position	β Position	Unhydrolyzed[1]
12:0[2]	1.54	4.89	3.25
13:0	1.86	4.00	6.66
14:0	1.12	4.44	2.03
15:0	1.08	3.78	3.82
16:0	3.08	9.99	4.71
17:0	2.00	3.89	3.41
18:0	59.88	18.32	9.67
18:1	12.95	18.82	19.18
18:2	14.18	16.10	16.90
18:3	1.39	2.22	1.18
20:0	0.92	----	----
20:4	----	2.89	1.22
22:1	----	5.55	19.85
23:0	----	3.89	----
24:1	----	1.22	8.12
Saturated	71.48	53.20	33.55
Unsaturated	28.52	46.80	66.45
Total	100.00	100.00	100.00

[1]Sample residue after enzymatic hydrolysis
[2]Carbon chain length : number of double bond (56)

Table 10 gives the fatty acid composition of the fractions resulting from the action of 1 ml of solution of snake venom phospholipase A on phosphatidyl serine. This also gave an incomplete hydrolysis. The total fatty acids in the α' position contained 71.48% of saturated acids which were chiefly stearic, oleic and linoleic acids, whereas in the β position, 46.80% of the total fatty acids were unsaturated which mainly consisted of oleic, stearic, linoleic and palmitic acids. The main fatty acids contained in the unhydrolyzed residual phosphatidyl serine were oleic, linoleic, decosaenoic, stearic and tetracosaenoic acids. The residual phosphatidyl serine seemed to contain more unsaturated fatty acids than saturated acids. This was contrary to what was observed in phosphatidyl ethanolamine.

The distribution between the α' position and the β position of saturated and unsaturated fatty acids varied among the three phosphoglycerides in chicken dark meat muscle. Lecithin had more of its saturated fatty acids in the α' position than did phosphatidyl ethanolamine or phosphatidyl serine. The greater amounts of unsaturated fatty acids in lecithin were therefore to be found in the β position whereas lesser amounts of those in phosphatidyl ethanolamine and phosphatidyl serine were in the β position. This indicated that the tendency to place the saturated fatty acids at the α' position and the unsaturated fatty acids at the β position was greater in lecithin than in the other two phosphoglycerides and there is not a completely fixed distribution

pattern in any of these three phospholipids. Specific positional distribution was once proposed on the basis that only saturated fatty acids are present in the α' position and the unsaturated acids are in the β position of egg lecithin (241). Later Privett et al. (195) found that was not wholly true in egg lecithin, instead, there are four variations of saturated and unsaturated fatty acid distribution in the α' and β positions, in which the α'-saturated-β-unsaturated type is the major form occurring in lecithin. The results obtained in this study on phospholipids from muscle are in agreement with the latter concept.

In comparing the fatty acid composition of intact and residual chicken dark meat phosphatidyl ethanolamine in Table 9, one may note that the longer-chain polyunsaturated fatty acids were not present in the residual sample, while they were found primarily in the fatty acid fraction from the β position as well as intact phosphatidyl ethanolamine. This demonstrated that snake venom phospholipase A was specific toward the β position, that the more highly unsaturated fatty acids were at the β position, and that the enzyme apparently showed selectivity toward those molecules with the greater unsaturation.

The following calculated figures, which were obtained by dividing each unsaturated fatty acid in the β position by the total percentage of the corresponding acid present in both α' and β positions, could provide further evidence of

the affinity of the enzyme for the β position which contained the more highly unsaturated fatty acids. In phosphatidyl ethanolamine (data from 2 ml of enzyme solution used in the hydrolysis), 100% of palmitoleic, 63.44% of oleic, 75.28% of linoleic, 100% of linolenic, and 93.40% of arachidonic acids were liberated by the enzyme from the β position. Lecithins had 79.19% of oleic, 90.24% of linoleic, and 100% of heptadecaenoic, eicosaenoic and arachidonic acids liberated from the β position. Phosphatidyl serine showed 59.24% of oleic, 53.17% of linoleic, 61.50% of linolenic, 100% of arachidonic, docosaenoic, and tetracosaenoic acids contained in the β position.

Since the structure pattern was determined in dark meat, only total fatty acid composition was determined on the white meat phospholipid components. It is probable that the positional distribution of the fatty acids was the same in phospholipids from either source.

Table 11 presents the total saturated and unsaturated fatty acids contained in three phospholipid components of chicken white meat as determined by GLC analysis. Phosphatidyl ethanolamine contained 72.37% of saturated fatty acids and 27.63% of unsaturated fatty acids in which the major fatty acids were stearic and arachidonic acids, which were also the major acids in dark meat phosphatidyl ethanolamine. Phosphatidyl serine had 31.17% of saturated and 68.83% of unsaturated fatty acids in which oleic acid was the main

Table 11. Fatty acid composition in white meat phospholipids (area %).

Fatty acid	Phosphatidyl ethanolamine	Phosphatidyl serine	Lecithins
10:0	1.30	3.62	2.88
10:1	----	2.57	2.15
11:1	0.79	2.51	2.10
12:0	1.16	----	----
13:0	4.29	1.46	8.75
14:0	----	1.52	----
15:1	2.60	0.64	1.73
16:0	7.51	1.69	30.20
16:1	----	----	1.63
17:0	----	0.70	----
17:1	----	1.34	----
18:0	58.10	10.21	9.65
18:1	7.72	42.54	18.24
18:2	4.31	1.52	12.21
18:3	0.15	----	----
20:4	12.15	10.91	9.38
24:1	----	1.28	1.07
25:0	----	0.64	----
25:1	----	5.52	----
26:0	----	11.32	----
Saturated	72.37	31.17	51.49
Unsaturated	27.63	68.83	48.51
Total	100.00	100.00	100.00

fatty acid, which agreed with its dominant position in the phosphatidyl serine in dark meat, then cerotic, arachidonic and stearic acids were the next most commonly found fatty acids. Lecithins consisted of 51.49% of saturated and 48.51% of unsaturated fatty acids of which palmitic acid was the predominant fatty acid in lecithins from white meat as well as those from dark meat, then oleic, linoleic, stearic and arachidonic acids were present in lesser amounts.

The sphingomyelins of dark and white meat were subjected to acid hydrolysis since they are resistant to basic hydrolysis. Dark meat sphingomyelins contained 51.40% of saturated fatty acids and 48.60% of unsaturated fatty acids, whereas white meat sphingomyelins consisted of 56.94% of saturated and 43.06% of unsaturated fatty acids as presented in Table 12.

Table 12. Fatty acid composition of sphingomyelins (area %).

Fatty acid	Dark meat	White meat
10:0	1.61	9.35
11:0	2.29	10.33
12:0	2.91	6.79
13:0	2.67	6.57
14:0	9.74	9.76
15:0	2.60	----
15:1	----	5.19
16:0	9.74	7.78
16:1	1.67	6.67
17:1	----	3.68
18:0	11.78	6.36
18:1	16.13	13.15
18:2	5.92	10.14
21:0	0.62	----
20:4	8.49	4.23
22:1	2.51	----
23:0	7.44	----
24:1	4.12	----
25:1	9.76	----
Saturated	51.40	56.94
Unsaturated	48.60	43.06
Total	100.00	100.00

Oleic acid was the fatty acid present in the greatest amount in dark and white meat sphingomyelins alike. In dark meat sphingomyelins the fatty acid in the next greatest amount was stearic acid accompanied by lesser amounts of pentacosaenoic, palmitic, myristic, arachidonic and tricosanoic acids, while undecanoic and linoleic acids were the next most prevalent fatty acids in white meat sphingomyelins, with myristic acid following. The types and quantities of fatty acids in these two sphingomyelins differed widely. Myristic acid was the only fatty acid present in the same amount in each. Dark meat sphingomyelins contained more longer-chain fatty acids such as C_{21} through C_{25}, whereas white meat sphingomyelins had none of them. On the basis of fatty acid composition, the sphingomyelins compare most favorably with phosphatidyl serine. Each type of phospholipid had longer-chain fatty acids up to C_{25} for the former and C_{26} the latter. The chief acid in each was oleic acid, and no short-chain fatty acids below C_{10} were present in either type.

Mention should be made here that the presence of plasmalogens had not been established when the fatty acid composition was determined for white meat phospholipids and when the fatty acids were determined in the α' position in dark meat phospholipids. Since the actual concentration of the sample injected into the GLC apparatus was not known, correction of results for the presence of plasmalogens was difficult to compute. To the best approximation, the exact

quantity of fatty acids and especially those in the α' position should be less than the reported figures.

Contamination was a problem in the analysis of fatty acid methyl esters by GLC. Once introduced, contaminants may accompany a sample all the way to the point of measurement and yield spurious and misleading peaks in the resulting gas-liquid chromatograms. Impure methanol was found as a potential source of contamination in that it gave 7 peaks from C_{12} through C_{18} on the GLC graph.

Regardless of the problems existing in GLC analysis, it was still found to be the most rapid and reproducible method for fatty acid analysis. Its great resolving power and sensitive response provide qualitative and quantitative information impossible to collect by other methods. The utility of the technique can be reinforced by other methods of fractionation (99).

Studies of Plasmalogens

The ether-containing phospholipids, plasmalogens, are now recognized as being almost as prevalent in some tissues as are diacyl phospholipids (205), since Feulgen et al. (62) discovered them. The structure of plasmalogens has been established by many scientists, but the significance of their presence in the muscle is still obscure. In their histochemical studies, Ferrans et al. (60) found that the plasmalogens were distributed in all muscles. By application of a colorimetric detection method using mercuric chloride

and diphenyl carbohydrazide, it was found that plasmalogens were present in phospholipids of chicken muscle tissues. This finding encouraged the study of more details of plasmalogens.

Qualitative Determination

The qualitative colorimetric determination of plasmalogens was based on the reaction of mercuric chloride with the enol-ether of plasmalogens which is analogous to the reaction of ionic mercury salts with normal olefins (33). Because of the low concentration of $HgCl^+$ ions, mercuric chloride reacts slowly or not at all with simple olefins (180). However, the reaction with the α,β-unsaturated ether is almost instantaneous because of the reactive property of this α,β-double bond due to resonance of the enol-ether structure which increases the negative charge in the β carbon making it much more reactive to electrophilic additions.

Since the molecular structure of a phospholipid and its corresponding plasmalogen are so similar, it is not surprising that the difference of one vinyl ether linkage in place of one ester linkage fails to separate these moieties in most fractionation systems and they almost invariably appear together. The ease and specificity of iodination of the double bond of the vinyl ether group permits the introduction of a considerable mass and also alters other properties of the molecule. It was expected that a plasmalogen molecule with two iodine atoms attached might exhibit a different

chromatographic behavior from that of a corresponding phospholipid, therefore, a study was initiated to determine if they could be separated by TLC or column chromatography.

Figure 3 shows a TLC separation of an iodinated plasmalogen-phospholipid mixture from some unknown compound on a silica gel G-10% (w/v) ammonium sulfate plate in a developing solvent consisting of 2 volumes of chloroform and 1 volume of methanol containing 5% 0.1N sulfuric acid. Detection was made by molybdate spray. The unknown compound which did not migrate gave a very faint blue color with molybdate. This compound could be either a phosphorylated glyceryl ether or a glyceryl ether (63). No attempt was made to identify it.

Since Figure 3 did show some indication that the iodinated plasmalogens and phospholipids had a tendency to separate, further experiments were required to prove this. If the plasmalogen portion moved at a different rate because iodine added to the α,β-double bond altered the polarity or acidity, it should possess the same rate of migration of plasmalogens and phospholipids if the added iodine were removed. Therefore, dehalogenation by treatment with a reactive metal, such as zinc in pure ether, to eliminate iodine from vic-dihalides in the iodinated plasmalogen molecule was carried out to regenerate the α,β-unsaturated double bond. When the static and mobile portions of the sample recovered from the TLC plate in Figure 3, before and after dehalogenation, were applied to TLC as shown in Figure 4, none of the

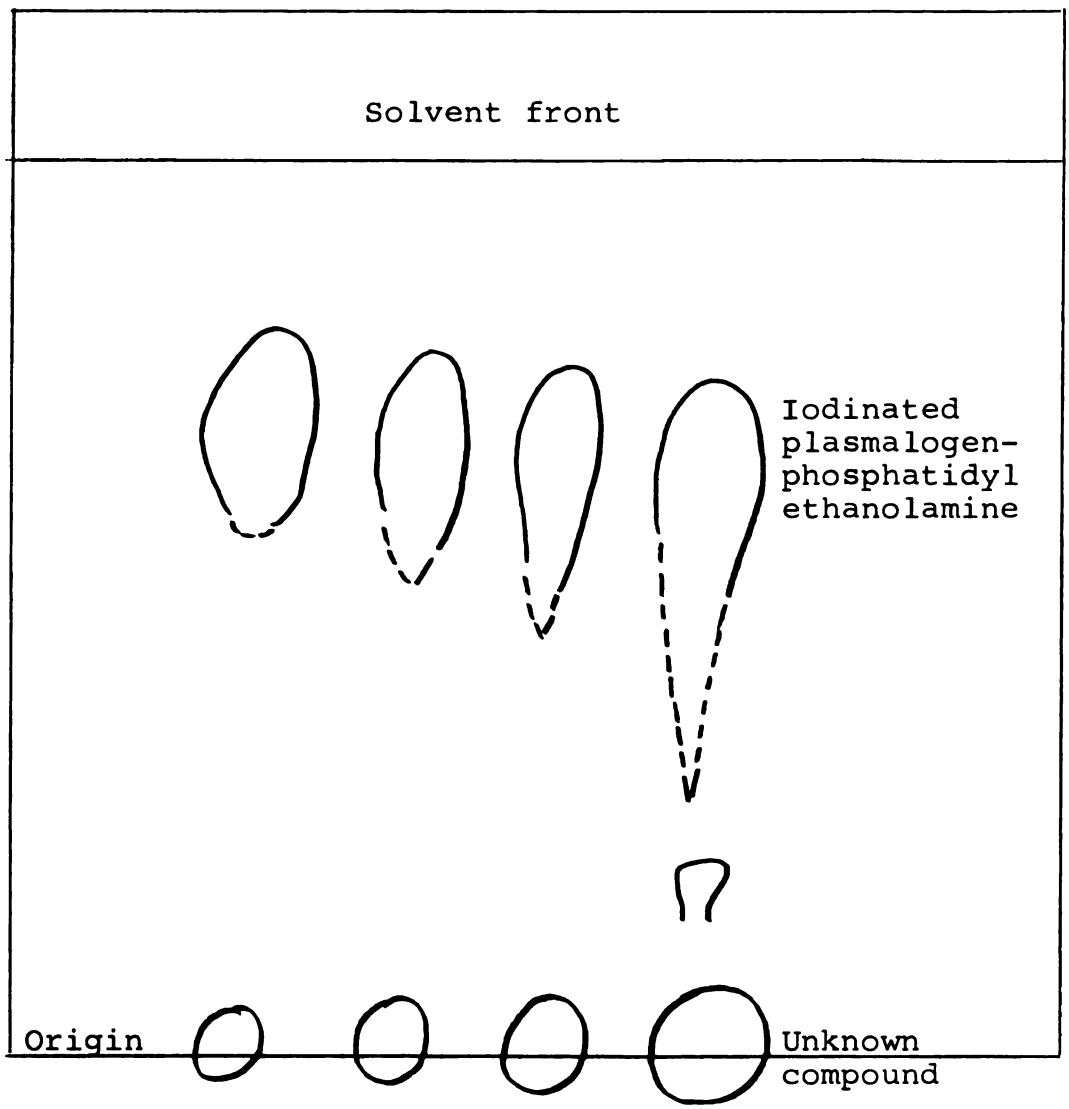
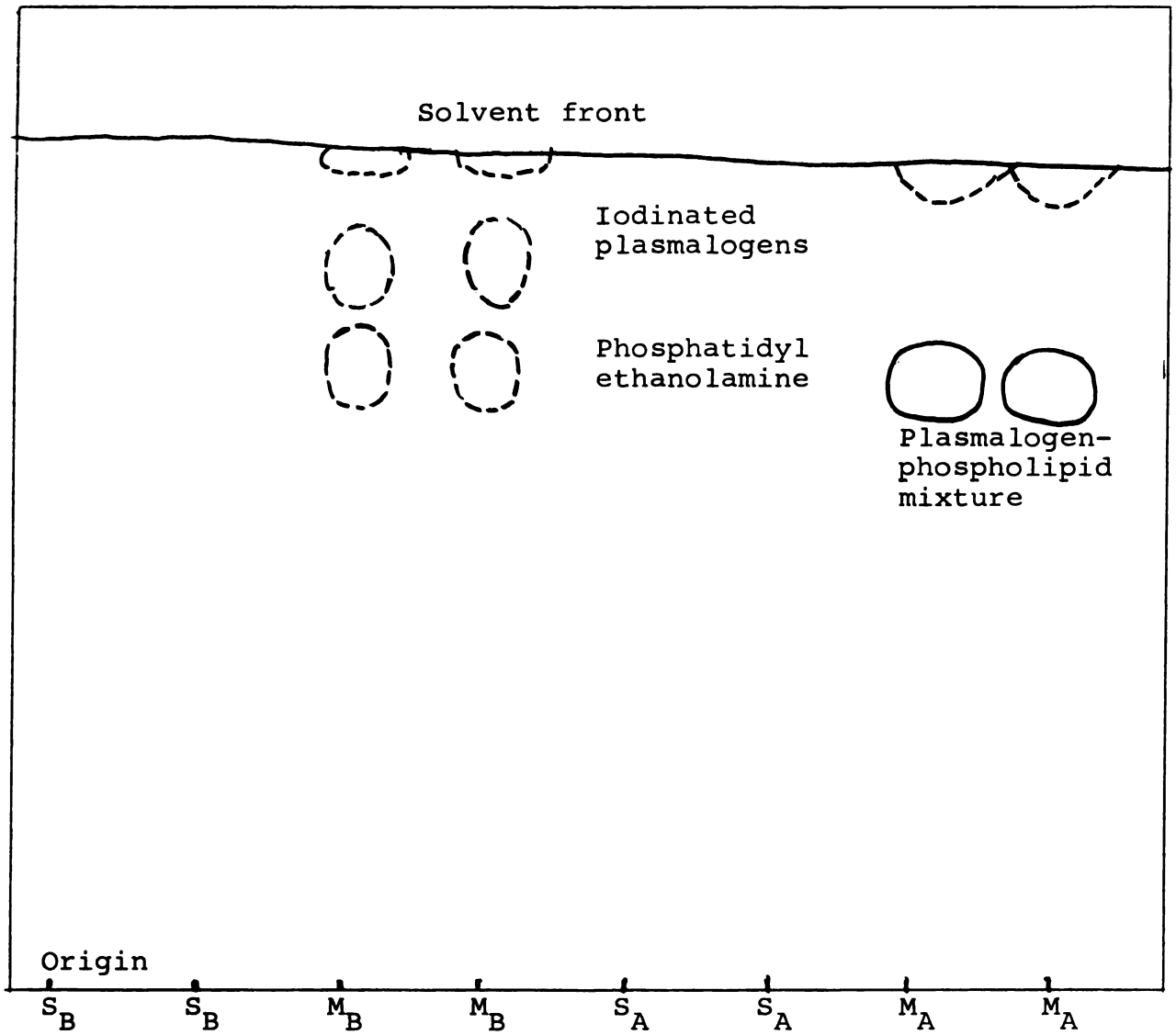


Figure 3. TLC separation of an iodinated plasmalogen-phospholipid mixture from some unknown compound.



S - Static portion from TLC plate in Figure 3
M - Mobile portion from TLC plate in Figure 3
B - Before dehalogenation
A - After dehalogenation

Figure 4. TLC chromatogram of an iodinated plasmalogen-phospholipid mixture before and after dehalogenation.

static portions (those which did not move on the plate described in Figure 3) before and after dehalogenation developed any color with molybdate spray, but the mobile portion of the iodinated sample developed two spots and the sample after dehalogenation showed only one spot with the same R_f value as the one with the lesser R_f in the iodinated samples. This was evidence that the sample with the higher R_f value must be due to iodinated plasmalogens which migrated at a different rate as a result of having different mass or acidity from phospholipids. This also illustrated that some separation of iodinated plasmalogens from phospholipids in Figure 3 did occur but not very distinctly. The brownish spots which moved with the solvent front were probably oxidized substances or free fatty acids.

The separation of iodinated plasmalogens from phospholipids on TLC or column chromatography was due probably to a different polarity or substituent effect. Since the α,β-unsaturated double bond exhibits a negative inductive effect (80), and since iodine has a very high mass, the adsorbability was consequently altered, and a different rate of migration was observed.

According to Mangold and Kammereck (154), strongly acidic lipids may be chromatographed on thin-layer by adding 10% of ammonium sulfate (w/v) in the silica gel and developing with a chloroform-methanol-aqueous sulfuric acid solvent system. This was apparently what made the iodinated

plasmalogens move more than the corresponding phospholipid because the electron-attracting property of halogens made the iodinated plasmalogen molecule more acidic than the corresponding phospholipids.

Chemical tests were also employed to confirm the dehalogenation process. In Table 13, all samples from the static portion (those which did not move on the plate described in Figure 3) gave negative tests before and after dehalogenation. A negative mercuric chloride reaction in the mobile portion (those which moved on the plate described in Figure 3) was obtained when the α,β-double bond in a plasmalogen was saturated by iodine, and a positive test when the iodine were removed in the mobile portion. Positive ninhydrin and molybdate tests in the mobile portion indicated that amino and phosphate groups were not affected by dehalogenation at all.

These findings apparently lead to a procedure for separating native plasmalogens from their corresponding phospholipids which involve the specific iodination of the plasmalogens in the plasmalogen-phospholipid mixture, removal of any contaminants by TLC, and then separating the mixture into the two components, iodinated plasmalogens and phospholipids, by the TLC system developed in this study. The separated iodinated plasmalogen may then be recovered by deiodination. Each step is checked by chemical tests.

Table 13. Chemical test for phosphatidal ethanolamine from TLC analysis

Test	Dehalogenation			
	Before		After	
	Static portion	Mobile portion	Static portion	Mobile portion
Mercuric chloride	-	-	-	+
Ninhydrin	-	+	-	+
Molybdate	-	+	-	+

+ Positive test
- Negative test

Quantitative Determination

The reaction of iodine with an α,β-unsaturated ether linkage is complex (205), and when it takes place in aqueous methanol, the product is reported to be the unsymmetrical methylacetals (230) which are capable of generating higher fatty aldehyde on hydrolysis. Studies with model compounds (201,243) and pure plasmalogens (76,201) show that the method suffers from its inherent inaccuracy. The sample left in the tubes after evaporation of the solvents in which they were dissolved were not uniformly or completely dissolved by methanol. These problems probably contributed to low and inconsistent results.

The phosphatidal content of phospholipid fractions from dark and white meat muscle was determined by iodination method of Williams et al. (261) (Table 14). The results were

quite inconsistent. Phosphatidal ethanolamine and phosphatidal serine of both dark and white meat muscle were apparently in greater quantity in the second determination than those found in the first, although the samples were the same in each analysis, while phosphatidal choline was apparently decreased. Fatty acid composition changes during low temperature storage have been reported (109). If oxidative changes were occurring in the fatty acids, one would have expected to see a change in the fatty aldehyde composition also. The degree of inconsistency was greater in dark meat, especially the cephalins fraction, than in white meat.

Table 14. Quantitative determination of plasmalogens by iodination.

Component	Dark meat (%)		White meat (%)	
	June 1964	March 1965	June 1964	March 1965
PE	3.51	10.08	5.19	9.03
PS	1.00	26.83	2.50	9.56
PC	13.19	9.04	9.97	3.88

PE - Phosphatidal ethanolamine
PS - Phosphatidal serine
PC - Phosphatidal choline

The gravimetric determination of plasmalogens in terms of dimethylacetals of each aldehyde component seemed to offer a better approach than the other methods, since methylated

samples were purified by eliminating all methyl esters through saponification to the sodium salts of the corresponding fatty acids. In the preparation of DMA, methylation must be accomplished before saponification. An experiment in reversing the above procedure indicated that all the respective peaks from gas-liquid chromatograms of ME and DMA were present but the relative height and areas were different due to apparent concentration differences. Generally, chicken white muscle tissues contained more plasmalogens than the dark meat. These findings were in agreement with those reported by Thiele et al. (248) who showed that in exercised skeletal muscle the plasmalogen concentration was decreased as the total phospholipid concentration increased. Phosphatidal ethanolamine was the most prevalent plasmalogen in the phospholipids of both dark and white meat as shown in Table 15, which agreed with the findings in ox-spleen by Gray (83). Phosphatidal choline was the least prevalent plasmalogen in dark meat and phosphatidal serine was the least prevalent in white meat.

GLC Analysis

The long-chain fatty aldehydes are rather unstable compounds. This instability makes the aldehyde itself unsuitable for GLC analysis. Fortunately, the aldehyde can easily be converted to a derivative, the aldehyde dimethylacetal, which is quite stable and has the added advantage of a much lower boiling point.

Table 15. Gravimetric determination of purified methylesters (ME) and dimethylacetals (DMA) in phospholipids from chicken muscle.

Component	Weight basis (g)					μMole basis				
	ME	DMA	Total	% ME	% DMA	ME	DMA	Total	% ME	% DMA
Dark meat										
PE	0.0198	0.0051	0.0249	79.52	20.48	82.60	18.00	100.60	82.11	17.89
PS	0.0012	0.0002	0.0014	85.71	14.29	4.80	0.73	5.53	86.80	13.20
PC	0.0553	0.0058	0.0611	90.51	9.49	231.40	22.00	253.40	91.32	8.68
White meat										
PE	0.0239	0.0167	0.0406	58.87	41.13	93.20	64.00	157.20	59.29	40.70
PS	0.0062	0.0006	0.0068	91.18	8.82	25.00	2.30	27.30	91.58	8.42
PC	0.0330	0.0204	0.0534	61.80	38.20	123.00	83.60	206.60	59.54	40.46

An aldehyde dimethylacetal and its corresponding acid methyl ester differ, for GLC analysis, in the degree of polarity exhibited by the end groups and by a mass difference of 16. The polar effect of the methyl ester is stronger than that of dimethylacetals. Therefore, the nonpolar and polar stationary phases which give excellent separations of the fatty acid methyl esters by GLC should also be suitable for separating mixtures of aldehyde dimethylacetals. Evidence has been given that the DMA are stable on a polar stationary phase (57).

Since commercially prepared DMA were not available, all standards for GLC of saturated and unsaturated DMA had to be prepared. In the preparation of fatty aldehyde dimethylacetals from corresponding fatty acids in the laboratory, the recovery was not 100%. The unchanged fatty acids, alcohols, methyl esters and other contaminants could possibly be carried along with dimethylacetals all the way to GLC analysis and yield misleading peaks in the resulting gas-liquid chromatograms. In order to have a more representative standard, all contaminants had to be removed. This was accomplished by taking advantage of the fact that DMA were alkali resistant while all others were susceptible to saponification.

Identification of DMA was accomplished in the same manner as ME determination by plotting logarithmic retention times versus carbon chain length or versus molecular weight of DMA.

The proportion of saturated to unsaturated component was much higher in DMA than was found in methyl esters. In dark meat, phosphatidal ethanolamine had 88.49%, phosphatidal serine contained 74.72%, and phosphatidal choline had 94.52% of saturated DMA. The saturation of white meat DMA was 93.51%, 93.14%, and 97.20% in the same order. The phosphatidal serine from dark meat contained the higher unsaturated DMA of those examined with 25.28% compared to 6.86% in white meat. The fatty aldehydes in the plasmalogens from dark and white meat are presented in Tables 16 and 17.

Table 16. Fatty aldehyde composition of dark meat plasmalogens (area %).

FADMA	Phosphatidal ethanolamine	Phosphatidal serine	Phosphatidal choline
8:0	----	----	0.94
9:0	6.60	----	3.22
10:0	11.71	5.96	4.63
11:0	4.66	5.06	3.25
12:0	3.30	6.48	4.33
13:0	----	5.35	----
14:0	2.47	5.19	2.49
15:0	2.47	7.29	2.98
16:0	27.46	16.21	62.96
16:1	----	8.10	----
17:0	4.54	5.35	2.55
17:1	----	----	0.72
18:0	25.28	17.83	6.08
18:1	7.26	8.10	4.76
18:2	1.88	5.51	----
21:0	----	----	1.09
20:4	2.37	3.57	----
Saturated	88.49	74.72	94.52
Unsaturated	11.51	25.28	5.48
Total	100.00	100.00	100.00

FADMA - Fatty aldehyde dimethylacetals

Table 17. Fatty aldehyde composition of white meat plasmalogens (area %).

FADMA	Phosphatidal ethanolamine	Phosphatidal serine	Phosphatidal choline
7:0	----	9.60	----
8:0	----	18.81	3.04
9:0	8.28	19.70	7.50
10:0	9.40	8.58	8.66
11:0	5.04	5.40	3.81
12:0	3.45	1.87	3.44
13:0	----	0.95	----
14:0	----	1.52	----
15:0	5.52	----	1.24
16:0	31.74	17.85	61.30
16:1	----	1.97	----
17:0	7.31	----	----
18:0	22.77	8.86	8.20
18:1	6.49	2.15	2.80
18:2	----	1.12	----
20:4	----	1.62	----
Saturated	93.51	93.14	97.20
Unsaturated	6.49	6.86	2.80
Total	100.00	100.00	100.00

FADMA - Fatty aldehyde dimethylacetals

The major aldehydes in each component were palmitaldehyde, stearaldehyde, oleylaldehyde, and capraldehyde which agreed in general with those found in pigeon muscle by Gray and MacFarlane (85), except for capraldehyde. The predominant aldehyde in both dark and white meat was palmitaldehyde. Since the area count is not strictly proportional to the concentration of the component in the issuing gas and not related to a simple molecular parameter of the component and carrier gas (124), no attempt has been made to correlate area percent

to mole percent in GLC analysis of both methylesters and dimethylacetals, because the peak area was considered to be more reliable (124). White meat showed a lesser content of oleylaldehyde than dark meat. This was probably due to a longer storage time of the white meat phospholipids before examination for aldehyde content and some oxidation or decomposition may have occurred since the white meat sample was prepared in January 1964 and the dark meat lipids were extracted in September of the same year.

By comparing corrected retention times of DMA with those of ME under the same operating conditions on the same column, they seemed to have a definite relationship in time of emergence from the column. The position of a chromatographic peak of a DMA was identical with the ME peak of the fatty acid with 2 carbons less than in the aldehyde responsible for the DMA peak. In other words, palmitaldehyde appeared in the position of myristic ME; pentadecanaldehyde DMA emerged in the same position as tridecanoic ME; and oleylaldehyde DMA overlapped with the palmitoleic ME peak. It appeared that saturated DMA's overlapped saturated ME's on the gas chromatogram and odd- and even-carbon number DMA and ME showed a definite overlapping pattern with the two-carbon difference and a similar effect was observed with monoenes. The Appendix B gives a comparison of positional relations in terms of corrected retention times in seconds. No attempt was made to speculate on the polyenes.

The presence of plasmalogens was also detected by observing the presence or absence of chromatographic peaks on two gas-liquid chromatograms. In Figure 5 are GLC traces for a sample of methylated dark meat phosphatidyl serine which was saponified, the soaps were acidified and remethylated, and analyzed by GLC and compared to the original trace for the ME-DMA mixture. Peaks of $C_{8:0}$, $C_{9:0}$, $C_{12:0}$, $C_{14:0}$, $C_{15:1}$, $C_{16:0}$, $C_{17:0}$, $C_{17:1}$, $C_{18:0}$, and $C_{18:1}$ appeared remarkably reduced in height, and the peak for $C_{17:1}$ was eliminated. This indicated that plasmalogens were present in the phospholipids in chicken muscle tissues, and by referring to the table in Appendix B, one may determine which aldehydes were present in the mixed sample and absent from the sample containing only methyl esters.

The presence of plasmalogens in appreciable amount in phospholipids was thus positively established. The fatty aldehydes of each plasmalogen component in phospholipids were found in quantities ranging from 8.82% fatty aldehydes in terms of dimethylacetals from phosphatidal serine in serine phosphatides up to 41.13% of fatty aldehydes from phosphatidal ethanolamine in ethanolamine phosphatides. These aldehydes were mainly saturated fatty aldehydes which are distributed similarly to the distribution of saturated fatty acids in phospholipids in this study. This relatively large quantity of plasmalogens present in the normal phospholipids could have a substantial effect of fatty acid composition, especially

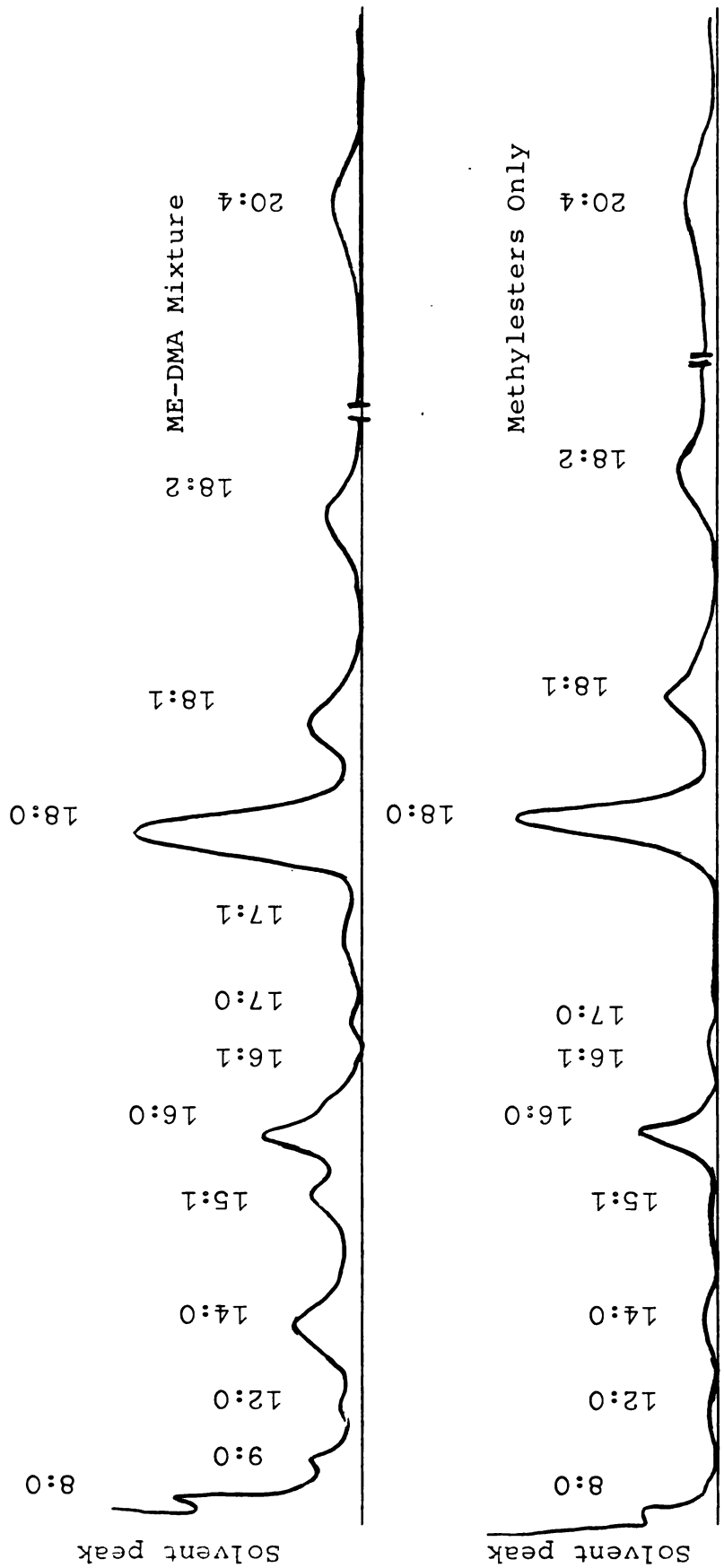


Figure 5. Comparison of gas-liquid chromatograms of a ME-DMA mixture and of ME alone from the same mixture.

relative to those in the α' position. The presence of plasmalogens was also illustrated in Figure 5 as the peak height and area decreased markedly when the GLC trace was obtained for ME alone.

Column Chromatography

An experiment was made in which white meat lecithins were applied to a Celite-2,4-dinitrophenylhydrazine-phosphoric acid column. The eluate, which presumably contained the 2,4-dinitrophenylhydrazones of the aldehydes of the plasmalogens in the phospholipid fraction placed on the column, was methylated by HCl-methanol and H_2SO_4-methanol. GLC results (Table 18) were inconsistent but showed that aldehydes could be removed from plasmalogens by this technique. The major difficulty associated with this method results from instability of the chromophore to fluorescent light while in hexane solution and from the fact that 2,4-dinitrophenylhydrazine forms products during the reaction which give high and variable blank values (205).

Phosphatidyl ethanolamine, phosphatidyl serine, lecithins, and sphingomyelins are the main components contained in the phospholipids, there are still other components present in lesser amounts which need to be analyzed and identified. Although plasmalogens have now been extensively investigated, further studies are necessary to explore their quantitative determination in order to make accurate calculations of the

Table 18. Gas-liquid chromatographic determination of the fatty aldehydes separated by Celite-2,4-dinitrophenylhydrazine from plasmalogens in lecithin from chicken white meat (area %).

FADMA	HCl-methanol	H_2SO_4-methanol
6:0	10.05	----
7:0	15.73	20.77
8:0	5.76	8.86
9:0	2.69	4.89
10:0	----	7.23
11:0	11.71	0.74
12:0	----	0.57
13:0	----	0.57
14:0	1.24	4.25
16:0	22.75	9.57
17:0	----	5.49
17:1	----	3.37
18:0	9.80	10.99
18:1	2.03	3.19
20:0	3.32	5.14
20:1	10.64	11.80
20:4	4.28	2.57
Total	100.00	100.00

FADMA - Fatty aldehyde dimethylacetals

fatty acid composition of a normal phospholipid, and to determine their significance in the muscle.

SUMMARY

The phospholipids from chicken muscle tissues have been studied from the standpoint of occurrence, nature, composition, and structure. The leg and breast muscles of two one-year old Cobb strain chickens on a standard MSU Z-4 diet were taken as raw material for these studies. Lipids were extracted by a chloroform-methanol mixture and fractionated by silicic acid column chromatography. Four main phospholipid components, phosphatidyl ethanolamine, phosphatidyl serine, lecithins, and sphingomyelins have been isolated and identified by thin-layer chromatography. Quantitative determination of each component was carried out by gravimetric analysis, phosphorus content determination, and infrared spectral analysis, and their results were compared. The phospholipid content of leg muscle (dark meat) lipids was higher than that in the breast muscle (white meat) lipids. Lecithins and phosphatidyl ethanolamine were found in relatively greater amounts than phosphatidyl serine and sphingomyelin. The fatty acid composition was analyzed by enzymatic, basic, or acid hydrolysis followed by gas-liquid chromatography of the fatty acid methyl esters. Chromatographic peaks were identified by using standards, and plotting the corrected retention times against carbon chain length. Stearic acid was the predominant fatty acid in

phosphatidyl ethanolamine, and palmitic acid was dominant in lecithin. The main fatty acid in phosphatidyl serine and sphingomyelin was oleic acid. These observations were comparable for fatty acid composition of the phospholipid moieties in both dark and white muscles with some variations of relative amounts of other fatty acids. Positional distribution of the fatty acids in lecithin and cephalins was accomplished by liberation of fatty acids from the β position by phospholipase A from snake venom. After separation of the fatty acids from the lysocompounds and any unhydrolyzed components, the free fatty acids were methylated for analysis by gas-liquid chromatography. The lysocompounds were hydrolyzed chemically and their fatty acids were converted to methyl esters for GLC analysis. It was found that the polyunsaturated fatty acids were located primarily at the β position and the saturated fatty acids mainly at the α' position. Sphingomyelins were acid hydrolyzed and their fatty acid composition determined by GLC of methyl esters of the fatty acids. The sphingomyelins of chicken dark meat contained fatty acids similar in kind and quantity to those in phosphatidyl serine.

The lecithin, phosphatidyl ethanolamine, and phophatidyl serine fractions were found to contain considerable quantities of plasmalogens. These plasmalogens were detected qualitatively by colorimetric methods and were tentatively separated from normal phospholipids by thin-layer and column

chromatography on a silica gel G-10% (w/v) ammonium sulfate solid phase using a chloroform-methanol-aqueous sulfuric acid solvent system. Iodination of the plasmalogens permitted higher R_f values than given by the corresponding phospholipids. The quantity of plasmalogens present in chicken muscle tissues were determined by an iodination method and by gravimetric analysis. The proportion of saturated to unsaturated fatty aldehydes in plasmalogens was much higher than that of fatty acids in the corresponding phospholipids. Phosphatidal ethanolamine was the most prevalent plasmalogen found. Chicken white meat was found to contain more plasmalogens than the dark meat. The fatty aldehyde composition was analyzed by gas-liquid chromatography of the fatty aldehyde dimethylacetals using standards prepared for this purpose. The major aldehydes in each plasmalogen component of chicken dark and white muscle were palmitaldehyde, stearaldehyde, oleylaldehyde, and capraldehyde. Palmitaldehyde was present in relatively greater quantity than the others.

Evaluation of the gas-liquid chromatographic peaks for dimethylacetals and methylesters in terms of retention times under the same conditions revealed that the DMA of an aldehyde traveled at the same rate as the methyl ester for the fatty acid having two carbons less than the aldehyde.

These studies have shown that although the total lipid content of chicken muscle is low, these lipids contain a

number of phospholipids in varying quantities. These phospholipids have a high percentage of the unsaturated fatty acids of those in the total lipids. While no fixed pattern of fatty acid distribution exists in phospholipids, the tendency is for the most highly unsaturated acids to occur at the β position of a given phospholipid moiety. A higher than expected amount of these phospholipids exist as plasmalogens. These findings are of interest to the nutritionist, the physiologist, and the food scientist. The concentration of polyunsaturated fatty acids in the phospholipids points to these as subjects for further study in processed foods and the various problems of stability and palatability associated with them.

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APPENDIX A

MSU Z-4 Formula

Material	Percentage
Ground yellow corn	34.5
Ground oats	20.0
Wheat bran	15.0
Flour midds	10.0
Alfalfa meal	3.0
Meat scraps	3.0
Dried skim milk	2.0
White fish meal	2.5
Soybean meal	2.5
Ground oyster shell flour	5.0
Steamed bone meal	1.5
Salt	0.6
Cod liver oil	0.4
Total	100.0

APPENDIX B

Positional relations between dimethylacetals and methyl-esters in terms of corrected retention times in seconds on a gas-liquid chromatogram.

DMA	Retention Times	ME	Retention Times
9:0	19	7:0	21
10:0	31	8:0	35
11:0	39	9:0	42
12:0	54	10:0	56
13:0	67	11:0	74
14:0	89	12:0	96
15:0	125	13:0	125
16:0	164	14:0	164
17:0	213	15:0	222
17:1	246	15:1	274
18:0	294	16:0	294
18:1	350	16:1	350

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Composition and Structure of Phospholipids in Chicken Muscle Tissues[1,2]

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Abstract

Lipids extracted from breast muscle and thigh muscle of one-year old chickens on a standard MSU-Z-4 diet have been fractionated by silicic acid column chromatography into nonphospholipids, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl choline (lecithin), and sphingomyelins. Phospholipid fractions were identified by thin-layer chromatography and the quantity of each determined by gravimetric analysis, analysis of the phosphorus content, and infrared spectra.

The phospholipid content of thigh muscle (dark meat) lipids was higher than that in the breast muscle (white meat). Phosphatidyl choline and phosphatidyl ethanolamine were found in relatively greater amts than phosphatidyl serine and sphingomyelins. Enzymatic hydrolysis followed by gas-liquid chromatographic analysis of the fatty acids liberated and those in the lysocompounds was used to establish the positional specificity of the fatty acids in the phosphoglycerides. The polyunsaturated fatty acids are located primarily at the β-position and the saturated fatty acids at the α'-position. The qualitative and quantitative determination of the plasmalogens was also accomplished.

Introduction

THE PHOSPHOLIPIDS of skeletal muscle have not been studied to the same extent as the phospholipids of organ and neural tissue and those of bacteria. Recent studies on the composition of the phospholipids of avian skeletal muscle have been reported by Davenport (3), and Gray and MacFarlane (5) on pigeon, and by Marion and Woodroof (11) on the broiler. The objective of this study was to investigate the composition and structure of the phospholipids of both dark (thigh) and white (breast) meat in chicken.

Experimental Procedures

Materials. Samples of muscle tissues were taken from two freshly killed one-year-old Cobb strain chickens on a standard MSU-Z-4 diet.

Silicic acid, cp, precipitated, from Fisher Scientific Co., Fair Lawn, N.J., was washed with methanol and dried 24 hr at 120C.

Chromatographic standards for gas-liquid chromatography (GLC) were obtained from California Corporation for Biochemical Research, Los Angeles, Calif.

Crotalus adamanteus venom, obtained from Ross Allen's Reptile Institute, Silver Springs, Fla., was used as a source of phospholipase A.

Silica Gel G, used for thin-layer chromatography, was purchased from Brinkmann Instruments, Inc., Chicago, Ill.

Chromosorb W, acid washed, mesh size 80/100, was obtained from Applied Science Laboratories, Inc., State College, Pa.

[1] Michigan Agriculture Experiment Station Journal Article No. 3527.
[2] Presented at the AOCS Meeting in Chicago, October, 1964.

TABLE I

Lipid Class Isolated from 100 g Chicken Muscle Tissues

Class	Dark meat		White meat	
	g/100g Sample	%	g/100g Sample	%
Nonphospholipids	1.0917	57.56	0.4413	48.82
Acetone elutable lipids	0.0639	3.37	0.0984	10.89
Phospholipids	0.7409	39.07	0.3642	40.29
Total	1.8965	100.00	0.9039	100.00

All chemicals were analytical reagent grades, and all solvents were freshly redistilled and made suitable for spectrophotometric use.

Lipid Extraction. The procedure for extraction of lipid from muscle tissues was essentially based on the method of Bligh and Dyer (2), modified by adopting the washing system from Folch et al. (4). Nitrogen was used to replace air throughout in extraction and separation while carbon dioxide was used in storage. The final proportion of chloroform, methanol and water was 8:4:3 by volume in the extract. Total lipids were determined by evaporating solvent in vacuo, drying overnight in a vacuum desiccator over calcium chloride, and weighing.

Column Chromatography. A column of activated silicic acid was prepared by pouring 40 g of silicic acid, slurried in an excess of chloroform, into a column (2.2 cm I.D.). The silicic acid was washed with acetone, methanol, and chloroform, respectively, to check whether undesirable channeling existed in the column and to remove colored materials. A 1 cm layer of powdered anhydrous sodium sulfate was then added. Total lipids, redissolved in a small portion of chloroform, were transferred onto the column in a ratio of 0.02 g per 1 g of silicic acid. Elution was accomplished by various solvent systems and successive 35 ml fractions were collected. The flow rate was adjusted to approx 3 ml/min by applying pressure with nitrogen. Nonphospholipids or neutral lipids were eluted by chloroform and monitored by the Salkowski test (9) until negative. Acetone, acting as a scavenger for oxidized materials as suggested by Nelson and Freeman (13), was used to remove pigmented materials. Cephalins were eluted by 15% methanol in chloroform (v/v) and detected in each fraction by ninhydrin. Phosphatidyl ethanolamine was found to come down the column faster than phosphatidyl serine as indicated by a decreasing color intensity of the ninhydrin test. Phosphatidyl choline was eluted by 35%methanol in chloroform (v/v) and its presence confirmed by negative ninhydrin and positive molybdate tests, while sphingomyelins were eluted by 100% methanol and checked by ninhydrin.

Phosphorus Determination. Phosphorus content of each fraction collected was determined by the method of Beveridge and Johnson (1).

Thin-layer Chromatography. Thin-layer absorption chromatography on Silica Gel G was used to check the identities of phospholipids (9). All components were applied in a solution of chloroform or chloroform-methanol. The developing solvent used for separation was chloroform: methanol:water (65:25:4) by volume (20). Lipid component spots were detected

with ninhydrin solution for amino phosphatides, molybdic acid for phosphatides, and Dragendorf reagent for choline (17). The relative R_f values were 0.79 for phosphatidyl ethanolamine, 0.54 for phosphatidyl serine, 0.64 for phosphatidyl choline, and 0.48 for sphingomyelins.

Infrared Spectra Analysis. All infrared (IR) spectra determinations have been made with a Beckman IR-5 double beam recording Spectrophotometer equipped with a sodium chloride prism, and an absorption cell with an optical path length of 0.0992 mm. All readings were taken from strong absorption bands; therefore, phosphatidyl ethanolamine and phosphatidyl serine were at 5.8 μ because they have been shown to exhibit similar (19) or identical (18) IR spectra, phosphatidyl choline at 10.3 μ and sphingomyelins were at 6.1 μ.

Hydrolysis. Phosphatidyl ethanolamine, phosphatidyl serine, and phosphatidyl choline were subjected to enzymatic hydrolysis in an aqueous medium at 37C, pH 7.3, with glycylglycine buffer and sodium deoxycholate (10) with phospholipase A in snake venom from *Crotalus adamanteus* (6). Sphingomyelins were hydrolyzed with sulfuric acid (12). The liberated free fatty acids, the unhydrolyzed phospholipides, and the lysocompounds were separated on a silicic acid column by chloroform, 25% methanol in chloroform (v/v), and 100% methanol, respectively. The unhydrolyzed phospholipids and the lysocompounds were subjected to basic hydrolysis with 0.5 M methanolic KOH (8).

Gas-Liquid Chromatography. The fatty acid composition of all phospholipid components was determined quantitatively by GLC of the methyl esters in an F and M Model 500 temperature programmed gas chromatograph (F and M Scientific Corporation, Avondale, Pa.) equipped with a thermal conductivity detector, Honeywell Electronik recorder, and the Series 200 DISC integrator. A coiled copper column (7.5 ft by ¼ in. O.D.) was used for methyl ester separation. It was packed with 20% by weight DEGS (Lac-728, F and M Scientific Corporation, Avondale, Pa.) and 1% by weight phosphoric acid on acid-washed chromosorb W, 80/100 mesh, as a support phase. The operating conditions used in this study were: Column temp, 210C; injection port temp, 265C; detector block temp, 250C; carrier gas, helium; carrier gas flow rate, 80 ml/min; reference gas flow rate, 120 ml/min; attenuator setting, 2; bridge current, 150 ma; chart speed, 80 sec/in. Identifications of the fatty acids on the chromatogram were made by comparing the semilog plots of retention times vs. carbon numbers of the chicken lipid methyl esters with those of known mixtures of methyl esters run on the same column under the same conditions. The fatty acid compositions were expressed as area percentage of the total area from all methyl esters.

Results and Discussion

The presence of phosphatidyl ethanolamine, phosphatidyl serine, and sphingomyelins were readily determined by the ninhydrin test. Phosphatidyl choline detection gave some trouble but a satisfactory method resulted from a combination of Beveridge's method for phosphorus (1) and Skidmore's determination of phosphatides (17). This involved adding 0.05 ml of coned sulfuric acid to 0.1 ml of eluate and heating two min at 130–140C in a sand bath, after which 0.05 ml of molybdic acid was immediately added. A blue color developed if phosphatides were present. The intensity of the blue color was a function of the conen of phosphatidyl choline in the sample. Therefore, the phosphatidyl choline fraction was identified with a negative ninhydrin test and a positive molybdate test.

Table I shows the lipid classes isolated from 100 g chicken muscle tissues by column chromatography. The total lipids in dark meat are twice those in the white meat; however, the percentage distribution of phospholipids in both is about the same at 40%. Table II compares three methods, phosphorus content determination, gravimetric analysis, and IR spectra analysis, which have been used to determine each component contained in white meat, and two methods, phosphorus content determination and gravimetric analysis, to determine each component in dark meat phospholipids. There is more of each phospholipid component on a weight basis in the dark meat than that of its counterpart in the white meat. This is probably due to more exercise by thigh than breast muscle. Phosphatidyl choline and phosphatidyl ethanolamine are present in relatively greater amts than the phosphatidyl serine and sphingomyelins; however, there are some discrepancies. The main difference is in the amt of sphingomyelins determined by IR spectra analysis when compared to the amt of sphingomyelin determined by the other methods. This is similar to the observations made in a study of pork muscle phospholipids by Kuchmak and Dugan (9). Since sphingomyelin is resistant to basic hydrolysis due to the amide linkage (15), one sample, containing phosphoglycerides and sphingomyelin from white meat, was purified by subjecting it to basic hydrolysis. The resulting products were separated on a silicic acid column and checked for purity of sphingomyelin by TLC. The sphingomyelins thus purified were determined by IR spectra and gave a value of 0.0053 g/100 g. This value was probably more precisely representative than those obtained for sphingomyelin by other analyses.

Table III lists a set of absorption coefficients of the measured phospholipid standards at their principal absorption band. The absorption coefficients, designated a, absorbance per mg per ml, and ϵ, molar absorptivity, were calculated by Lambert-Beer's Law.

TABLE II

Phospholipid Components Isolated from 100 g Chicken Muscle Tissues

Component	Dark meat				White meat					
	Phosphorus content		Gravimetry		Phosphorus content		Gravimetry		IR spectra	
	g/100g	%	g/100g	%	g/100g	%	g/100g	%	g/100g	%
PE[a]	0.2303	30.02	0.1833	24.74	0.0652	15.40	0.0563	15.46	0.0573	12.16
PS[b]	0.0681	8.88	0.0519	7.00	0.0394	9.30	0.0330	9.06	0.0279	5.92
PE and PS[c]	0.0429	5.59	0.0467	6.30	–	–	–	–	–	–
PC[c]	0.3992	52.04	0.4305	58.11	0.2157	58.01	0.2243	61.59	0.3100	65.80
PC and SP[c]	–	–	–	–	0.0594	14.03	0.0403	11.06	0.0706	14.99
SP[d]	0.0266	3.47	0.0285	3.85	0.0138	3.26	0.0103	2.83	0.0053[f]	1.13
Total	0.7671	100.00	0.7409	100.00	0.4235	100.00	0.3642	100.00	0.4711	100.00

[a] Phosphatidyl ethanolamine.
[b] Phosphatidyl serine.
[c] Phosphatidyl choline.
[d] Sphingomyelin.
[e] Fraction in which both components were present as determined by TLC.
[f] Determined on sample from which phosphoglycerides had been removed by mild basic hydrolysis.

TABLE III

Absorption Coefficients

Component	Solvent	Wavelength (μ)	Absorbance per mg per ml (a)	Molar[a] absorptivity (ϵ)
PE	CS_2	5.8	0.908	67656
PS	$CHCl_3$	5.8	0.671	53175
PC	$CHCl_3$	10.3	0.449	36008
PC	$CHCl_3$	5.8	0.773	61666
SP	$CHCl_3$	6.1	0.596	44019

[a] Assuming following relative mol wts: phosphatidyl ethanolamine, 743; phosphatidyl serine, 787; phosphatidyl choline, 786; and sphingomyelins, 728.

Since different materials have definitely different absorption coefficients due to different fatty acid composition and location (16), it was deemed preferable to prepare our own standards from chicken muscle tissues by chromatography. Solvents selected for this study were based principally on the solubility of the various lipid classes in them. Hence, phosphatidyl ethanolamine was measured in carbon disulfide, while phosphatidyl serine, phosphatidyl choline and sphingomyelins were measured in chloroform. It should be emphasized that these values are specific only for the material, cells, and instruments used in this study.

TABLE IV

Percentage of Plasmalogens in Each Component

Component	Dark meat	White meat
Phosphatidal ethanolamine	3.51	5.19
Phosphatidal serine	1.00	2.50
Phosphatidal choline	13.19	9.97

The plasmalogens were first detected qualitatively by the reaction of mercuric chloride with an α,β-unsaturated ether and the color developed with diphenyl carbohydrazide (14). Phosphatidyl ethanolamine, phosphatidyl serine, and phosphatidyl choline gave positive tests for the presence of plasmalogens while sphingomyelins were negative. Plasmalogen content shown in Table IV was determined quantitatively by the uptake of iodine because of the sensitivity of the method and the linear relationship between iodine uptake and sample size (21). The relative mol wt of plasmalogen was calculated on the basis of linoleic acid and stearyl aldehyde attached to the β- and α'-positions, respectively. Thus, the mol wt of plasmalogen in the phosphatidyl ethanolamine form is 727, it is 771 in the phosphatidyl serine form and 770 in the phosphatidyl choline form. Plasmalogens in chicken muscle tissues are most prevalent in the phosphatidyl choline form and least prevalent in the phosphatidyl serine form.

After enzymatic or basic or acid hydrolysis of phospholipid components, all liberated free fatty acids were converted to methyl esters by the method of Hornstein et al. (7). GLC analysis of the methyl esters derived from dark meat phospholipids is shown in Table V and that from white meat phopholipids in Table VI. In dark meat the predominant fatty acid in phosphatidyl ethanolamine is stearic acid. At the α'-position the chief component, stearic acid, is accompanied by lesser amts of linoleic, oleic and arachidonic acids; while arachidonic acid is the main component at the β-position, with linoleic, stearic and oleic acids in decreasing order. The question marks indicate components for which no standard was available to identify them on the chromatogram. The main fatty acids contained in phosphatidyl serine are stearic, oleic, linoleic, docosaenoic and tetracosaenoic acids. At the α'-position, the chief acids are stearic, oleic and linoleic acids while oleic, stearic, linoleic and palmitic are the main fatty acids at the β-position. Palmitic acid is predominant in phosphatidyl choline, followed by stearic, oleic and linoleic acids. Saturated fatty acids, stearic, palmitic, tricosanoic and lignoceric, are found mainly at the α'-position while the unsaturated linoleic, oleic and arachidonic acids predominate at the β-position. Sphingomyelins contain chiefly oleic, and stearic acids, followed by lesser amts of myristic, palmitic, arachidonic, pentacosaenoic and tricosanoic acids.

Since the structure pattern was determined in dark meat, only basic hydrolysis was carried out on the white meat components. It is probable that the positional distribution of the fatty acids is the same in phosphatides from either source. The major fatty acid of phosphatidyl ethanolamine is stearic acid, which was also the major fatty acid in phosphatidyl ethanolamine in the dark meat, while arachidonic acid is the next. This differs from the observations made on dark meat phosphatidyl ethanolamine. Phosphatidyl serine contains mainly oleic acid which

TABLE V

Fatty Acid Composition of Phosphatidyl Ethanolamine, Phosphatidyl Serine, Phosphatidyl Choline, and Sphingomyelins in Dark Meat (Area %)

FA[a]	PE			PS			PC			SP
	α'-FA	β-FA	Unhydrolyzed[b]	α'-FA	β-FA	Unhydrolyzed[b]	α'-FA	β-FA	Unhydrolyzed[b]	
6:0	–	–	–	–	–	–	–	–	–	Trace
8:0	–	–	2.09	–	–	–	–	–	–	Trace
9:0	–	–	–	–	–	–	6.69	1.91	1.51	–
10:0	–	–	–	–	–	–	5.18	2.19	7.52	1.61
11:0	–	–	–	–	–	–	4.11	1.06	–	2.29
12:0	3.78	2.37	1.91	1.54	4.89	3.25	3.16	2.95	4.09	2.91
?	–	0.51	–	–	–	–	–	–	–	–
13:0	1.05	–	0.97	1.86	4.00	6.66	–	1.20	2.00	2.67
?	1.08	–	–	–	–	–	–	–	–	–
14:0	2.24	1.25	2.16	1.12	4.44	2.03	–	1.16	1.56	9.73
14:1	–	–	–	–	–	–	–	–	1.27	–
?	1.75	0.77	1.32	–	–	–	–	–	–	–
15:0	3.00	0.80	2.36	1.08	3.77	3.82	–	1.74	1.01	2.60
16:0	6.84	5.16	6.61	3.08	9.99	4.71	19.50	9.23	21.18	9.73
16:1	2.62	1.68	2.78	–	–	–	–	–	–	1.67
17:0	2.12	0.79	1.63	2.00	3.89	3.41	–	–	–	–
17:1	–	–	–	–	–	–	–	0.62	1.89	–
?	1.95	–	–	–	–	–	–	–	–	–
18:0	35.79	17.22	58.61	59.88	18.32	9.67	21.07	4.21	18.67	11.78
18:1	10.68	13.51	10.08	12.95	18.82	19.17	7.77	29.60	14.36	16.12
18:2	12.54	22.25	9.48	14.18	16.10	16.90	3.85	35.60	14.25	5.92
18:3	0.90	2.06	–	1.39	2.22	1.18	–	–	–	–
20:0	–	–	–	0.92	–	–	–	–	–	–
20:1	–	–	–	–	–	–	0.40	0.37	2.69	–
21:0	3.20	–	–	–	–	–	–	–	1.36	0.62
20:4	10.47	31.63	–	–	2.89	1.22	–	8.17	3.46	8.49
22:1	–	–	–	–	5.55	19.85	–	–	1.12	2.51
23:0	–	–	–	–	3.89	–	14.12	–	1.05	7.44
24:0	–	–	–	–	–	–	14.17	–	1.03	–
24:1	–	–	–	–	1.22	8.12	–	–	–	4.12
25:1	–	–	–	–	–	–	–	–	–	9.67

[a] Fatty acids. [b] Unhydrolyzed refers to unchanged portion of sample separated after enzymatic hydrolysis.

TABLE VI

Fatty Acid Composition of Phosphatidyl Ethanolamine, Phosphatidyl Serine, Phosphatidyl Choline, and Sphingomyelins in White Meat (Area %)

FA	PE	PS	PC	SP
10:0	1.30	3.62	2.88	9.35
10:1	–	2.57	2.15	–
11:0	–	–	–	10.33
11:1	0.70	2.51	2.10	–
12:0	1.16	–	–	6.79
13:0	4.29	1.46	8.75	6.57
14:0	–	1.52	–	9.76
15:1	2.60	0.64	1.73	5.19
16:0	7.51	1.69	30.20	7.78
16:1	–	–	1.63	6.67
17:0	–	0.70	–	–
17:1	–	1.34	–	3.68
18:0	58.10	10.21	9.65	6.36
18:1	7.72	42.54	18.24	13.15
18:2	4.31	1.52	12.21	10.14
18:3	0.15	–	–	–
20:4	12.15	10.91	9.38	4.23
24:1	–	1.28	1.07	–
25:0	–	0.64	–	–
25:1	–	5.52	–	–
26:0	–	11.32	–	–

agrees with its composition in dark meat, then cerotic, arachidonic and stearic are the next most commonly found fatty acids. Palmitic acid is the predominant fatty acid in phosphatidyl choline in white meat as well as in dark meat; oleic, linoleic, stearic and arachidonic acids follow. In the sphingomyelin molecule oleic acid is present in the greatest amt, the same as in dark meat; next are undecanoic, linoleic and myristic acids.

The predominant fatty acid contained in phosphatidyl ethanolamine is stearic acid. Oleic acid is the main acid in both phosphatidyl serine and sphingomyelins, while palmitic acid is dominant in phosphatidyl choline. These observations hold both in dark and white muscle tissues. Some variations of amts of other fatty acids do exist. The polyunsaturated fatty acids are located primarily at the β-position and the saturated fatty acids at the α'-position as has been observed in phopholipids from other sources.

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