PLASMALOGEN COMPOSITION AND METHODS OF DETERMINATION

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
Ricardo R. del Rosario
1967

ABSTRACT

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METHODS OF DETERMINATION

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The modified 2, 4-dinitrophenylhydrazine method for plasmalogen determination was compared with the iodine-uptake and p-nitrophenylhydrazine methods. Phosphatidyl ethonolamine and phosphatidyl choline extracts from beef brain lipids and hog brain lipids and total lipid extracts of beef liver and brain were examined. The values obtained for plasmalogen concentration by the 2, 4-dinitrophenylhydrazine method were slightly higher than those obtained by the other two procedures.

After treatment of a phospholipid fraction containing plasmalogen with 2, 4-dinitrophenylhydrazine, it is possible to use phosphorous assay for the analysis of plasmalogen after thin-layer chromatographic separation of the lyso- and diacyl phospholipids.

Gas chromatography was also applied to the determination of aldehydes and fatty acids in plasmalogens. Preliminary tests showed incomplete reactions were obtained with the three

methods used, namely, the low temperature-sulfuric acid, hydrochloric acid-methanol, and boron trifluoride-methanol procedures for methylation. The reaction conditions were modified and complete reactions were obtained after testing with thin-layer chromatography.

PLASMALOGEN COMPOSITION AND METHODS OF DETERMINATION

By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Food Science

1967

ACKNOWLEDGMENTS

The author expresses his sincere thanks and gratitude to his major adviser, Dr. LeRoy Dugan, Jr., for suggesting the problem and for his help and guidance throughout the course of the work; to Dr. P. Markakis, of the Department of Food Science, and Dr. R. Evans, of the Department of Biochemistry, for their helpful suggestions in the preparation of the manuscript and acting as members of the examining committee.

A special thanks goes to Dr. B. S. Schweigert, head of the Department of Food Science, for his continuous encouragement during the study.

The author's appreciation goes to his companions in the laboratory for their help and cooperation and especially to Dr. Ki Soon Rhee.

And finally the author is grateful to the National Institutes of Health, which supported the study from Grant No. HE-09441.

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INTRODUCTION

Plasmalogens are a group of compounds that belong to the lipids classified as phospholipids. They differ from the ordinary phospholipids by the presence of a vinyl ether linkage on the α' carbon of the glycerol moiety instead of an ester bond. Acid cleavage of the vinyl ether yields an aldehyde.

The first report on these compounds was made by Feulgen and Voit (1924), who noted the presence of aldehydogenic compounds in tissue slices treated with fuchsin-sulfurous acid. When the tissue was previously treated with alcohol, no reaction was obtained in the tissue, but color was found in the alcohol which indicated the fatty character of the compound.

Studies in the structural determination of plasmalogens were hindered by the lack of suitable analytical techniques. The identity of the compound remained obscure for some time. Meanwhile, studies on their distribution in other sources were carried out. They were found widely distributed from sources such as one-celled yeast (Thiele, 1955) to the tissues of higher forms of animals such as horses (Christl, 1953) and even in man (Stepp et al., 1927).

Feulgen and Bersin (1939) isolated from bovine muscle a crystalline acetal phospholipid which was thought to have a cyclic structure. This feat led the way for more intensive studies on the elucidation of the structure of plasmalogens. Further investigations showed that it contained a fatty acid molecule in addition to the aldehyde (Klenk and Debuch, 1954). The fatty aldehyde was found mostly present in the α' carbon (Marinetti et al., 1957; 1958) of the glycerol molecule linked with a vinyl ether bond (Debuch, 1957).

The exact biological function of plasmalogens has not been established. Plasmalogens have been implicated in fat deposition (Möckel, 1943; Yarbro and Anderson, 1956), but this has not yet been confirmed. Another notable observation is its high concentration in certain active tissues such as the heart (Klenk et al., 1952; Gray and MacFarlane, 1958) and the brain (Christl, 1953) but low in the liver (Klenk et al., 1952; Christl, 1953). Why this is so has not been clarified.

Earlier reports on plasmalogens indicated that they were only present in the phospholipid fractions. With the use of better analytical separation and testing procedures, it was shown that they were also present among the triglycerides (Schogt et al., 1965; Eichberg, 1961).

Among the phospholipids, phosphatidyl ethanolamine has the greatest proportion of plasmalogens. Since the vinyl ether

bond is very susceptible to hydrolysis, giving rise to aldehydes, speculation is aroused relative to its possible role in non-enzymatic browning reactions. Phosphatidyl ethanolamine as well as phosphatidyl serine have free amino groups in the ethanolamine and serine residues which may interact with the fatty aldehydes obtained from the hydrolysis of the vinyl ether bond to give rise to non-enzymatic browning. With the increasing use of dehydration as a method of food preservation, information on this type of reaction is important.

In order to study the role of plasmalogens in biological materials, it would be desirable to have a satisfactory analytical tool which is both rapid and accurate for their determination.

Earlier workers in plasmalogens used the fuchsin-sulfurous acid method of Fuelgen and Imhauser (1927). This was a sensitive method, but was subject to interference. In the presence of surface-active compounds, the color which developed faded rapidly resulting in low values. Attempts to improve the method (Erlich et al., 1948; Leopold, 1953; Waelsch, 1951; Warner and Lands, 1963) met with little success as low values were still obtained. In spite of this difficulty, however, it remained in use because of the absence of better methods.

A number of methods have been proposed and some have been found promising. These were the iodine-uptake method

(Siggia and Edsberg, 1948; Williams et al., 1962), 2,4-dinitrophenyldrazine (Katz and Keeney, 1966; Rhee et al., in press), and p-nitrophenyldrazine (Pries and Böttcher, 1965; Wittenberg et al., 1956) methods, gas chromatography of methylated products (Gray, 1958; 1960; Farquhar, 1960; 1960a), and thin-layer chromatography (Owen, 1966).

Iodine-uptake as a method for plasmalogen determination is highly desirable as the reaction is specific for the vinyl ether linkage. While many favor its use for its accuracy (Rapport and Lerner, 1959; Rapport and Alonzo, 1960), observations to the contrary are not absent (Camejo et al., 1964; Peng, 1966).

The p-nitrophenyldrazine method suffered from certain difficulties. Like the iodine-uptake method, it has been used by a number of workers in spite of the generally accepted fact that incomplete recovery of the hydrazones occurs, especially in the case of short chain aldehydes.

The use of 2,4-dinitrophenyldrazine was a later development. Although it has long been used for the separation and purification of aldehydes, it was only in early 1966 that Katz and Keeney described the use of 2,4-dinitrophenylhydrazine for the determination of plasmalogen aldehydes involving the simultaneous methylation and hydrazone formation of the fatty acids and aldehydes, respectively.

Esterification and subsequent gas chromatographic analysis of dimethylacetals were introduced by Gray (1958; 1960) and Farquhar (1962; 1962a). It is one of the more powerful tools for plasmalogen analysis. Problems encountered in esterification procedures, especially of biological materials, are still under consideration.

Rhee et al., (in press) proposed a modified 2, 4-dinitrophenylhydrazine method wherein selective cleavage of the vinyl ether bond is effected using a different set of conditions. This also made possible the determination of plasmalogen by phosphorous analysis after thin-layer chromatographic separation of the lyso-compound formed, after the cleavage of the vinyl ether linkage, and the unreacted diacyl compound. Owen (1966) later reported the use of phosphorous determination for plasmalogen analysis using two-dimensional thin-layer chromatography and mercuric chloride reagent.

In view of the diverse difficulties encountered in the use of various methods, further studies were made on the modified 2, 4-dinitrophenylhydrazine method as proposed by Rhee et al. Comparison with the existing procedures, namely iodine-uptake and p-nitrophenylhydrazine, was conducted and additional studies on esterification and gas-liquid chromatographic analysis for plasmalogen aldehydes were accomplished.

REVIEW OF LITERATURE

Early Method

The use of fuchsin-sulfurous acid as a quantitative test for plasmalogens was introduced by Feulgen and Imhauser (1927). It was based on the fact that aldehydes, liberated by acid hydrolysis, react with the fuchsin reagent, resulting in pink color the intensity of which is determined spectrophotometrically. The method is very sensitive and therefore highly appropriate for the analysis of plasmalogen which is present in small quantities in the lipid of the tissue and in even more minute quantities in the individual component. The high sensitivity of the method, however, is coupled with a low degree of selectivity. Color development was found to be suppressed by surface-active substances (Erlich et al., 1948). It was shown also that addition of detergents after color development resulted in rapid decoloration; but, if the reaction were carried out in acidic media, the effect of the detergent was nullified.

Iodine-Uptake Method

Vinyl alkyl ethers have been analyzed previously (Orton and McKie, 1916) by hydrolyzing the vinyl ether to acetaldehyde and alcohol. The aldehyde was distilled off and reacted with sodium

bisulfite. The excess sodium bisulfite was then titrated with standard iodine solution. This required hours to accomplish and could not be used in routine analysis.

In 1945, Siggia and Edsberg proposed a simple and rapid method for vinyl ether determination. The vinyl ether sample was dissolved in methanol and incubated under vigorous shaking with a standard iodine solution in a closed container. After the reaction, the excess iodine was titrated with thiosulfate solution. It was found that the method worked for almost all vinyl ethers tried except for lauryl- and octadecyl-vinyl ether. Further studies showed that the low results obtained were due to the low solubility of the compounds in the media used. Efforts were made to increase the sensitivity of the reaction. Changes in the concentration of the iodine solution were proposed to enable the use of smaller samples while mixtures of chloroform, methanol and water were tried to improve the solubility (Gottfried and Rapport, 1962; Rapport and Lerner, 1959; Rapport and Alonzo, 1960; Norton, 1960). Custer and Natelson (1949) reported on microdetermination of iodine by spectrophotometric means. They studied the absorption spectra of iodine in solutions of water, toluene, potassium iodide, chloroform and benzene and recommended the extraction of iodine prior to the measurement of absorbance. Concurrently, Sloane-Stanley (1949), recognizing the difficulty encountered in the routine analysis of iodine, suggested the use of a

spectrophotometric method for end-point determination and proposed several wavelengths.

In 1962, Williams et al. devised a procedure which was both more sensitive and simple. The sample was incubated with iodine in a buffer solution. The excess iodine was then extracted with butyl acetate and the absorbance determined at 363 mu.

2, 4-Dinitrophenylhydrazine and p-Nitrophenylhydrazine Methods

Because of the difficulty encountered in the use of fuchsin-sulfurous acid, it became necessary to find another method to evaluate correctly the amount of plasmalogen in the lipid samples. Attention was directed to the use of 2, 4-dinitrophenylhydrazine which has been used widely for characterizing aldehydes and ketones. As early as 1942, Jones et al. showed that 2, 4-dinitrophenylhydrazones have absorption spectra characteristic of the solvent and the unsaturation of the fatty chain. It was shown further (Jones et al., 1957) that the extinction coefficient of the hydrazones of different aldehydes were almost constant irrespective of the chain length. Although the possibility appeared good, initial application to plasmalogen determination was not successful. High blank values and variable results were obtained (Rapport and Alonzo, 1962). This was later traced to impurities present in the 2, 4-dinitrophenylhydrazine reagent (Schwartz and Parks, 1961).

Schwartz et al. (1963) reported a direct quantitative isolation of the 2, 4-dinitrophenylhydrazones of carbonyl compounds from oxidized oils. It was accomplished by passing oil through a column of celite impregnated with 2, 4-dinitrophenylhydrazine and phosphoric acid. The hydrazones were eluted and the concentration determined spectrophotometrically. Later Katz and Keeney (1966) reported a quantitative microdetermination of plasmalogen aldehydes as hydrazones patterned after the method of Wittenberg et al. (1956) for p-nitrophenylhydrazine. The sample was incubated with the 2, 4-dinitrophenylhydrazine dissolved in methanol in the presence of sulfuric acid for 15 hours. After incubation, the hydrazones and methyl esters were separated by thin-layer chromatography. The hydrazones were eluted with hexane and the absorbance determined at 339 mu.

Rhee et al. (in press) modified the procedure and employed 55% phosphoric acid and 2, 4-dinitrophenylhydrazine. The sample was dissolved in 1:1 (v/v) chloroform: methanol and mixed with the reagents in a test tube. The tube was kept in the dark and shaken occasionally. Reaction was found to be complete in 1.5 hours. The hydrazones were extracted with carbonyl-free hexane and the combined extracts were washed with distilled water to remove excess reagents. After drying the washed extract over anhydrous sodium sulfate, the absorbance was determined using a spectrophotometer set at 338 mu. The procedure allows a check analysis by phosphorous

determination. This is possible since the cleavage of the aldehyde from the plasmalogen gives rise to a lysocompound. On thin-layer chromatography the diacyl analogue of the plasmalogen has a different R_f value from that of the lysocompound. By spotting the sample on a thin-layer plate and developing with a suitable solvent system, the plasmalogen may be determined by analyzing the phosphorous content of the spots.

Owen (1966) described the use of two-dimensional thin-layer chromatography for the determination of plasmalogen. Initial separation of the phospholipid on thin-layer chromatography was made with the use of 65:43:3:1 (v/v) chloroform:methanol:water:acetic acid and then redeveloped with 80:20:1 (v/v) light petroleum ether:diethyl ether:acetic acid in the same direction. The plates were dried and sprayed with mercuric chloride which cleaved the vinyl ether bond of the plasmalogen molecules. The plate was then developed in the direction perpendicular to the first dimension to effect the final separation of the diacyls from the lysocompounds. Owen was able to determine the plasmalogen content of each of the individual lipid fractions from the phosphorous content of the spots.

Esterification and Gas Chromatography

The gas chromatographic separation and identification of lipids were initiated by James and Martin (1941; 1956). They separated, through a silicone column, fatty acids ranging from C_1 to C_{14}.

Resolution was, however, far from satisfactory except for the short chain acids. Even with some of the later developments and improvements in the solid supports and liquid phases (Smith, 1959; Smith et al., 1961; Jackson, 1966; Metcalfe, 1960; Ralls, 1960) difficulties were encountered in the separation of the longer chain fatty acids as well as those of the unsaturated fatty acids. It was not until Cropper and Heywood (1953) introduced the concept of using methylated components that satisfactory results were obtained. Since then, together with the introduction of better column materials, excellent separation of methyl esters of fatty acids up to C_{30} has been made possible.

Fatty aldehydes, being volatile, should have been suitable for gas chromatographic analysis. However, because of the instability of the aldehydes at the elevated temperatures that prevail in the column, it was not generally appropriate to determine these by gas chromatography. Gray established the feasibility of the gas chromatographic separation of aldehyde derivatives. He reacted aldehydes with methanol by refluxing in the presence of hydrochloric acid to form dimethylacetals which are more heat stable than the corresponding aldehydes and which could be analyzed chromatographically. A systematic method for analysis of aldehydes as dimethylacetals was established. The difficulty of identification of these components in the presence of methyl esters of fatty acids was especially noted. Another problem encountered in natural fats is

the fact that aldehydes, formed by lipid oxidation, are sometimes present and make evaluation more difficult. Gray identified unknown aldehydes by plotting the logarithm of retention time against the carbon number of standard aldehydes and referring the retention times of the unknown samples to these plots. Farquhar (1962; 1962a) published results of an extensive study on the identification of aldehydes. He employed several techniques to differentiate saturated from unsaturated aldehydes, branched chain from straight chain compounds and, in addition, utilized two columns, one polar and another non-polar, to check the behavior of the dimethylacetals.

Hand in hand with the development of chromatographic separation was the development of suitable methylation techniques for the formation of methyl esters. Aldehydes, when present with the fatty acids, form dimethylacetals. Esterification may be accomplished either by liberation of the fatty acids from the lipid and subsequently methylating them or by direct interesterification resulting in the formation of the methyl esters directly from the lipid.

Methylation of the fatty acids has been accomplished in a number of ways. Stoffel et al. (1959) used hydrochloric acid in anhydrous methanol to convert the fatty acids to methyl esters. Diazomethane was used extensively (Schlenk and Gellerman, 1960; Metcalfe and Smith, 1961) in spite of its toxicity and dangerous preparation procedure. In addition, it is known to form pyrazoline

compounds with the unsaturated acids. Hornstein et al. (1960) successfully used ion exchange resin to methylate the fatty acids liberated from lipids.

Direct conversion of the fatty acids from the lipids to methyl esters has been accomplished with both basic and acidic catalysts. Boron trifluoride dissolved in anhydrous methanol was originally used for fatty acids (Metcalfe et al., 1960). Morrison and Smith (1964) reported that it could be used for efficient interesterification of most phospholipid fractions, with the exception of sphingomyelin. Sodium methoxide (Luddy et al., 1960) has been used for esterification by controlling the temperature and the amount used but, like most basic catalysts, it has the ability to cause isomerization of unsaturated fatty acids and it is also ineffective with sphingomyelin which is resistant to basic hydrolysis.

Sulfuric acid has been used as a catalyst (Downing et al., 1960) for interesterification at elevated temperatures. McGinnis and Dugan (1965) proposed a low temperature methylation procedure using sulfuric acid as catalyst. Reaction was effected at -60 C which is ideal for esterification especially of labile substances like lipids. No side reactions have been noted (Mahadevan et al., 1966).

MATERIALS AND METHODS

Sample Preparation

Hog and beef brain and beef liver were used as source materials in this work. They were obtained from the Michigan State University Meat Laboratory and from a local slaughter house. The brains, obtained a short time after the animals were killed, were washed with cold water to remove the blood. A hundred gram sample of each was weighed and extracted using the method of Bligh and Dyer (1959), combined with the washing procedure of Folch et al. (1957). The excess samples were placed in polyethylene bags with distilled water which was saturated with nitrogen by bubbling from a tank. The bags were sealed and stored in a -25 C room.

Extraction of the lipids was achieved by the addition of 200 ml chloroform and 100 ml methanol and homogenizing for 2 minutes. Then 100 ml of water were added and the mixture again homogenized for an additional 30 seconds. The homogenate was filtered through Whatman No. 4 filter paper on a Büchner funnel using moderate suction. The sample was kept under a nitrogen atmosphere by inverting a funnel connected to a nitrogen tank over

the sample (Fig. 1). The filtrate was transferred to a 500 ml separatory funnel and allowed to separate into two phases.

After 12 hours, the lower phase, consisting mainly of chloroform and lipids, was removed and washed with 0.9% aqueous sodium chloride and the solvent removed under reduced pressure with a rotary evaporator. The dry sample was taken up with 1:1 (v/v) chloroform:methanol solution and stored under nitrogen in a freezer until used for further studies.

Column Chromatography

Preparation of column materials

The 100 mesh silicic acid (Mallinkrodt Chemical Works) used for column work was stirred in distilled water and allowed to stand for about 25 minutes until the bulk of the silicic acid settled, leaving the fines in suspension. The turbid supernatant was poured off and additional water was added. The process was repeated until the silicic acid settled uniformly and a clear supernatant was obtained. Excess water was removed by suction filtration and the silicic acid washed once with methanol. After the removal of the residual solvent by suction, the silicic acid was activated at 105 C over a 16 hour period.

Fifty grams of the activated silicic acid were slurried in chloroform and poured in a 2.5 × 40 cm column provided with a fritted glass and a teflon stopcock.

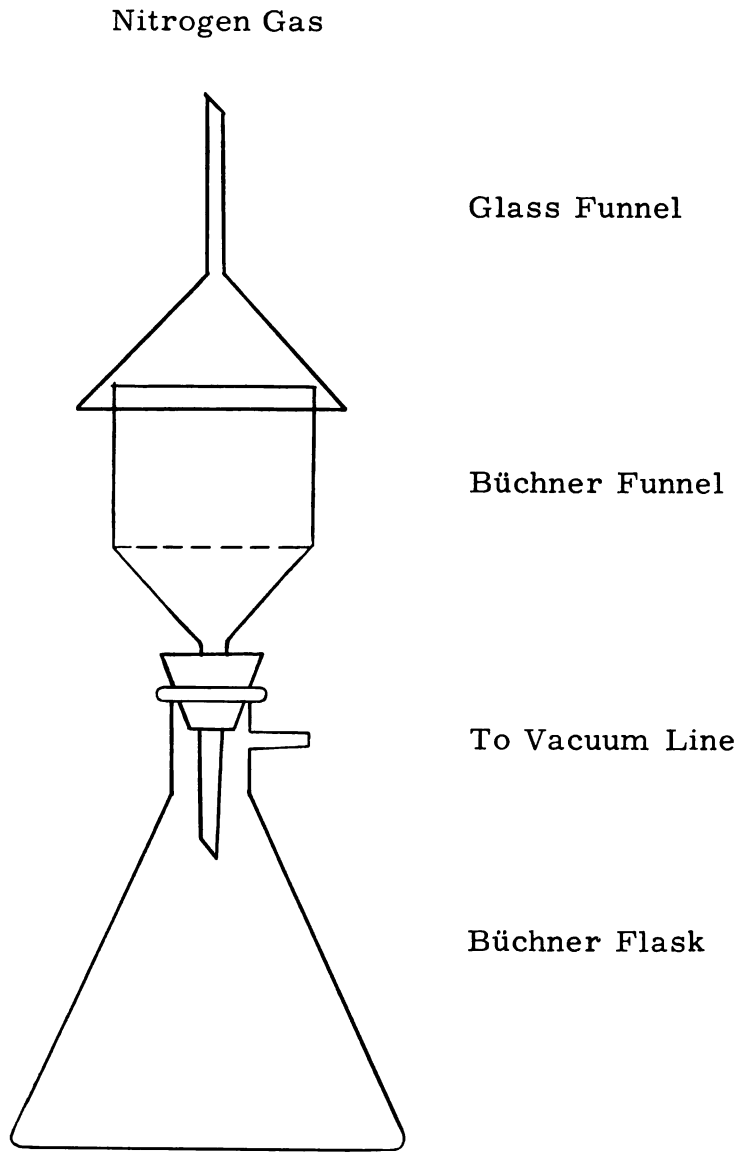


Fig. 1. Schematic diagram of apparatus used for filtration of lipid samples.

The diethylaminoethyl cellulose was treated according to the method of Rouser et al., (1961; 1963). The material was placed in a Büchner funnel with Whatman No. 1 filter paper and washed with 3 bed volumes of 1N hydrochloric acid and by distilled water until the filtrate was neutral. This was followed by 3 bed volumes of 1N potassium hydroxide and then with distilled water. This constituted one washing cycle, which was repeated 3 times. Excess moisture was removed by application of suction and the diethylaminoethyl cellulose was air dried prior to storage.

To prepare the column, approximately 15 g of diethylaminoethyl cellulose were slurried in glacial acetic acid. The column material was added to the column in 5 or 6 portions and packed uniformly after each addition by tamping with a glass rod. Before using the column the acid was removed by passing methanol through the column until a neutral eluate was obtained. Chloroform was passed through the column to remove the methanol.

Separation of phospholipid fractions

The phospholipid fractions used in the study were obtained using the diethylaminoethyl cellulose method of Rouser et al. (1963).

The lipid extract was concentrated under a stream of nitrogen and then taken up with about 2 ml of chloroform. Elution was started with 9:1 (v/v) chloroform:methanol which elutes neutral

lipids, sphingomyelin, lecithin, cholesterol, lysolecithin and cerebrosides. Elution of the phospholipids was monitored with the use of a molybdate test for phosphorous. The front fraction was collected in small tubes and a sample from each tube was tested for phosphorous. Collection of the front fraction was stopped when a negative molybdate test was obtained or when a positive ninhydrin test was obtained. Ninhydrin was used for detecting the free amino groups (Rouser et al., 1961) present in phosphatidyl ethanolamine, which is the second fraction. At this point, the solvent was changed to 7:3 (v/v) chloroform:methanol, which was used until a negative ninhydrin test was observed. Elution of the remaining phospholipids in the column was achieved with 4:1 (v/v) chloroform:methanol saturated with ammonium hydroxide.

To obtain the lecithin used for the study, the first fraction obtained from the diethylaminoethyl cellulose column was concentrated under a stream of nitrogen and introduced into the silicic acid column. Separation was made using the elution scheme of Kuchmak and Dugan (1965). The first fraction to be eluted consisted mainly of neutral lipids. The presence of lipid in the eluate was followed by the application of the Salkowski test (Kuchmak and Dugan, 1965). When a negative test was obtained, the volume of the eluant was measured and this was designated as a unit volume. An equal volume of chloroform was added to insure complete removal of the

neutral lipids. The cerebrosides were then eluted with one volume of 1:9 (v/v) methanol:chloroform followed by 1.5:5.6 (v/v) methanol: chloroform. Collection was terminated when a negative Salkowski test was obtained. Lecithin, the next component, was eluted with 3.5:5.6 (v/v) methanol:chloroform while sphingomyelin was eluted with methanol. Elution of the last fractions was followed by thin-layer chromatography and molybdate spray instead of the tube test because of the erratic results obtained earlier. The aliquot used for the test was concentrated and spotted on a thin-layer chromatography plate and then sprayed with molybdate solution according to Dittmer and Lester (1964).

The fractions collected were concentrated by warming and evaporating the solvent under nitrogen and the purity was assessed using thin-layer chromatography. The different fractions were spotted and the thin-layer chromatographic plate developed with 65:25:4 (v/v) chloroform:methanol:water solvent system.

Purification of Reagents

The 2,4-dinitrophenylhydrazine used in the experiment was purified by the method of Schwartz and Parks (1961). Carbonyl-free hexane was added to 2.5 g 2,4-dinitrophenylhydrazine and the mixture was refluxed for 15 minutes. While still warm, the partially purified 2,4-dinitrophenylhydrazine was removed by filtration and refluxed once more with another 500 ml of fresh carbonyl-free

hexane. The purified 2, 4-dinitrophenylhydrazine was air dried and then stored in a brown bottle.

The p-nitrophenylhydrazine was purified by double crystallization in ethyl alcohol (Pries and Böttcher, 1965).

Carbonyl-free solvents were prepared by the use of 2, 4-dinitrophenylhydrazine. For benzene, chloroform and hexane, 2, 4-dinitrophenylhydrazine and trichloroacetic acid were used (Henick et al., 1959) at the rate of 5 g and 1 g per liter of solvent respectively. The mixture was refluxed for 1 hour and the solvent distilled off. The first 20 ml or so were discarded and the remainder collected in a brown bottle. In the case of methanol and ethanol, 2 ml of phosphoric acid were used instead of trichloroacetic acid (Schwartz and Parks, 1961).

Gas Chromatography

The gas chromatographic analysis was performed with a dual column Beckman Gas Chromatograph Model GC-5 with a thermal conductivity detector.

The columns were packed with 20% diethylene glycol succinate with 1% phosphoric acid on 80-100 mesh Chromosorb W. The required amount of high temperature stabilized diethylene glycol succinate and phosphoric acid were dissolved in acetone. Chromosorb W was added to the acetone mixture and stirred

thoroughly. The solvent was removed with the use of a rotary vacuum evaporator.

After the columns were packed, they were conditioned at 220 C for 48 hours. The columns were connected and the chromatograph was operated using the following set of conditions: helium flow, 30 ml per minute; column temperature, 190 C; injection port temperature, 210 C; feed line temperature, 230 C; detector temperature, 250 C; and the detector current, 350 ma.

Chemical Methods

Iodine-uptake method

The method used was that developed by Williams et al. (1962). Aliquots containing 1 umole of plasmalogen were placed in uncapped vials that were fitted for screw caps. The solvents were evaporated under a stream of nitrogen and the residue was taken up in 0.9 ml methanol. Whenever necessary, the mixture was heated in a 70 C water bath to completely dissolve the sample. Then 3.2 ml of 0.094 M citrate buffer were added to all tubes. A volume of 0.9 ml 3 M potassium iodide solution followed by 0.5 ml of 0.005 M iodine in potassium iodide solution were added to the investigated samples. The mixture was shaken and incubated for 45 minutes in the capped vials, after which the unreacted iodine was extracted by shaking the mixture with 10

ml butyl acetate. The absorbance of the butyl acetate layer was determined at 365 mu using a Beckman DU spectrophotometer.

P-nitrophenylhydrazine method

The method used was essentially that of Wittenberg et al. (1956). Samples were placed in bottles fitted with screw caps and the solvent removed by evaporating under a stream of nitrogen. The sample was dissolved in 8 ml of carbonyl-free methanol and 1 ml of 1N sulfuric acid was added followed by 1 ml of 0.02M p-nitrophenylhydrazine. The mixture was incubated for 20 minutes at 70 C on a water bath. The hydrazones formed were extracted twice with 5 ml petroleum ether. The combined extracts were washed twice with 10 ml of distilled water. An aliquot was taken and evaporated to dryness under subdued light by using a stream of nitrogen. The hydrazone residue was taken up with ethyl alcohol and the absorbance measured at 395 mu. A blank containing all of the reagents was used.

2, 4-dinitrophenylhydrazine method

Aliquots of samples for plasmalogen determination were placed in test tubes and the solvents removed using a stream of nitrogen. The residue was taken up with 0.2 ml of 1:1 chloroform: methanol. One ml of 2, 4-dinitrophenylhydrazine reagent was added

and the mixture was incubated in the dark for 1.5 hours with occasional shaking. After the required time, 10 ml of distilled water were added and the reaction mixture extracted once with 10 ml and twice with 5 ml portions of carbonyl-free hexane. The combined extracts were washed with distilled water to remove excess reagents and then dried over anhydrous sodium sulfate. The absorbance was determined using a spectrophotometer at 338 mu. The concentration was determined using the molar extraction coefficient of 2.15 $\times 10^4$.

Phosphorous Determination

The procedure of Parker and Peterson (1965) was followed for phosphorous determination. This allowed the determination of phospholipid from thin-layer chromatography scrapings (Rouser et al., 1966; Rhee and Dugan, 1967) without eluting the lipids from the silica gel.

The phospholipid spot on the thin-layer chromatography was aspirated into a Kjeldahl flask containing 0.9 ml perchloric acid as a trap. (See diagram Fig. 2.) Equal areas of silica gel were removed from every spot as much as possible. Digestion of the sample was carried out using a well-regulated heater which insured uniform heating and minimum evaporation of the acid. After the flasks were cooled, the necks were rinsed with 5 ml of distilled

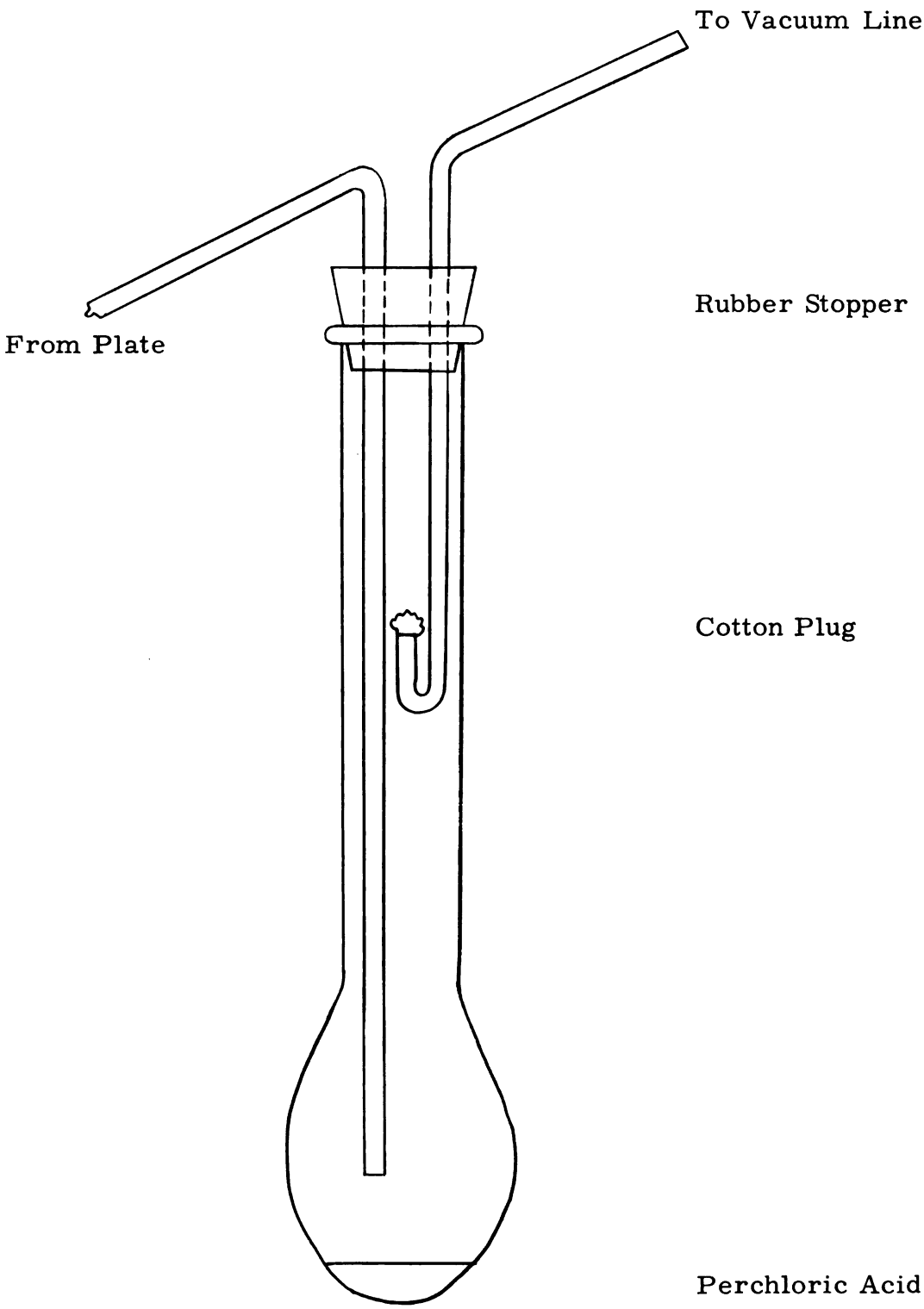


Fig. 2. Diagram of apparatus used for aspirating sample spots from thin-layer chromatographic plates.

water. The contents of the flasks were mixed with 1 ml of 2.5% ammonium molybdate and 1 ml of 10% ascorbic acid. Another 2 ml of distilled water were added and the mixture heated in a boiling water bath for 5 minutes. The solution was centrifuged to bring down any suspended silica gel and then the transmission of the clear supernatant was measured at 720 mu. The concentration of phosphorous in the sample was determined by measuring the absorbance of a series of standard phosphorous solutions and then the values obtained for the sample were referred back to the graph of concentration versus absorbance of the standard solutions.

Methylation Procedures

Boron trifluoride-methanol

This procedure was based on the report of Morrison and Smith (1964). The lipid sample was placed in vials fitted for screw caps and the solvents evaporated under nitrogen. A 14% solution of boron trifluoride in anhydrous methanol was added to the sample at the rate of 1 ml per 8 to 16 mg sample. The mixture in capped vials was placed in a boiling water bath for 30 minutes and cooled in an ice bath. The BF_3 was neutralized with 1 ml of 0.5N sodium hydroxide and the solution extracted with three 5 ml portions of petroleum ether. The combined extracts were washed once with water

and dried over anhydrous sodium sulfate and concentrated in a centrifuge tube.

Hydrochloric acid-methanol

An aliquot of the lipid solution was placed in a 200 ml round bottom flask. The sample was transferred into 20 ml of methanolic hydrochloric acid after removal of the original solvent. The mixture was refluxed for 2 hours, cooled, and then extracted once with 15 ml and twice with 10 ml portions of petroleum ether. All the extracts were combined, washed with water, and dried over anhydrous sodium sulfate.

Low temperature-sulfuric acid method

The lipid sample was dissolved in 25 ml of diethyl ether and the mixture cooled to -60 C. Under constant stirring, 2 ml of concentrated sulfuric acid were added at the rate of 1 ml per minute. The temperature was allowed to come up to -10 C in 10 minutes and then cooled again to -60 C. Methanol was added in the amount of 15 ml per sample. The reaction was allowed to proceed for another 20 minutes and was stopped by the addition of 13 ml of 35% KOH in methanol. The reaction mixture was transferred into a 500 ml separatory funnel with about 250 ml of distilled water. Extraction

of methyl esters was accomplished with one 15 ml and two 10 ml portions of petroleum ether.

Saponification Procedure

To recover the dimethylacetals formed in the methylation step, a portion of the methylated samples from the three methylation procedures was saponified (Farquhar, 1962). The samples were placed in round bottom flasks and the solvents removed by evaporation. Methanolic KOH was added in the amount of 20 ml per sample. After refluxing for 2 hours at 85 C, the mixture was cooled and extracted three times with 5 ml of petroleum ether. The extracts were washed with 4:1:0.1 (v/v) H_2O: ethyl ether:0.3 N NaOH and dried over anhydrous Na_2SO_4. The dimethylacetals recovered were stored under nitrogen until used for gas chromatographic analysis.

RESULTS AND DISCUSSION

Chemical Methods

The modified 2, 4-dinitrophenylhydrazine procedure for plasmalogen determination appeared to be a promising method. Rhee et al. (in press) showed that cleavage of the vinyl ether bond appeared to be complete and that the reagent acted specifically on the vinyl ether bond without affecting the acyl bonds.

In order to determine the efficiency of the method, the recovery was tested using standard aldehydes. Known amounts of a series of standard aldehydes ranging from C_{10} to C_{18} were treated with the reagent to form hydrazones. The concentrations of the hydrazone solutions were determined by the use of a spectrophotometer. Low recovery was noted with long chain fatty aldehydes. This was shown later to be due to impurities present in the samples. To determine the impurities, samples of the aldehydes were methylated and analyzed on a gas chromatograph. Extra peaks which correspond to certain fatty acid esters showed up in the chromatograms. Because of this, correction factors were introduced in some of the recovery values calculated. Purity was calculated from the peak areas of the different components in the chromatogram.

The values obtained after introducing the corrections are shown in Table 1. The recoveries compare favorably with those reported by Schwartz et al. (1963) using pure aldehydes passed through a column packed with 2, 4-dinitrophenylhydrazine-treated celite.

Table 1. Recovery of the standard aldehydes as hydrazones using the 2, 4-dinitrophenylhydrazine method.

Carbon Number of Aldehyde	Amount Used (umole)	Amount Recovered (umole)	% Recovery
C_{10}	1	0.952	95.20[a]
C_{12}	1	0.975	97.50[a]
C_{14}	1	0.734	99.37[b]
C_{16}	1	0.847	101.00[b]
C_{18}	1	0.940	98.87[b]

[a]Based on original sample.

[b]Corrected with purity calculated from gas chromatographic analysis of the standard aldehyde.

This is an advantage compared to the p-nitrophenylhydrazine method where recovery is quite low for short chain aldehydes (Wittenberg et al., 1956).

To determine the reliability of the method, comparison was made with two other methods which are now being used, namely,

the iodine-uptake and the p-nitrophenylhydrazine methods. Two pure phospholipid fractions from beef brain and liver were used for the study. The use of pure fractions and crude extracts would enable us to ascertain the behavior under conditions using natural samples.

Table 2 shows the results obtained. It may be seen that the values obtained by the 2, 4-dinitrophenylhydrazine method are slightly higher than those by the other two methods, and apparently it is more consistent. This is especially true of the pure phospholipid fractions, which is to be expected, since the samples were pure and therefore not subject to the difficulties encountered with total extracts. The presence of large amounts of neutral lipids and other non-lipid materials in the total extract may have affected the results of the determinations. Neutral lipids reduce the solubility and dispersion of the phospholipid. With p-nitrophenylhydrazine the low results may be attributed to incomplete reaction and loss of hydrazones in the extraction step or changes due to the presence of light and oxygen during the analyses.

Statistical treatment of the results showed that the differences between the values obtained for the 2, 4-dinitrophenylhydrazine and the two other methods were not significant even at the 90% confidence level except in the case of phosphatidyl choline where a

Table 2. Comparison of the 2,4-dinitrophenylhydrazine method (2,4-DNPH), p-nitrophenylhydrazine method (p-NPH), and the iodine-uptake procedure for plasmalogen determination.

Sample	Moles Plasmalogen/l Lipid Solution[a]		
	2,4-DNPH	p-NPH	Iodine-Uptake
PE[b] (beef brain)	$(2.170 \pm 0.005) \times 10^{-3}$	$(2.158 \pm 0.008) \times 10^{-3}$	$(2.164 \pm 0.014) \times 10^{-3}$
PC[b] (beef brain)	$(0.603 \pm 0.010) \times 10^{-4}$	$(0.560 \pm 0.020) \times 10^{-4}$	$(0.566 \pm 0.170) \times 10^{-4}$
Total beef liver lipids	$(8.618 \pm 0.031) \times 10^{-4}$	$(8.460 \pm 0.051) \times 10^{-4}$	$(8.570 \pm 0.031) \times 10^{-4}$
Total beef brain lipids	$(2.256 \pm 0.031) \times 10^{-4}$	$(2.205 \pm 0.046) \times 10^{-4}$	$(2.197 \pm 0.037) \times 10^{-4}$

[a]Each value represents the average of three determinations.

[b]PE: phosphatidyl ethanolamine; PC: phosphatidyl choline.

[c]Spectrophotometric determination.

significant difference was obtained between the 2, 4-dinitrophenylhydrazine and p-nitrophenylhydrazine methods.

Since the differences obtained were not significant, it was felt that the differences may be due only to the presence of free aldehydes resulting from autoxidation or cleavage of the plasmalogen. Two samples were treated with a saturated solution of sodium bisulfite before analyzing for plasmalogen. Sodium bisulfite has the ability to react with any free aldehyde in the sample which could interfere with the analysis.

Table 3 gives the results of the analysis. This showed that there were negligible quantities of free aldehydes in the samples. This was also confirmed by the gas chromatographic patterns of the dimethylacetals obtained from the same sample lot. Only three major dimethylacetal peaks were found which corresponded to aldehydes with chain length greater than twelve carbon atoms. Since it is generally considered that carbonyl compounds with carbon numbers lower than twelve result from lipid oxidation, the possibility of the presence of free aldehydes from autoxidation was discounted.

A feature of the method is the ability to check the results of the 2, 4-dinitrophenylhydrazine method by phosphorous assay. This was possible since the liberation of the aldehyde molecule from the plasmalogen results in the formation of a lysocompound, while the

diacyl analogue is not acted upon by the reagent. On thin-layer chromatography, the lysocompound behaves differently from the diacyl compound. It is adsorbed more strongly and travels more slowly. By scraping the spots from the plate and determining the phosphorous contents, one may determine the relative amount of plasmalogen in the sample. This results from the assumption of 1 mole phosphorous per mole of phospholipid which permits the relation:

$$\% \text{ plasmalogen} = \frac{100 \times \text{phosphorous of lysocompound}}{\text{phosphorous of lysocompound} + \text{phosphorous of diacyl compound}}$$

Table 3. Effect of sodium bisulfite treatment on apparent plasmalogen content.[a]

Lipid Sample	Moles Plasmalogen/l Lipid Solution	
	Control	$NaHSO_3$ Treated
PE[b] (Beef Brain)	2.171×10^{-3}	2.167×10^{-3}
Liver (Beef)	2.960×10^{-4}	2.950×10^{-4}

[a]Each value represents the average of three determinations.

[b]Phosphatidyl ethanolamine.

The results of the phosphorous and 2, 4-dinitrophenylhydrazine analyses in Table 4 closely match each other while the

Table 4. Plasmalogen concentration[a] of phosphatidyl choline and phosphatidyl ethanolamine by 2, 4-dinitrophenylhydrazine (2, 4-DNPH) and p-nitrophenylhydrazine (p-NPH) methods and by phosphorous determination.

Sample	Moles Plasmalogen/l Lipid Solution			
	P-method	2, 4-DNPH	P-method	p-NPH
PE[b] (beef brain)	2.162×10^{-3}	2.169×10^{-3}	2.063×10^{-3}	2.150×10^{-3}
PC[b] (beef brain)	0.594×10^{-4}	0.603×10^{-4}	0.560×10^{-4}	0.537×10^{-4}

[a]Each value represents the average of three determinations.

[b]PE: phosphatidyl ethanolamine; PC: phosphatidyl choline.

phosphorous and p-nitrophenylhydrazine analyses showed a slight discrepancy. This was found to be due to incomplete extraction of the mixture. The reaction was carried out in alcoholic solution. After the required reaction time, the mixture was extracted with hexane to recover the hydrazones and the unreacted lipids. During the process not all of the diacyl compounds and the lysocompounds from the plasmalogens went with the extract. This was especially true of the lysocompounds which apparently have a greater affinity for the alcoholic medium. Evidence for this was shown when the extracted medium was spotted on a thin-layer chromatography plate and developed by the appropriate solvent system. Spots corresponding to the lyso- and diacyl compounds showed up.

Methylation and Gas Chromatography

Farquhar (1962a) suggested the use of gas-liquid chromatography for the determination of plasmalogens from phospholipids. From the percentage composition of the methyl esters and dimethylacetals and their individual molecular weights, it is possible theoretically to calculate the total number of moles of plasmalogens in a given sample. In view of this, a study was made of the use of the low temperature methylation procedure of McGinnis and Dugan (1965) for plasmalogen determination and compared with hydrochloric acid-methanol and boron trifluoride-methanol transesterification methods.

Hog brain total lipid extracts were methylated using the three methods. Each methylated sample was evenly divided and

one-half was saponified to recover dimethylacetals for analysis. The methyl esters and dimethylacetals were identified using a plot of the logarithm of retention time against the carbon number obtained from standard methyl esters and dimethylacetals.

The percentage composition of the methyl esters and dimethylacetals calculated from peak height and retention time (Bartlet and Iverson, 1966) are shown in Tables 5 and 6. The results were comparable in all three cases.

Table 5. A comparison of three different methylation techniques for fatty aldehyde analysis of hog brain plasmalogens.

Carbon Number of DMA Aldehyde	Percentage Composition		
	BF_3-Methanol	HCl-Methanol	Low Temperature-H_2SO_4
16:0	29.94	25.13	29.03
?	0.77	2.74	trace
16:1	4.71	2.74	1.61
18:0	32.94	28.20	32.26
18:1	34.64	38.14	37.10

Calculation of the percentage of plasmalogens gave erratic results. Because of this, the methylated samples were checked by spotting on thin-layer chromatographic plates and developing in 65:25:4 (v/v) chloroform:methanol:water for the separation of

Table 6. Percentage composition of fatty acids from hog brain lipids found by using three different methylation procedures.

Carbon Number of Methyl Esters	Percentage Composition		
	BF_3-Methanol	HCl-Methanol	Low Temperature-H_2SO_4
14:0	1.34	0.80	0.98
16:0	16.78	15.35	18.48
18:0	22.70	21.84	28.61
18:1	28.81	26.62	29.92
18:2	0.17	0.50	0.17
20:0	0.03	trace	0.30
18:3	4.08	2.52	3.25
20:2	0.34	1.09	0.28
20:3	0.79	0.93	0.31
22:0	1.02	1.24	0.38
20:4	6.10	5.98	5.81
22:2	0.87	2.67	.52
24:0	1.13	1.79	.38
?	6.23	2.92	4.76
24:1	0.85	7.56	4.83
26:0	8.70	7.98	5.96

phospholipids and 90:10:1 (v/v) petroleum ether:diethyl ether:acetic acid to resolve the neutral lipids and sterols. When the plates were sprayed, both ninhydrin and molybdate positive spots appeared which should not have been present if the esterification reaction were complete. A complete esterification reaction would yield only dimethylacetals and methyl esters in the extracts and the water soluble glycerol, partial glycerides and glycerophosphate residue would remain in the aqueous medium.

A study was made of the conditions of reaction in order to effect better esterification specifically of the low temperature-sulfuric acid method. From the original report of Newman (1941), it was postulated that an organic acid in the presence of sulfuric acid and at low temperatures forms methyl esters through the following steps:

$$RCOOH + 2H_2SO_4 \rightleftharpoons RCO^+ + 2HSO_4^- + H_3O^+$$

$$RCO^+ + ROH + HSO_4^- \rightleftharpoons RCOOR + H_2SO_4$$

He showed that it involves equilibrium and that the presence of the bisulfate in the reaction reduced the yield of methyl esters. In view of this, the mode of addition of the alcohol was slowed down in the hope that it could drive the reaction to completion. However, this was not the case and the results were still the

same. The addition of the potassium hydroxide was not studied since its only apparent function was to neutralize the acids in order to prevent the reversion of the fatty esters to the acids form. Since the original procedure called for 100% sulfuric acid and only 98.0% acid was being used, the acid was changed to 100% sulfuric acid. The absolute sulfuric acid was prepared according to the method of Newman (1941) and methylation conducted, but the yield was still unsatisfactory. Finally the amount of the acid was varied and so was the time of reaction. One methylation was made with double the amount of acid while another was run for one hour. In another trial the temperature was raised from the original low temperature of -60 C to 0 C keeping other conditions the same. With boron trifluoride-methanol, the sample was dissolved in about 1 ml of benzene and the mixture refluxed for 1 hour, while for the hydrochloric acid-methanol treated sample the reaction was conducted for 2.5 hours.

Methylated samples of hog brain total lipids were then spotted on thin-layer chromatographic plates and developed in the solvent systems used in the previous trial. Upon spraying, the chromatograms appeared as shown in Fig. 3 and Fig. 4. The chromatograms indicated incomplete methylation at the low temperature of the dry ice-acetone bath and also at 0 C. Incomplete methylation also occurred in these samples methylated with

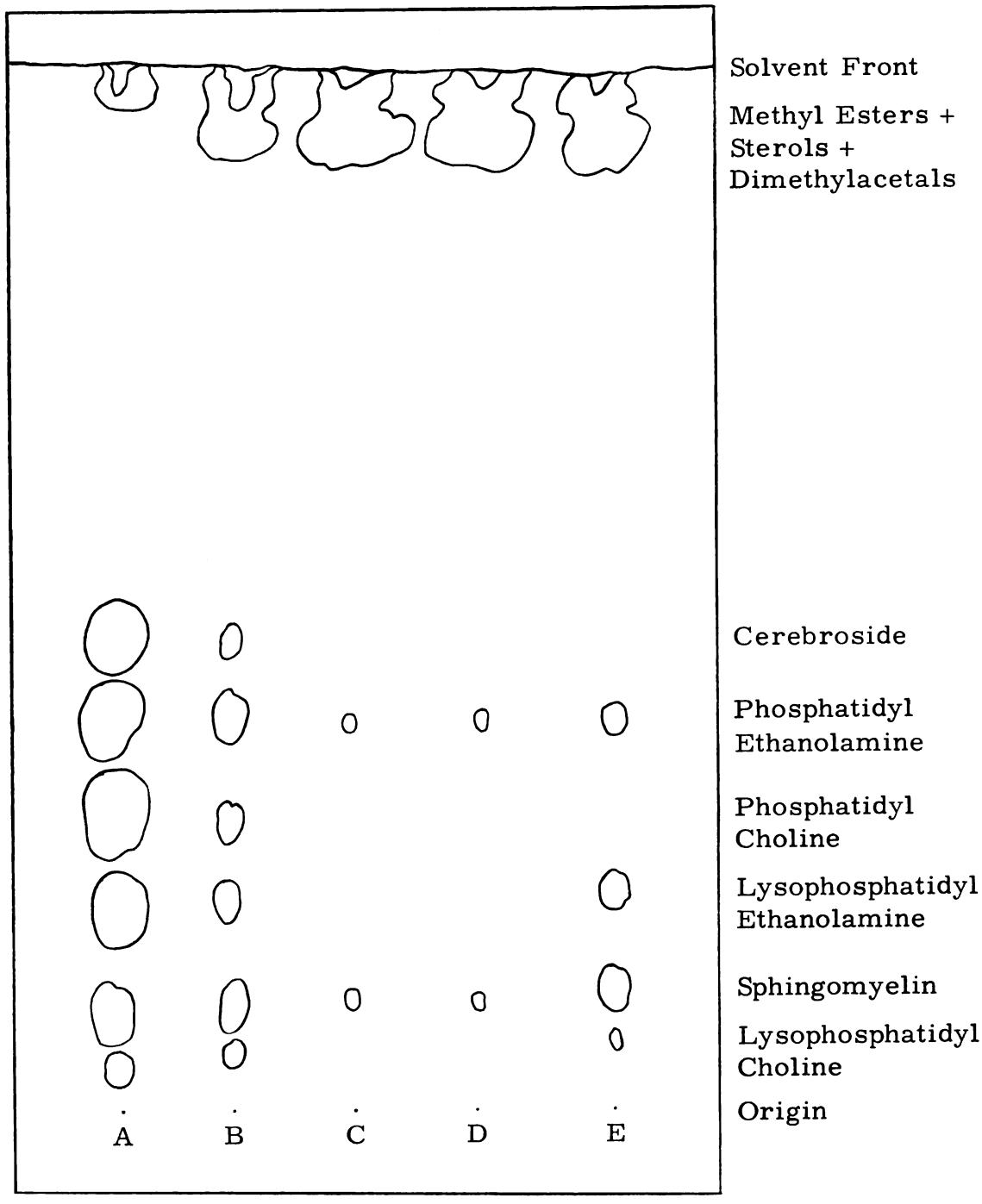


Fig. 3. Thin-layer chromatographic separation of phospholipid components from methylated hog brain lipids. Solvent system: 65:25:4 (v/v) chloroform:methanol:water. Treatments: A. Control, B. Ice temperature-H_2SO_4 (20 min.), C. Low temperature-H_2SO_4 (1 hr.), D. Low temperature-H_2SO_4 (4 ml), E. Low temperature-H_2SO_4 (20 min.).

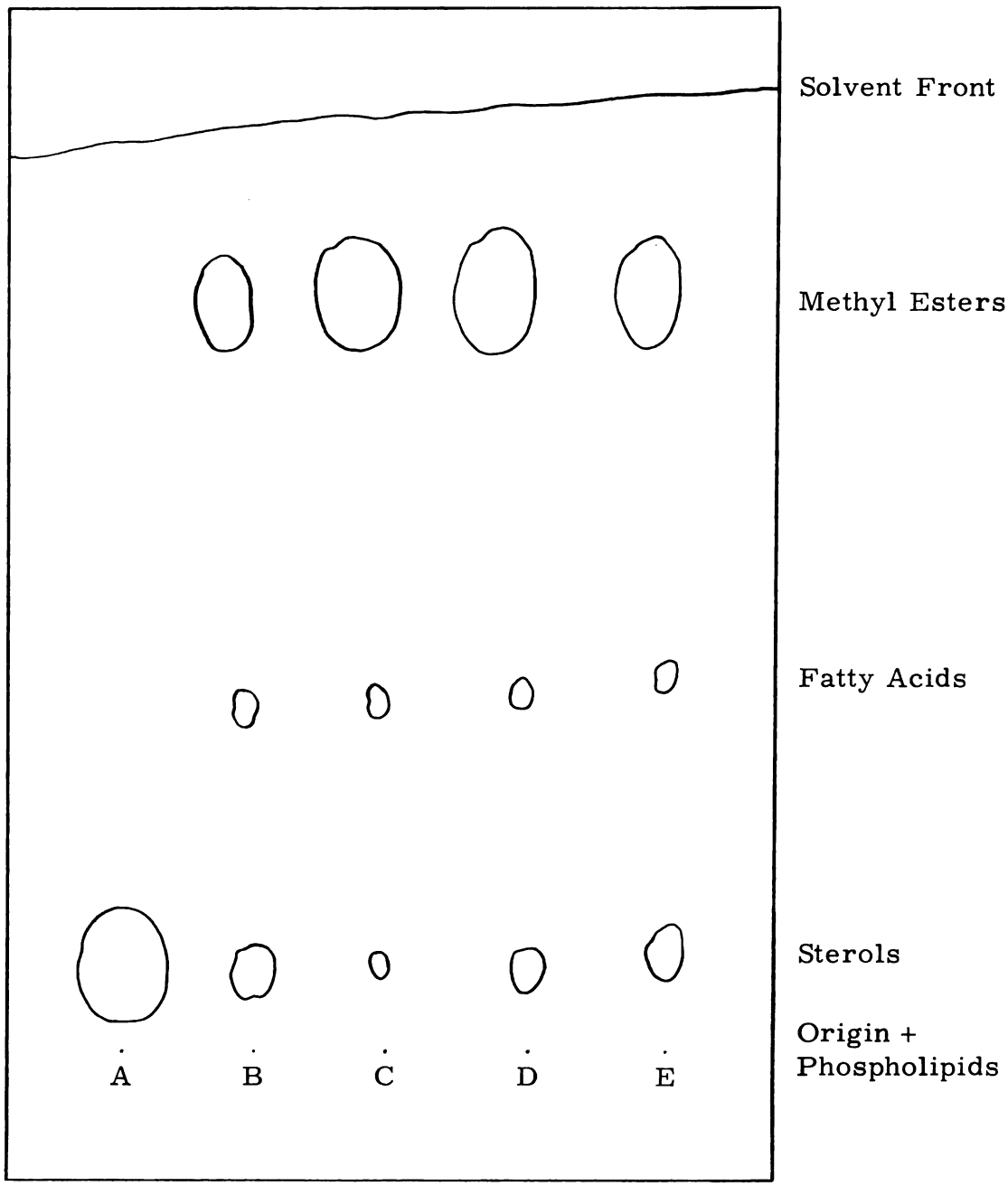


Fig. 4. Thin-layer chromatographic separation of neutral lipid components from methylated hog brain lipids. Solvent system: 90:10:1 (v/v) petroleum ether:diethyl ether:acetic acid. Treatments: A. Control, B. Ice temperature-H_2SO_4 (20 min.), C. Low temperature-H_2SO_4 (1 hr.), D. Low temperature-H_2SO_4 (4 ml), E. Low temperature-H_2SO_4 (20 min.).

hydrochloric acid-methanol. The boron trifluoride-methanol showed almost complete interesterification, as did the methylation at low temperature for 1 hour and with 4 ml of sulfuric acid except for traces of unreacted sphingomyelin. Apparently the time of reaction and the amount of acid are important for completeness of reaction. It is believed that with proper amounts of acid and sample, the reaction may be made to go to completion. In the trials that were performed where the amount of phospholipid was measured in terms of the phosphorous content of the sample, it was shown that about 2 mg of phosphorous, in terms of phospholipids, would be good enough for the original condition of reaction. In terms of sphingomyelin, which seems to be resistant to interesterification, it should not exceed more than 5% of the total phospholipids in order to insure complete methylation.

SUMMARY AND CONCLUSIONS

The method for plasmalogen determination proposed by Rhee et al. (in press) which makes use of 55% phosphoric acid and 2, 4-dinitrophenylhydrazine was studied. It was shown previously that cleavage of the vinyl ether bond is quantitative and that the reaction involved is specific for vinyl ether. A series of standard aldehydes varying from C_{10} to C_{18} was used to determine the yield as a function of chain length. The recovery was found quantitative for the whole range of aldehydes used after correcting for the purity, which was determined by gas chromatography

Comparison of the method was made with the iodine-uptake and p-nitrophenylhydrazine methods. Total lipid extracts of beef brain and liver were used together with phosphatidyl ethanolamine and phosphatidyl choline from beef brain. More consistent results were obtained using the proposed method. The concentrations found were slightly higher for the 2, 4-dinitrophenylhydrazine than the other two methods, although the differences were not significant.

Using liver extract and phosphatidyl choline as samples, the determination was repeated after treating the samples with a

saturated solution of sodium bisulfite to remove whatever free aldehydes that might be present. The results showed almost no differences in the values obtained before and after treatment, indicating that the differences found by the various methods are not due to free aldehydes in the sample.

Subsequent to treatment with 2, 4-dinitrophenylhydrazine of a phospholipid fraction containing plasmalogen, it is possible to use phosphorous assay for the analysis of plasmalogen after separation of the lyso- and diacyl phospholipid by thin-layer chromatography. Using phosphatidyl ethanolamine and phosphatidyl choline, it was shown that phosphorous determination agrees well with the 2, 4-dinitrophenylhydrazine method of determining plasmalogens.

The use of gas-liquid chromatography for plasmalogen determination was studied in relation to three methylation procedures: the low temperature-sulfuric acid, hydrochloric acid-methanol, and boron trifluoride-methanol methods. After methylating the hog brain sample, it was divided evenly and one portion was saponified to recover the dimethyl acetal in the sample. The gas-liquid chromatography data showed almost comparable analysis for fatty acids and aldehydes in the three methods. Tests to determine the completeness of reaction using thin-layer chromatography showed incomplete interesterification in all three methods.

In order to be able to use gas liquid chromatography for analysis of plasmalogen aldehydes and fatty acids, the methylation should be complete. The conditions of reaction were studied. Some factors were found to have no effect on the extent of the reaction. For the low temperature-sulfuric acid, the amount of acid was found to be critical as well as the time of reaction. Raising the temperature to 0 C did not influence the yield. Even sphingomyelin, which showed resistance in the other two methods, was hardly detectable in the low temperature methylation with modified methylation conditions. Improved yields were obtained with hydrochloric acid-methanol when the reaction time was increased, while for the boron trifluoride-methanol method the yield was increased when the sample and the reagent were refluxed instead of being incubated in a screw capped vial in a water bath.

The spectrophotometric method of determining plasmalogen by the 2, 4-dinitrophenylhydrazine and phosphoric acid mixture could be used conveniently and accurately with any lipid sample. In cases wherein the plasmalogen content of the individual lipid fractions are desired, one may employ the method of Owen (1966) or, in the case wherein preliminary separations have been accomplished or pure phospholipids are involved, the 2, 4-dinitrophenylhydrazine may be used or the phosphorous assay, especially if the condition of the sample is not known. In combination with methylation and gas

chromatography, it is a powerful tool for the structural analysis of plasmalogens. From the 2, 4-dinitrophenylhydrazine treated sample, it is possible to determine the fatty acid composition of the plasmalogen by determining the fatty acids in the diacyl analogue and the lysocompound formed from plasmalogen.

With this combination of analytical tools, it may be possible to study the role that plasmalogen plays in biological materials, and in dehydrated meat and other foods in relation to browning and rancidity development. In addition, one may also utilize the procedures in studying the function of plasmalogen in relation to fat metabolism and other metabolic processes.

LITERATURE CITED

Bligh, B. G., and W. J. Dyer. 1958. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911-917.

Bartlet, J., and C. Iverson. 1966. Estimation of fatty acid composition by gas chromatography using peak height and retention time. J. Assoc. Offic. Agric. Chem. 49:21-27.

Braude, F. A., and E. R. H. Jones. 1945. Studies on light absorption. II. 2, 4-Dinitrophenylhydrazones. J. Chem. Soc. 1945:498-504.

Camejo, G., M. M. Rapport and G. A. Morril. 1964. The structure of plasmalogens. VII. Analysis of mammalian liver lipids and the interference of vitamin A in the analysis. J. Lipid Res. 5:75-80.

Christl, H. 1953. Quantitative bestimmung der acetalphosphatide in organen. Z. Physiol. Chem. 293:83-84.

Cropper, F. R., and A. Heywood. 1953. Analytical separation of methyl esters of C_{12}-C_{22} fatty acids by vapor phase chromatography. Nature 172:1101-1102.

Custer, J. J., and S. Natelson. 1949. Spectrophotometric determination of microquantities of iodine. Anal. Chem. 21:1005-1009.

Debuch, H. 1957. Nature of the linkage of the aldehyde residue in the natural plasmalogen. Biochem. J. 67:27P.

Dittmer, J., and R. L. Lester. 1964. A simple specific spray for the determination of phospholipid on thin-layer chromatography. J. Lipid Res. 5:126-127.

Downing, D. T., Z. H. Kranz and K. E. Murray. 1960. Studies in waxes. XIV. An investigation on the aliphatic constituents of hydrolyzed wool wax by gas chromatography. Australian J. Chrom. 13:80-92.

Erlich, G., H. E. Taylor and H. Waelsch. 1948. The effect of surface active agents on the fuchsin reaction of fatty aldehydes. J. Biol. Chem. 173:54-61.

Eichberg, J. J., R. Gilbertson and M. L. Karnovsky. 1961. Neutral plasmalogen analogous to neutral triglyceride. J. Biol. Chem. 236:PC15.

Farquhar, J. W. 1962. Identification and GLG behavior of plasmalogens and their acetal, alcohol and acetylated alcohol derivatives. J. Lipid Res. 3:21-30.

Farquhar, J. W. 1962a. Human erythrocyte phosphoglyceride. I. Quantitative analysis of plasmalogen fatty acids and fatty aldehydes. Biochem. Biophys. Acta. 60:80-89.

Feulgen, R., and T. Bersin. 1939. Zur kenntnis des plasmalogen. IV. Mitteilung eine neuartige gruppe von phosphatiden (acetalphosphatide). Z. Physiol. Chem. 260:217-245.

Feulgen, R., and K. Imhauser. 1927. Die quantitative bestimmung des plasmals in serum. Biochem. J. 81:30-48.

Feulgen, R., and K. Voit. 1924. Über einen weitvertreiteten festen aldehyde seine enstehung aus einer vorstufe, sein mikrochemischer and mikroskopisch-chemisher nachweis und die wege zu zeiner preparaten darstellung. Pflüg. Arch. Ges. Physiol. 206:389-410.

Folch, J., M. Lees and G. H. Sloane-Stanley. 1957. A simple method of the isolation and purification of the total lipids from animal tissues. J. Biol. Chem. 226:497-509.

Gottfried, E. L., and M. M. Rapport. 1962. The biochemistry of plasmalogen. I. Isolation and purification of phosphatidyl choline, a pure native plasmalogen. J. Biol. Chem. 237:320-330.

Gray, G. M. 1958. The structure of plasmalogen of ox heart. Biochem. J. 70:425-431.

Gray, G. M., and M. G. MacFarlane. 1958. Separation and composition of the phospholipids of ox heart. Biochem. J. 70: 409-413.

Henich, A. S., M. F. Benca and H. J. Mitchell. 1959. Estimating carbonyl compounds in rancid fats and feeds. J. Am. Oil Chem. Soc. 31:38-43.

Hornstein, I., J. A. Alford, L. E. Elliot and P. F. Crowe. 1960. Determination of free fatty acids in fats. Anal. Chem. 32:540-542.

Imhauser, K. 1927. Über das vorkommen des plasmalogen. II. Mitteilung:Uber das vorkommen des plasmalogen bei tierens. Biochem. Z. 186:360-375.

Jackson, R. B. 1966. Gas-liquid chromatography of volatile fatty acids from formic acid to valeric acid. II. The instability of silicone oil-fatty acid stationary phases. J. Chrom. 22:251-257.

James, A. T., and A. J. P. Martin. 1956. Gas-liquid chromatography. The separation and identification of methyl esters of saturated and unsaturated acids from formic to n-octadecanoic acid. Biochem. J. 50:679-690.

Jones, L. A., J. C. Holmes and R. B. Seligman. 1956. Spectrophotometric studies of some 2, 4-dinitrophenylhydrazones. Anal. Chem. 28:191-196.

Katz, I., and M. Keeney. 1966. Quantitative microdetermination and isolation of plasmalogen aldehydes as 2, 4-dinitrophenylhydrazones. J. Lipid Res. 7:170-174.

Klenk, E., and E. Friedrichs. 1952. Über den aldehydgehalt der glycerinphosphatide verscheidener organe. Z. Physiol. Chem. 290:167-171.

Klenk, E., and H. Debuch. 1954. Zur kenntnis der acetalphosphatide. Z. Physiol. Chem. 296:179-188.

Kuchmak, M., and L. R. Dugan, Jr. 1963. Composition and positional distribution of fatty acids in phospholipids isolated from pork muscle tissue. J. Am. Oil Chem. Soc. 42:45-49.

Leupold, F., H. Büttner and K. Ranninger. 1953. Die bestimmung der acetalphosphatide im vollblut. Z. Physiol. Chem. 294:107-110.

Luddy, F. E., R. A. Barford and R. W. Reimenschneider. 1960. The transesterification of cholesterol esters to methyl esters without degradation and alteration of the sterols. J. Am. Oil Chem. Soc. 37:447-450.

Mahadevan, V., C. V. Viswanathan and W. O. Lundberg. 1966. Interconversion of fatty aldehydes and dimethylacetals at low temperatures. Lipids 1:349-352.

Marinetti, G. V., and J. Erbland. 1957. The structure of pig heart plasmalogens. Biochem. Biophys. Acta. 26:429-430.

Marinetti, G. V , J. Erbland and E. Stotz. 1958. The structure of pig heart plasmalogen. J. A. Chem. Soc. 80:1624-1628.

Martin, A. J. P., and R. L. M. Synge. 1941. A new form of chromatogram employing two liquid phases. I. Theory of chromatography. II. Application of the microdetermination of higher monoamino acids in proteins. Biochem. J. 35:1358-1368.

McGinnis, G. W., and L. R. Dugan, Jr. 1965. A rapid low temperature method for preparation for methyl esters of fatty acids. J. Am. Oil Chem. Soc. 42:305-309.

Metcalfe, L. D. 1960. Chromatography of unesterified fatty acids using polyester column treated with phosphoric acid. Nature 188:142-143.

Metcalfe, L. D., and A. A. Schmitz. 1961. The rapid preparation of fatty acid esters for gas chromatographic analysis. Anal. Chem. 33:363-364.

Metcalfe, L. D., A. A. Schmitz and J. R. Pelka. 1966. Rapid preparation of fatty acid esters from lipids for GLC analysis. Anal. Chem. 38:514-515.

Möckel, G. 1943. Topochemische untersuchungen über das verhalten des fettgewebes zur plasmalreaktion. Hoppe-Seyler Z. 277:135-146.

Morrison, W. R., and L. M. Smith. 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with BF_3-methanol. J. Lipid Res. 5:600-609.

Newman, M. D. 1941. A new method for the esterification of certain sterically hindered acids. J. Am. Chem. Soc. 63:2431-2435.

Norton, W. T. 1960. Potentiometric iodometric determination of plasmalogen. Biochem. Biophys. Acta. 38:340-342.

Orton, K. J. P., and P. V. McKie. 1961. The estimation of mixtures of paracetaldehyde and acetal. J. Chem. Soc. 109:184-190.

Owen, K. 1966. A two-dimensional thin-layer chromatographic procedure for the estimation of plasmalogens. Biochem. J. 100:354-359.

Parker, F., and N. D. Peterson. 1965. Quantitative analysis of phospholipid and phospholipid fatty acids from silica gel TLC. J. Lipid Res. 6:455-460.

Peng, C. Y. 1966. Some studies on the composition and structure of phospholipids in chicken muscle. Ph. D. Thesis. Michigan State University.

Pries, C., and C. J. F. Böttcher. 1965. The determination of free and bound aldehydes in lipid fraction. Biochem. Biophys. Acta. 98:329-334.

Ralls, J. W. 1960. Rapid method for semi-quantitative determination of volatile aldehydes, ketones and acids. Flash exchange chromatography. Anal. Chem. 32:332-336.

Rapport, M. M., and W. T. Norton. 1962. Chemistry of lipids. Ann. Rev. Biochem. 31:103-108.

Rapport, M. M., and B. Lerner. 1959. The structure of plasmalogens. IV. Lipids in normal and neoplastic tissues of man and in normal tissues of rabbit and rat. Biochem. Biophys. Acta. 33:319-325.

Rapport, M. M., and N. Alonzo. 1960. The structure of plasmalogens. V. Lipids of marine vertebrates. J. Biol. Chem. 235:1953-1956.

Raupp, G. R. 1959. Gas chromatographische trennung niederes fettsaüren. Angew. Chem. 71:284-285.

Rhee, K. S., and L. R. Dugan, Jr. 1967. The implications of acid concentration in lipid phosphorous determination. Anal. Biochem. 19:157-168.

Rhee, K. S., R. R. del Rosario and L. R. Dugan, Jr. (In press.) Determination of plasmalogens after treating with a 2, 4-dinitrophenylhydrazine-phosphoric acid reagent. Lipids.

Rouser, G., A. J. Bauman, G. Kritchevsky, D. Heller and J. S. O'Brien. 1961. Quantitative chromatographic separation of complex lipid mixtures: Brain lipids. J. Am. Oil Chem. Soc. 38:554-563.

Rouser, G., G. Kritchevsky, D. Heller and E. Lieber. 1963. Lipid composition of beef brain, beef liver and sea anemone. Two approaches to quantitative fractionation of complex lipid mixture. J. Am. Oil Chem. Soc. 40: 425-442.

Rouser, G., J. S. O'Brien and D. Heller. 1961. The separation of phosphatidyl ethanolamine and phosphatidyl serine by column chromatography. J. Am. Oil Chem. Soc. 38: 14-19.

Rouser, G., A. N. Siakotos and S. Fleischer. 1966. Quantitative analysis of phospholipids by thin-layer chromatography and phosphorous analysis of spots. Lipids 1:85-87.

Schlenk, H., and J. L. Gellerman. 1960. Esterification of fatty acids with diazomethane on a small scale. Anal. Chem. 32:1412-1414.

Schwartz, D. P., H. S. Haller and M. Keeney. 1963. Direct quantitative isolation of monocarbonyl compounds from fats and oils. Anal. Chem. 35:2191-2195.

Schwartz, D. P., and O. W. Parks. 1961. Preparation of carbonyl free solvents. Anal. Chem. 33:1396-1400.

Schogt, J. C. N., P. Haverkamp-Begemann and J. Koster. 1965. Nonphosphatide aldehydogenic lipid in milk fats, beef tallow and ox heart. J. Lipid Res. 2:142-147.

Siggia, S., and R. L. Edsberg. 1948. Determination of vinyl ether and acetals and of amyl alcohol, acetaldehyde and water contained therein. Anal. Chem. 20:762-765.

Sloane-Stanley, G. H. 1949. Aspects of spectrophotometric iodometry. Biochem. J. 55:8P.

Smith, B. 1959. Analysis of aqueous mixtures by gas chromatography. Acta. Chem. Scand. 13:480-488.

Smith, E. E., G. D. Goetsch and H. D. Jackson. 1961. Oxidation of fatty acids by rumen epithelium and by liver from ketotic sheep. Archiv. Biochem. Biophys. 95:256-561.

Stoffel, W., F. Chu and A. H. Ahrens, Jr. 1959. Analysis of long chain fatty acids by gas-liquid chromatography. Micromethod of preparation of methyl esters. Anal. Chem. 31:307-309.

Thiele, O. W. 1955. Über den "Waelsch-Effect" und über die hemmwirkung von primaren aminen bei der plasmalbestimmung. Z. Physiol. Chem. 300:157-166.

Waelsch, H. 1951. Zur quantitative bestimmung der acetal phosphatide bermerkung zu der arbeit von "Feulgen, Bögutti und Andressen." Z. Physiol. Chem. 288:123-129.

Warner, H. R., and W. E. M. Lands. 1963. Metabolism of plasmalogen. II. The determination of alkenyl ethers in the presence of free aldehydes. J. Lipid Res. 4:216-220.

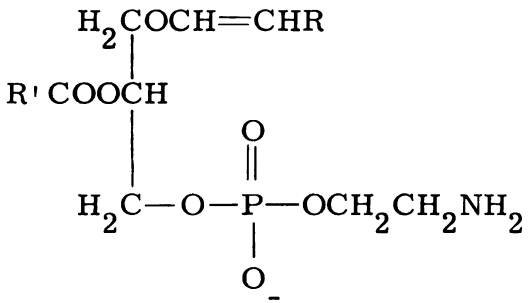
Williams, J. N., C. E. Anderson and A. D. Jasik. 1962. A sensitive and specific method for plasmalogen determination and other enol ethers. J. Lipid Res. 3:378-382.

Wittenberg, J. B., B. S. Korey and F. H. Swenson. 1956. The determination of higher fatty aldehydes in tissues. J. Biol. Chem. 219:39-46.

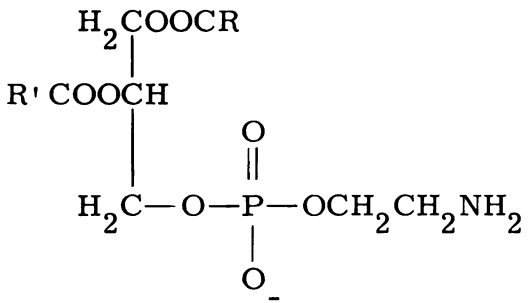
Yarbo, C. L., and C. E. Anderson. 1956. Acetal phospholipid in the adipose tissue of newborn rats. Proc. Soc. Exptl. Biol. Med. 91:408-409.

APPENDIX I

STRUCTURES OF PLASMALOGENS AND THEIR DIACYL ANALOGUES



Phosphatidal Ethanolamine



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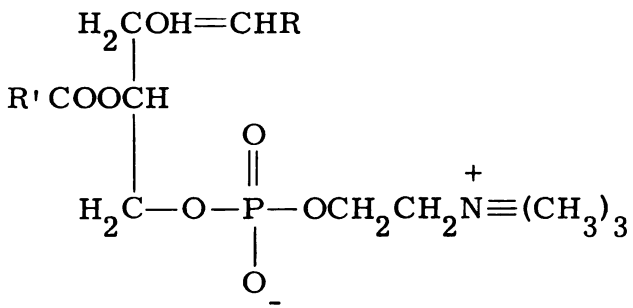
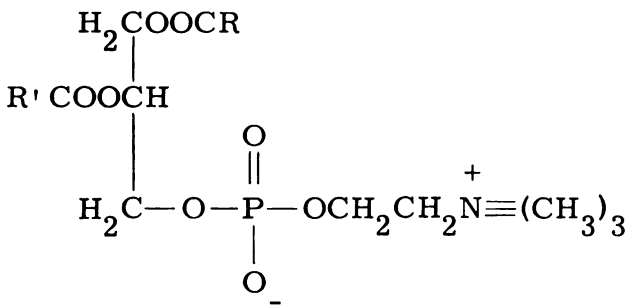


Phosphatidal Choline



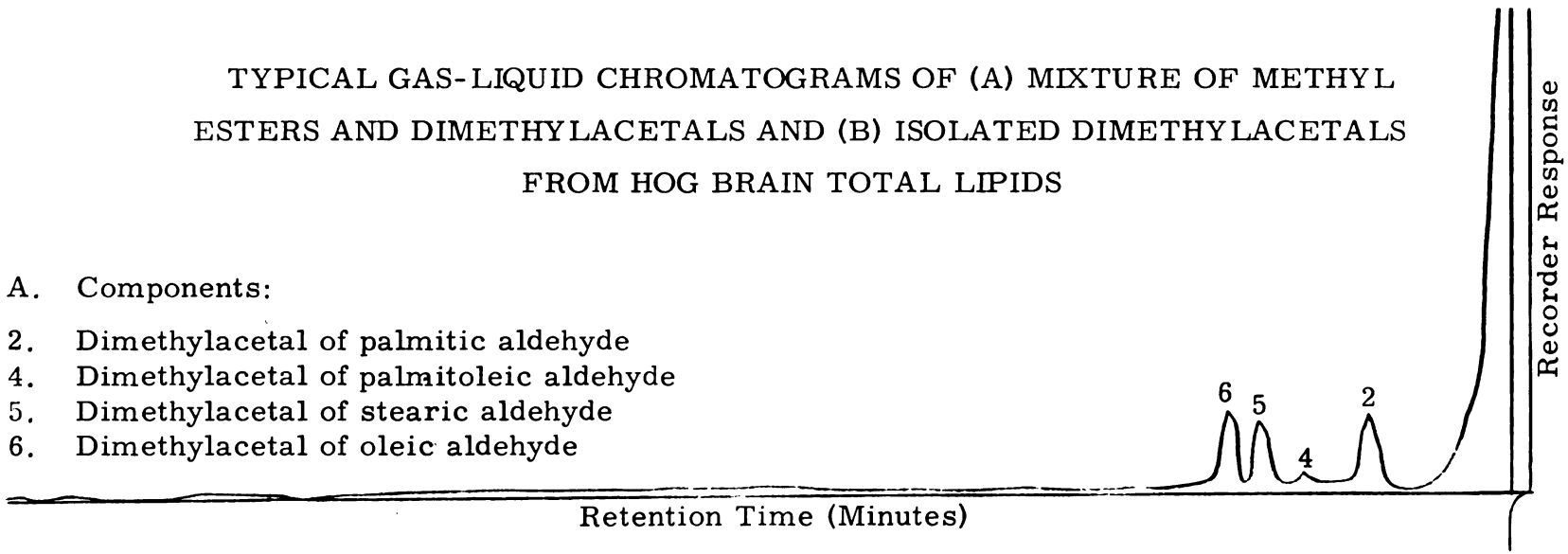
Phosphatidal Choline

APPENDIX II

TYPICAL GAS-LIQUID CHROMATOGRAMS OF (A) MIXTURE OF METHYL ESTERS AND DIMETHYLACETALS AND (B) ISOLATED DIMETHYLACETALS FROM HOG BRAIN TOTAL LIPIDS

A. Components:

2. Dimethylacetal of palmitic aldehyde
4. Dimethylacetal of palmitoleic aldehyde
5. Dimethylacetal of stearic aldehyde
6. Dimethylacetal of oleic aldehyde



B. Components:

1. Methyl laurate
2. Dimethylacetal of palmitic aldehyde
3. Methyl palmitate
4. Dimethylacetal palmitoleic aldehyde
5. Dimethylacetal of stearic aldehyde
6. Dimethylacetal of oleic aldehyde
7. Methyl stearate
8. Methyl oleate
9. Methyl linoleate
10. Methyl arachidate
11. Methyl arachidonate