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FRACTIONATION OF A NATURAL MIXTURE OF ALKENYL ACYL- AND DIACYL CHOLINE PHOSPHATIDES BY ARGENTATION ADSORPTION THIN-LAYER CHROMATOGRAPHY

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SUMMARY

Argentation adsorption thin-layer chromatography has been used for the fractionation of a natural mixture of alkenyl acyl- and diacyl choline phosphatides. This produced not only concentrates of alkenyl acyl choline phosphatides but also species of alkenyl acyl- and diacyl choline phosphatides that varied in unsaturation in the fatty acid moieties.

INTRODUCTION

The first attempt to separate intact plasmalogens (alkenyl acyl phosphatides) from the corresponding ester (diacyl) phosphatides was made by GRAY AND MACFARLANE[1] on a mixture of alkenyl acyl- and diacyl choline phosphatides obtained from beef heart muscle. They used partition chromatography with secondary cellulose acetate as a support and cyclohexane–methanol as a solvent system. Although they concluded that it was difficult to obtain pure native plasmalogen by this technique, they obtained a fraction in 20 % yield from the original mixture, which contained 75–85 % plasmalogen, whereas the original material had a plasmalogen content of only 50 %. They noted also that the plasmalogens had a very low content of saturated fatty acids, and that polyene fatty acids predominated. They attributed their fractionation to the degree of unsaturation, and, as a further possibility, to the chain length of the fatty acids.

Other attempts to prepare native plasmalogens from natural mixtures of alkenyl acyl and diacyl phosphatides by selective enzymatic[2,5] and alkaline hydrolysis[3,4] were successful, although the corresponding ester phosphatides were destroyed.

Fractionation of egg and soy diacyl choline phosphatides into species varying in their degree of unsaturation by argentation adsorption thin-layer chromatography (TLC) was reported by KAUFMANN, WESSELS AND BANDOPADHYAYA[6]. ARVIDSON[7] used a similar technique to fractionate diacyl choline phosphatides from egg yolk and livers of various animal species, and also reported the quantitative fatty acid composition of the components. Recently, ARVIDSON[8] subfractionated by reversed-phase partition TLC four fractions obtained by argentation TLC of intact rat liver

diacyl choline phosphatides, and obtained eight components, each containing one saturated and one unsaturated fatty acid.

The observations of GRAY AND MACFARLANE[1], GOTTFRIED AND RAPPORT[2], and KLENK AND KRICKAU[9] that alkenyl acyl phosphatides generally contain very small amounts of saturated fatty acids, and that the main fatty acids are polyenes and the fact that an additional unsaturation due to vinyl linkage is present in the alkenyl acyl phosphatides as compared to the diacyl phosphatides, pointed to the possibility exploited in the study of argentation adsorption TLC for the fractionation of a mixture of alkenyl acyl- and diacyl choline phosphatides. This produced not only concentrates of alkenyl acyl choline phosphatides, but also species of alkenyl acyl- and diacyl choline phosphatides that varied in unsaturation in the fatty acid moieties.

EXPERIMENTAL

Preparation of phosphatides

Fresh beef heart was freed of adipose and connective tissues and ground to a paste. The lipids were extracted with chloroform–methanol (2:1) by the method of FOLCH as modified by DAS AND ROUSER[10]. The total lipids (50 g) were dissolved in 200 ml of chloroform, and the phosphatides were precipitated with 2 l of acetone. Phosphatides free of neutral lipids were obtained by redissolving the precipitate in 200 ml of chloroform and reprecipitating the phosphatides with 2 l of acetone. A final repetition of this procedure yielded 25 g of an essentially 100 % phosphatide fraction. Absence of neutral lipids in the phosphatide fraction was confirmed by TLC. In the TLC system Silica Gel G/chloroform–methanol–ammonia (70:30:5), nothing was seen to migrate close to the solvent front where lipids less polar than cerebrosides migrate.

Isolation of a mixture of alkenyl acyl- and diacyl choline phosphatides

Two grams of this phosphatide mixture were fractionated into different classes of phosphatides by preparative TLC on Silica Gel G plates (20 × 20 cm with a silica gel layer thickness of 250 μ activated for 1 h at 110°) with chloroform–methanol–ammonia (70:30:5) as the solvent. Fifty mg of phosphatides were spotted per plate on 40 plates. The bands of different phosphatides were made visible under ultraviolet light after spraying the plates with 0.2 % alcoholic solution of 2′,7′-dichlorofluorescein. The fraction containing alkenyl acyl- and diacyl choline phosphatides was scraped off each plate with a razor blade and eluted three times with 100 ml of chloroform–methanol (2:1). After removing the chloroform–methanol under reduced pressure at 40° on a rotary evaporator, the residue was redissolved in chloroform, filtered and evaporated to dryness. This yielded 500 mg of a mixture of alkenyl acyl- and diacyl choline phosphatides. The purity of this mixture was tested in the above mentioned system where a single spot was obtained having the same R_F value as a sample of diacyl choline phosphatide synthesized in the laboratory and purified by preparative thin-layer chromatography. The synthetic sample was homogeneous as shown by thin-layer chromatography on Silica Gel G with chloroform–methanol–ammonia (70:30:5) as the developing solvent and the infrared spectrum of this sample was identical with those reported in the literature.

Fractionation of a mixture of alkenyl acyl- and diacyl choline phosphatides by argentation TLC

The silver nitrate-impregnated plates were prepared by developing activated Silica Gel G plates (silica gel thickness, 250 μ, plates, 20 × 20 cm) in a saturated solution of silver nitrate in 95 % ethanol contained in a chromatographic chamber. After the solution rose to the top of the plates, the plates were removed, air dried for 10 min, and activated for 2 h at 100°. Subfractionation of a mixture of alkenyl acyl- and diacyl choline phosphatides (10 mg per plate) into several species was carried out on 45 silver nitrate–impregnated Silica Gel G plates with chloroform-methanol–water (70:25:3) as the developing solvent. The different bands, which were visible under ultraviolet light, after spraying the plates with 0.2 % alcoholic solution of 2′-7′-dichlorofluorescein, were scraped off separately into separate 125 ml Erlenmeyer flasks and eluted three times with 50 ml of chloroform–methanol (1:2). The purity of the fractions was verified by argentation adsorption TLC in the same system when they showed single spots and their identities confirmed by plain adsorption TLC (system Silica Gel G/chloroform–methanol–ammonia (70:30:5)) where all the fractions gave single spots with the same R_F values and their R_F values were the same as that of a synthetic sample of diacyl choline phosphatide.

Methanolysis of alkenyl acyl- and diacyl choline phosphatides

Individual fractions (20 mg of each) were dissolved separately in 20 ml of diethyl ether in 125 ml Erlenmeyer flasks and immersed in a dry ice–acetone bath maintained at —30°. While the contents of each flasks were being stirred with a magnet, 2 ml of 100 % sulfuric acid[11] were added and stirring was continued for 10 min, during which time the temperature of the bath rose to 0°. The bath temperature was lowered again to —30°, 15 ml of absolute methanol were added and the stirring was continued for 10 min. The reaction mixture was carefully neutralized with 20 ml of 35 % KOH in methanol, followed by continuous rapid swirling until the jelly-like mixture turned into a white, milky solution. After 10 min of stirring, the contents of the flasks were transferred to a separatory funnel containing 200 ml of cold, distilled water, and the flasks rinsed twice with 20 ml of ether. The products obtained by methanolysis were extracted completely with 150 ml of ether, the ether extract washed free of alkali with distilled water and dried over anhydrous sodium sulfate. The silica gel containing adsorbed alkenyl acyl- and diacyl choline phosphatides may also be methanolyzed directly by the above cited technique after suspending the silica gel in 20 ml of chloroform–methanol (1:2) and extracting the products obtained by methanolysis with ether.

Separation of fatty acid methyl esters (ME) and dimethyl acetals (DMA) by TLC and their quantitative analysis by gas–liquid chromatography (GLC)

The ME and DMA obtained from different fractions of alkenyl acyl- and diacyl choline phosphatides were separated by preparative TLC, using Silica Gel G/toluene system. The ME and DMA were extracted from silica gel with diethyl ether and, after concentrating, were analyzed by GLC. The analyses were made with a Beckman GC-2A instrument equipped with a hydrogen flame ionization detector containing an aluminum column (6 ft long, 1/8 in. outer diameter) packed with Gas Chrom P, 80–100 mesh, impregnated with 20 % ethylene glycol succinate and 2 % phosphoric

acid. The temperature of the column was 190°, and helium flow rate was 30 ml/min. Peaks of ME and DMA were identified by comparing their retention values with authentic and synthetic standards, and the chain lengths of the unsaturated components were verified by GLC after hydrogenation. The peaks were quantitated by triangulation. The fatty acid methyl ester standards were supplied by The Hormel Institute Lipids Preparation Laboratory. The dimethyl acetals used as standards were synthesized and their purity confirmed by TLC, GLC and I.R. analysis as reported earlier from this laboratory[12].

Infrared analysis

The I.R. spectra of the samples, as liquid films, were obtained with a Perkin-Elmer Model 21 double beam spectrophotometer equipped with sodium chloride optics.

Determination of the plasmalogen content in different fractions

The percent plasmalogen content of the original mixture of alkenyl acyl- and diacyl choline phosphatides and the fractions thereof obtained by argentation TLC was determined by the use of the formula 2(amounts of DMA) × 100/(amounts of DMA + amounts of ME) after estimating the amounts of DMA and ME in methanolysis products of lipids by either densitometry or by quantitative weighing after preparative TLC separation and extraction. The densitometry was carried out as described by BLANK, SCHMIT AND PRIVETT[13] after running the products of methanolysis of lipids in the TLC system, Silica Gel G/toluene. The quantitative weighing of methyl esters and dimethyl acetals required larger amounts and hence the original mixture and fraction B (on argentation TLC) were subjected to methanolysis in five 20 mg batches.

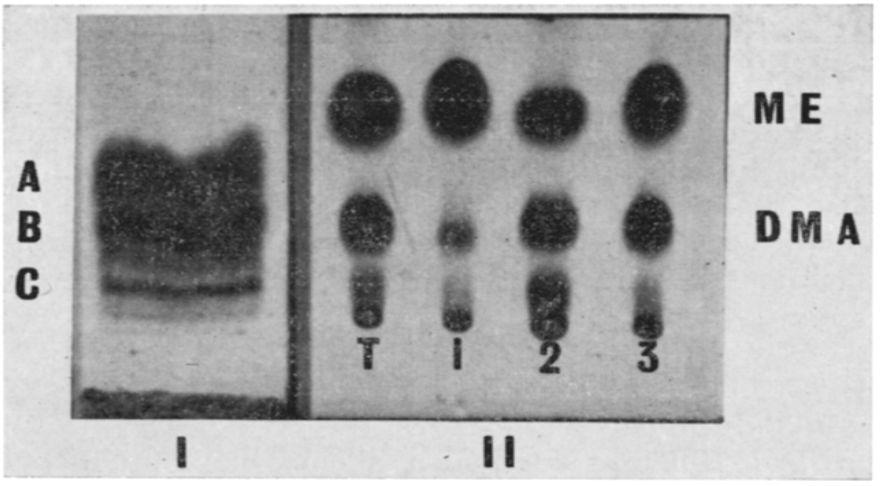

Fig. 1. (I) Fractionation of a mixture of alkenyl acyl- and diacyl choline phosphatides from beef heart. Adsorbent: Silica Gel G impregnated with silver nitrate. Activation: 2 h at 100°. Solvent $CHCl_3$–CH_3OH–H_2O (70:25:3). Spray reagent: 50% aqueous sulfuric acid (plate charred for 10 min at 160°). (II) Methanolysis products of original mixture and different fractions thereof obtained by argentation adsorption TLC of the original mixture. (T) Methanolysis products of original mixture of alkenyl acyl- and diacyl choline phosphatides. (1) Methanolysis products of fraction A. (2) Methanolysis products of fraction B. (3) Methanolysis products of fraction C.

RESULTS

The argentation fractionation of mixtures of alkenyl acyl- and diacyl choline phosphatides is demonstrated in Fig. 1 (I). Three major fractions were collected, designated A, B and C. Fractions A and B showed a tendency to separate into two parts but, under the experimental conditions employed, it was not possible to separate them completely. There were two other fractions in trace amounts, one above and one below fraction C, but their small amounts precluded their further investigation. The products of methanolysis of the original mixture (T) and those obtained from fractions A, B and C (designated as 1, 2 and 3, respectively) separated into ME and DMA (Fig. 1 (II)), indicating that the different fractions, as well as the original mixture, had alkenyl acyl choline phosphatides, though in different amounts (Table I). The original

TABLE I

THE PERCENT PLASMALOGEN CONTENT OF AN ORIGINAL MIXTURE OF ALKENYL ACYL- AND DIACYL CHOLINE PHOSPHATIDES AND THE FRACTIONS THEREOF OBTAINED BY ARGENTATION TLC

Estimation	*Percent plasmalogen content*			
	Original mixture	*Fraction A*	*Fraction B*	*Fraction C*
Densitometry	55.6	8.0	84.4	65.0
Quantitative weighing	59.8	—	88.6	—

mixture had a plasmalogen content of 55 to 60 % which agrees fairly well with the values reported in literature[1,2]. Fraction A had only 8 % of plasmalogen content, whereas fraction B had a plasmalogen content of 80 to 90 %, a very concentrated plasmalogen fraction. Fraction C had a plasmalogen content of 65 %.

The high concentration of plasmalogen in fraction B was confirmed further by I.R. studies. The ratio of absorbance of vinyl ether (at 1668 cm^{-1}) to ester (at 1735 cm^{-1}) for the original mixture was 0.27, and for fraction B it was 0.34, which confirmed our earlier findings that fraction B was a plasmalogen concentrate.

The composition of fatty acids and aldehydes in the original mixture and different fractions thereof are given in Table II. There was a steady increase in unsaturation of the fatty acids with a steady decrease in R_F value of the fraction. The composition of aldehydes in all the three fractions was nearly the same.

DISCUSSION

The technique described in this communication fractionated a mixture of alkenyl acyl- and diacyl choline phosphatides into different species, due to varied unsaturation in the fatty acid moieties and the additional unsaturation due to vinyl linkage present in the alkenyl acyl choline phosphatides. Unsaturation due to vinyl linkage helped further the concentration of alkenyl acyl choline phosphatides in fractions B and C (Fig. 1 (1)). The technique described by GRAY AND MACFARLANE[1] fractionated a similar mixture on the basis of unsaturation and chain length of the fatty acid moieties. Though they did not mention the effect of vinyl unsaturation on their separation, it must have had some effect on their separations. Their technique, however, will lead

TABLE II

FATTY ACID AND FATTY ALDEHYDE COMPOSITION OF BEEF HEART ALKENYL ACYL- AND DIACYL CHOLINE PHOSPHATIDES

	Percent fatty acid composition				*Percent fatty aldehyde composition*			
	Original sample	*Fraction A*	*Fraction B*	*Fraction C*	*Original sample*	*Fraction A*	*Fraction B*	*Fraction C*
13:0	—	—	0.5	—	2.4	1.5	—	—
14:0	tr	0.5	—	0.8	2.6	—	2.6	—
14:1	tr	tr	0.6	1.2	2.1	2.4	4.1	—
15:0	tr	0.7	tr	tr	2.6	8.3	3.7	12.3
15:1	tr	tr	1.1	1.2	2.1	1.8	2.7	1.3
16:0	30.8	38.8	6.7	14.6	69.4	64.6	66.3	55.0
16:1	1.7	3.1	0.5	1.3	3.1	4.2	3.8	3.5
17:0	tr	0.8	1.5	tr	1.2	2.2	2.5	6.8
17:1	tr	1.4	—	—	—	—	—	—
18:0	5.8	7.5	14.8	5.9	12.1	11.8	11.4	19.8
18:1	19.1	23.4	12.8	3.1	2.4	3.1	3.0	1.2
18:2	32.3	24.0	57.6	8.6	—	—	—	—
20:0	—	—	—	0.9	—	—	—	—
20:1	tr	—	tr	3.9	—	—	—	—
20:3	4.2	—	4.0	14.8	—	—	—	—
20:4	6.2	—	—	32.1	—	—	—	—
Unidentified	—	—	—	1.0	—	—	—	—
20:5	—	—	—	2.1	—	—	—	—
24:0	—	—	—	2.5	—	—	—	—
Unidentified C22?	—	—	—	1.4	—	—	—	—
Unidentified C22?	—	—	—	4.7	—	—	—	—

to the formation of "critical pairs" since the unsaturation and chain length have opposite effects as far as their partitioning properties between two phases are concerned. Thus, a fractionation by combination of argentation TLC, followed by partition chromatography (normal or reversed-phase) will give the maximum number of fractions as indicated by ARVIDSON[3]. Another advantage of argentation adsorption TLC over partition chromatography is that the former technique requires less time and can fractionate larger amounts.

In a recent communication, OWENS[14] mentioned that slight structural differences between diacyl phosphatides and analogous alkenyl acyl phosphatides militated against the chromatographic separation of intact plasmalogens. However, the present technique seemed to overcome this problem. This technique resulted also in recovery of all the components of the original mixture in their native form.

Work is in progress to obtain different molecular species of phosphatidal cholines, -ethanolamines and -serines by a combination of argentation adsorption and partition TLC to investigate their role in blood coagulation.

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