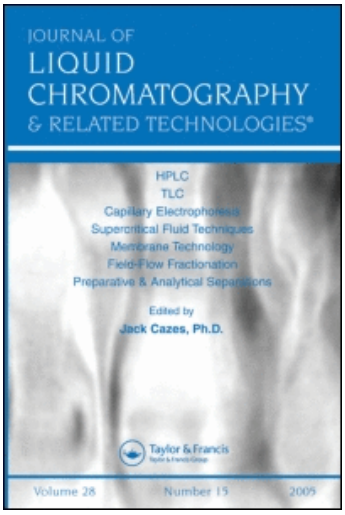

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CHARACTERIZATION OF PHOSPHOGLYCERIDES BY CHEMICAL AND ENZYMATIC HYDROLYSIS ON THIN LAYER PLATES IN SITU

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ABSTRACT

The use of chemical and enzymatic reactions in situ on thin layer chromatograms for characterization of phosphoglycerides is described. Acid and alkaline hydrolysis under specific conditions can differentiate acyl, alkyl and alkenyl moieties. Phospholipases A_2, C and D were shown to give complete and specific hydrolysis of phospholipids directly on the chromatograms.

INTRODUCTION

The need for methods for partial characterization of the small amounts of phospholipids available in studies dealing with mammalian spermatozoa has led to development of in situ reactions on thin layer chromatograms. Earlier procedures showed the potential for performing reactions in this manner, but the methodology was not fully developed (1). One example is analysis of alk-1-enyl-

phosphoglycerides by acid hydrolysis of the vinyl ether bond with HCl vapor to produce aldehydes (2). This method proved in our hands to be relatively non-selective for the alkenyl bonds as compared with acyl ester bonds. Another example is the method of Hack and Ferrans (3) which involves hydrolysis of the alkenyl moiety of plasmalogens on thin layer chromatograms with mercuric chloride, followed by the Schiff reagent to detect aldehydes formed. However, we have found that the mercuric chloride method resulted in reaction with unsaturated phospholipids to give multiple cleavage products.

Dinitrophenylhydrazine was used to derivatize the aldehyde released by acid hydrolysis on the thin layer. The hydrochloric acid and phenylhydrazine are made up in a single solution (4). Goldfine et al., have used 90% acetic acid to hydrolyze plasmenyl-ethanolamine (5).

A number of methods have been reported for alkaline hydrolysis of phospholipid acyl groups (6-8), but these have the disadvantages of requiring large samples, or of requiring multiple manipulations including preliminary separation of the components prior to final separtion by TLC.

Dutta and coworkers have reported the use of lipase and phospholipases A_2 and C directly on thin layer plates for lipid hydrolysis (9). However the pH during hydrolysis was not considered and for anaylsis the products were eluted from the chromatogram.

MATERIALS

The synthetic standards 1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine (DPPE), 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine (DPPE), lysophosphatidylethanolamine (LPE), phosphatidic acid (PA), dipalmitoyl glycerol (DPG), 1,2-dipalmitoyl-sn-glycerol-3-phosphoglycerol (DPPG), cardiolipin (CL),

sphingomyelin (SP),lysophosphatidylcholine (LPC) and 1-palmitoyl 2-oleoyl-sn-phosphocholine (POPC), were obtained from Avanti Biochemicals (Birmingham, Alabama). PE from bovine brain (PEB) was obtained from Supelco Co. (Bellefonte, Pennsylvania). Glycerylphosphoethanolamine(GPE), glycerylphosphocholine (GPC), phospholipase A_2 from snake venom (Naja Naja) (E.C.3.1.1.4), phospholipase C, type XII from Cl. Perfringens (E.C.3.1.4.3) and phospholipase D type V from cabbage (E.C.3.1.4.4), were purchased from Sigma Chemical Co. (St.Louis, Missouri). The Schiff reagent was obtained from Accra-Lab, Inc. (Bridgeport, New Jersey). Phospholipid standard solutions were prepared at concentrations between 0.5 and 2 mg/ml in chloroform/methanol (1:1, v/v). The purity of these standards was verified by TLC with different mobile phases. Solvents were EM Science chromatographic grade. Precoated Silica LK5 plates (250 μm thick with preadsorbent zone of 500 μm thickness were obtained from Whatman, Inc. (Clifton, New Jersey). Inorganic salts were from J. T. Baker (Phillipsburg, New Jersey) and of the highest purity available.

METHODS

Direct Analysis of Phospholipids in Cell Suspensions

Whatman LK5 silica gel layers (20 x 20 cm; 250 μm thick with preadsorbent zone) were washed by continuous development overnight in chloroform/methanol (1:1, v/v). Those plates used for in situ chemical and enzymatic hydrolysis experiments were scored on a Schoeffel scoring device to give 1 cm lanes prior to the washing procedure. Development was carried out in standard size tanks. Samples of cell suspensions were applied directly to the preadsorbent zone in 125 μl aliquots as a thin band on the preadsorbent zone of unscored plates. Four plates were used in each determination. Synthetic or purified phospholipid standards were applied to the two edges of the plate for location of the unknown

phospholipids. The plates were dried under a stream of warm air for 10 min. After drying, the layers were predeveloped three times in chloroform/methanol (1:1, v/v) to the interface of the preadsorbent zone. Between each predevelopment, the layers were air dried for a time sufficient to assure complete evaporation of the solvent. This procedure extracts the phospholipid from the sample and deposits it as a line on the starting point of the chromatogram (11).The mobile phase was chloroform /ethanol /triethylamine /water (30:34:30:8, v/v/v/v). Development proceeded until the mobile phase reached 2 cm from the top of the plate; this usually required 1.5 hr. After development, the plates were dried and 1-inch strips from both edges of the plates, where the standards had been applied, were cut and dried in an oven at 170°C for two min to remove residual solvent. Half of these strips were sprayed with a 10% solution of $CuSO_4$ in 8% H_3PO_4; the other half were sprayed with a 0.2% solution of ninhydrin in acetone. The $CuSO_4$-sprayed plates were dried for five min at room temperature, heated in an oven at 100°C, and finally placed in an oven at 170°C for 10 min (10). Ninhydrin-sprayed plates were placed directly in an oven at 110°C for five min. These procedures gave optimal development of the chromatograms.

The developed chromatograms were scanned in a Kontes Fiber Optic Scanner (Model 800) using a 440 nm filter. A Hewlett-Packard 3390A integrator provided integration of the absorbance bands. The scanning was carried out in the transmission mode using double beam operation. DPPE, DPPC, LPE, LPC, PEB, LPEB, GPE and palmitic acid were used to obtain standard curves. Amounts of phospholipid between 0.5-5 μg gave linear standard curves, so procedures were adjusted so that the amounts applied to the plates were in this range. Amounts of the unknowns were interpolated directly from the standard curves and so are referred to the standards. Phospholipid phosphorous was calculated from these amounts, using the conversion factor 1 μg phospholipid = 0.04 μg P.

RESULTS AND DISCUSSION

Plasmalogen Analysis by Acid Hydrolysis

Phospholipids were subjected to mild acid hydrolysis, to which the alkenylether groups are very sensitive. Products are the corresponding glycerol and aldehyde derivatives. This provides a rapid method for plasmalogen identification (12). The reaction was carried out in situ by streaking 25 µl of each phospholipid at 2 mg/ml in chloroform/methanol (1:1, v/v) on the preadsorbent zone of scored LK5 plates in eight adjacent lanes. Then 25 µl of a mixture of 2% trichloroacetic acid and 8% hydrochloric acid (TCA/HCl) (1:1, v/v) was added to this zone of each lane. Plates remained at room temperature for 10 min to assure complete hydrolysis of the alkenylether group by the TCA/HCl mixture. Then two predevelopments and the final development of the chromatogram were carried out as described above. After development, the plates were dried and one strip was cut from each edge of the plate in such a way that two lanes would be on each strip. These strips were dried in an oven at 170°C for two min. One strip was sprayed with copper sulfate and the other with ninhydrin reagent, as described above. The solvent front area was sprayed with a solution of the Schiff reagent (1) for aldehyde identification. Quantitation of the aldehyde was done only on the $CuSO_4$-sprayed plate. The products of hydrolysis then were scraped individually from the remaining unstained plate and subjected to either a second acid or enzymatic hydrolysis. The identical procedure was used for purified PEB.

Treatment of sperm phospholipids with TCA/HCl reagent for determination of plasmalogen gave the result shown in the densitometer trace of the $CuSO_4$-stained plate in Figure 1a and 1b. The peak for PC decreased by about half, with a corresponding increase in LPC, as expected for a monoplasmalogen. The peak for PE was nearly eliminated, with no appearance of the corresponding lysophospholipid. None of the other phospholipids were affected by the TCA/HCl

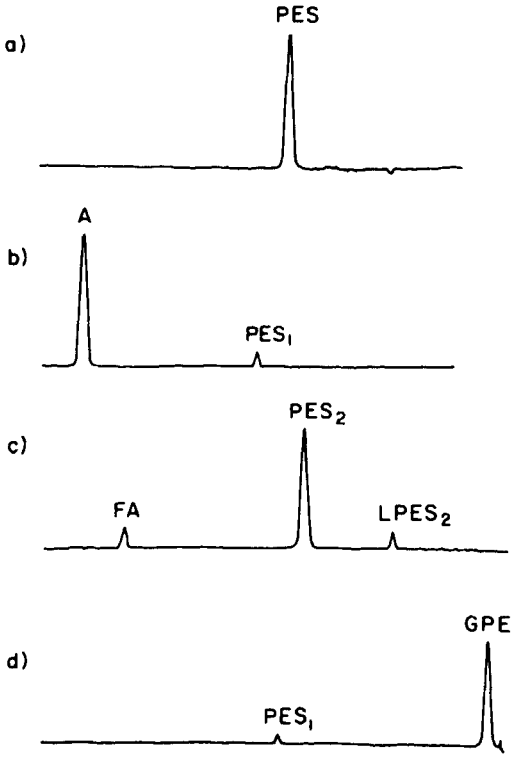

FIGURE 1. Densitometric traces of TLC plates with isolated rabbit epididymal sperm phosphatidylethanolamine (PES) after various treatments, and stained with $CuSO_4$ or ninhydrin. The profile of untreated PES is shown in trace a; that of PES treated with the TCA/HCl reagent is shown in trace b; staining with $CuSO_4$ is shown in trace c; band FA is fatty acid. The profile obtained by treatment with the TCA/HCl reagent and stained with ninhydrin is shown in trace d; band GPE is glycerylphosphoethanolamine.

reagent within the limits of detectability, indicating that these do not contain plasmalogen. (13)

The sperm PE, designated PES prior to any treatment, was isolated from the plate and rechromatographed. It gave a single sharp peak after $CuSO_4$ staining, as shown in Figure 2a. Treatment with the TCA/HCl reagent, followed by $CuSO_4$ staining, yielded only a fatty aldehyde peak (Fig. 2b) and a small peak designated PES_1. The latter amounts to 10% of the original PES. When the chromatogram corresponding to that of Figure 2b was treated with ninhydrin to identify the ethanolamine moiety of the PE molecule, only a small peak corresponding to GPE was found (Fig. 2d). These results are consistent with PES containing 90% of the PE component as diplasmalogen with alkenylether moieties at the 1 and 2 positions and 10% PE as diacyl PE. The diacyl PE appears as PES_1 (Fig. 2b) while the diplasmalogen appears as PES_2 (Fig. 2c). The amount of monoplasmalogen must be at or below the limit of detection, since no lyso form was found in the chromatogram of Figure 2b, obtained by TCA/HCl treatment. Monoplasmalogens would be converted by this treatment to a lyso form of PE with the acyl moiety on either the 1 or 2 positions.

Purified bovine plasmalogen from bovine brain, designated PEB, was subjected to identical experimental conditions for mild acid hydrolysis used with PES to check the method of analysis. Results are shown in Figure 2. PEB (Fig. 2a) subjected to TCA/HCl hydrolysis resulted in PEB_1 (15%), $LPEB_1$ (65%), fatty aldehyde (85%) (Fig. 3b, $CuSO_4$ staining) and GPE (20%) (Fig. 2c, ninhydrin staining). (13)

Acyl Ester Analysis by Alkaline Hydrolysis

Aliquots of 5 to 25 µl of the phospholipid solutions (0.5 to 2 mg/ml) were streaked on the preadsorbent zone of the scored LK5 plates. Then 25 µl aliquots of

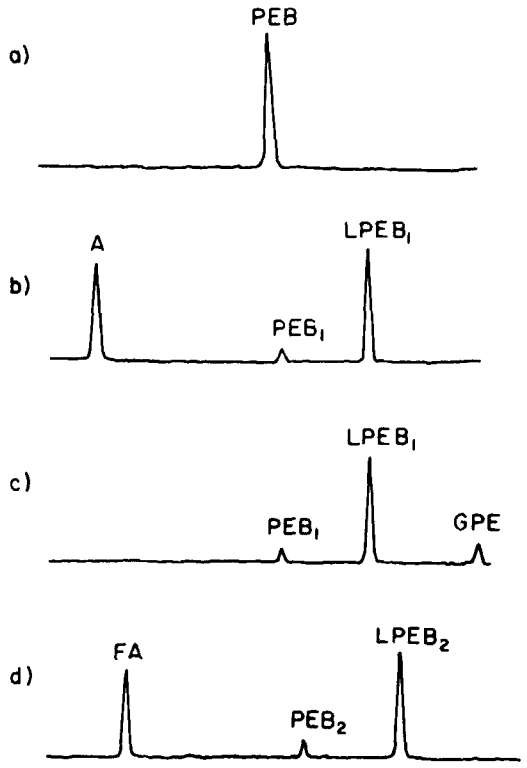

FIGURE 2. Densitometric traces of TLC plates with purified phosphatidylethanolamine from bovine brain (PEB), stained with $CuSO_4$ or ninhydrin after treatment as in Fig. 2. The profile of untreated PEB is shown in trace a; that of PEB treated with the TCA/HCl reagent is shown in trace b;. staining was with the $CuSO_4$ reagent. The profile of PEB treated with the TCA/HCl reagent and stained with ninhydrin is shown in trace c; that of PEB treated with phospholipase A_2 and stained with $CuSO_4$ is shown in trace d.

methanolic sodium hydroxide (NaOH) with final concentrations 0.2, 0.4, 0.6, 0.8 and 1.00 N were applied over the areas of sample. These solutions were made by dilution of 2.4 N aq. NaOH in methanol. (14) When POPC was used, GPC, PLC, and palmitic acid were added to contiguous lanes for the identification of the products of hydrolysis. If PE or PEB were used, GPE, LPE, palmitic acid, and octadecenal were added to the contiguous standard lanes.

Five ml of a methanol-water mixture in a ratio equivalent to that chosen for the NaOH reagent (from 1:1.2 to 1:11 aq. NaOH:methanol v/v) were placed in standard size tanks and then incubated in an oven at 37°C to saturate the air with methanol-water vapor. Immediately after addition of the 25 μl of the NaOH/methanol mixture to the phospholipid sample, plates were placed in the presaturated tanks and incubated at 37°C for 20 min. This step avoids preferential evaporation of methanol and maintains constant the NaOH concentration of the methanolic reagent throughout the incubation, which is critical to the procedure. (14) After incubation, plates were predeveloped once in chloroform/methanol 1:1 to the zone juncture, then air dried for a time sufficient to complete evaporation of the solvent. The chromatograms were then developed in a mobile phase of chloroform/ethanol/triethylamine/water (30:34:30:8 v/v/v/v) (8). Development proceeded until the mobile phase reached 2 cm from the top of the plate. After development, the plates were air dried, followed by heating in an oven at 170°C for 2 min to remove residual solvent.

Treatment of POPC, PEB and PCS with methanolic NaOH at concentrations between 0.2 and 1.0 N NaOH resulted in different degrees of hydrolysis of the acyl groups, as shown in Fig. 3. Complete hydrolysis was observed between 0.8 and 1.0 N. At 1.0 N NaOH, attack on the vinyl ether was detected, but at 0.8 N, this reaction was below the limit of detection. Methanolic NaOH obtained by diluting

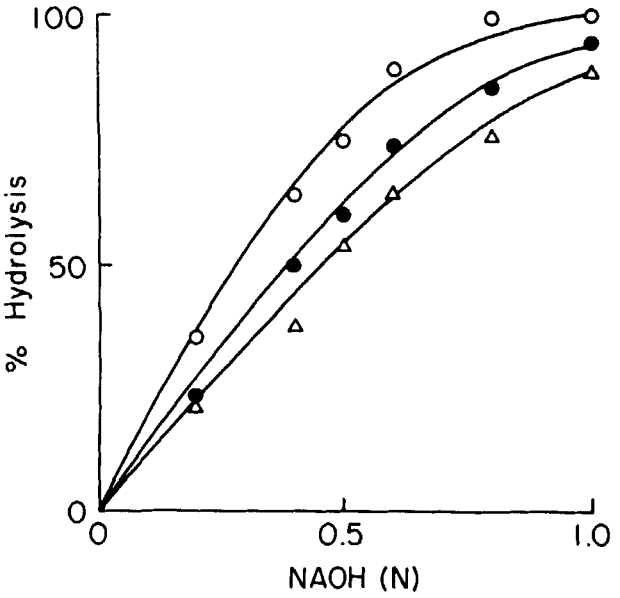

FIGURE 3. Percent hydrolysis of the three phospholipids POPC (o), PEB (●), and PCS (▲) as a function of NaOH concentration upon treatment with methanolic NaOH directly on the TLC plate. Hydrolysis conditions were 37° C for 20 min. in a chamber saturated with aqueous methanol vapor to maintain constant the NaOH concentration (see Materials and Methods).

2.4 N aqueous NaOH with methanol to 0.8 N (1:2 v/v) provided the desired selective hydrolysis of the acyl groups. When POPC was treated with 0.8 N methanolic NaOH reagent, free fatty acid (FA) (Fig. 4) and glyceryl phosphocholine (GPC), as identified by $CuSO_4$ reaction (14), resulted as the major products. In addition, a third component was found which comigrated with fatty acyl methyl ester (FME) in hexane/ether (96:4) (data not shown); this is the other solvolysis product expected in this system. Its migration with the solvent front in

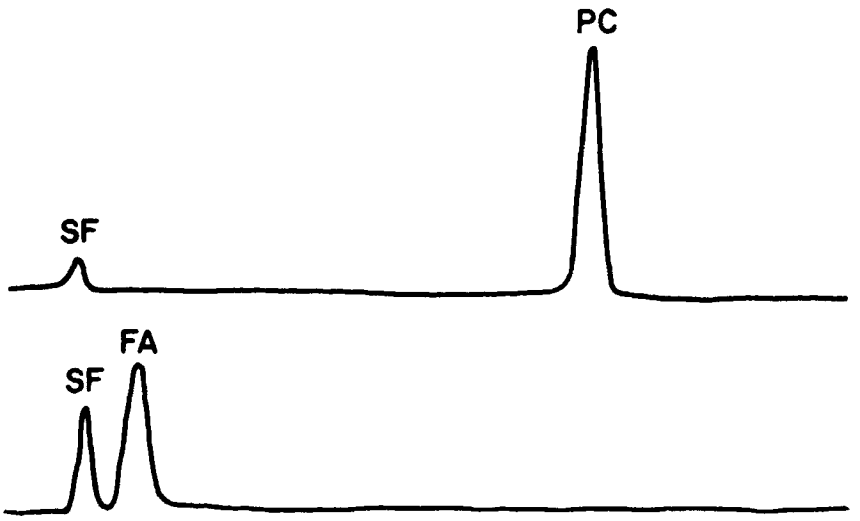

FIGURE 4. Densitometric traces of TLC plates with 1-palmitoyl-2-oleoyl-sn-3-phosphocholine (POPC) separated and stained with $CuSO_4$. The untreated sample gave the upper trace with the single component indicated by PC and the solvent front by SF. The lower trace was obtained by treatment with 0.8 N methanolic NaOH as described in Materials and Methods, followed by separation and staining with $CuSO_4$. The original phospholipid has been quantitatively hydrolyzed; the free fatty acid (FA) was the only component detected on the plate.

the standard solvent system (Fig. 4) conveniently removes it from the chromatogram. (14)

Treatment of PEB with 0.8 N methanolic NaOH, followed by chromatography (Fig. 5), gave the following products as mole percent long chain acyl and alkyl groups: PEB_1, corresponding to previously identified 1,2-di(0-1′-akenyl)PE, 19%; FME, 29%; free FA, 20%; lyso PE corresponding to $LPEB_2$ previously identified as 1-(0-1′-alkenyl)-lyso PE, 32%. The 19% recovery of the

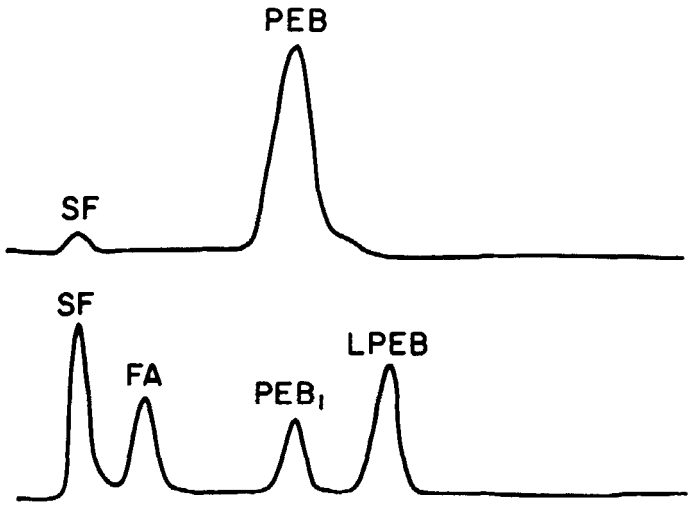

FIGURE 5. Densitometric traces of TLC plates with phosphatidylethanolamine from bovine brain (PEB) separated and stained with $CuSO_4$. The upper trace shows only the untreated phospholipid as a single, somewhat broad peak and the solvent front (SF). The lower trace shows the effect of treatment of PEB with 0.8 N methanolic NaOH. Hydrolysis has occurred to give three peaks: unreacted displasmalogen PE (PEB_1), lysophosphatidylethanolamine (LPEB), and free fatty acid (FA).

dialkenyl plasmalogen as compared to 20% by acid hydrolysis shows that this method cleaves less than 5% of diplasmalogens. From the percentages of monoacyl and diacyl PE in PEB, one calculates that the sum of FME and free FA should amount to 48%, compared to 49% observed; and that $LPEB_2$ should account for 33%, compared to 32% observed. These results gave evidence that 0.8 N methanolic NaOH cleaved the acyl chains of phospholipid plasmalogens in yields sufficient for quantitation, while leaving the ether links intact. A further test of the

specificity of the methanolic NaOH reagent was carried out with the neutral lipids, tripalmitin and cholesteryl palmitate. Triglycerides, cholesterol esters or sphingomyelin were not cleaved under the conditions which gave quantitative cleavage of the acyl groups from the phospholipids. (14)

Treatment of PCS with 0.8 N methanolic NaOH, followed by chromatography and staining with $CuSO_4$ (Fig. 6), gave the following products: FME, 30%; free FA, 45%, and lyso PC (LPCS), 25%. These are products to be expected for hydrolysis of the acyl groups while leaving the 2-(0-1′-alkenyl) group intact on LPCS. Were 1-(0-1′-alkyl) groups to be present in a significant proportion of the plasmalogen molecules, a lower yield of FME plus free FA and a higher yield of LPCS, comprising two different compounds, would have been observed. (14) A modification of this method was found to give complete and selective hydrolysis of cholesteryl palmitate. Aliquots of 1 to 15 µl of the cholesteryl palmitate solutions (0.1 mg/ml) in chloroform were streaked on the preadsorbent zone of scored LK5 plates. Then 25 µl of 2 N aqueous NaOH were applied over the areas of the sample. Cholesterol and palmitic acid were added to contiguous lanes for the identification of the products of hydrolysis. Then plates remained at room temperature for 20 min to assure complete hydrolysis of the ester group by the NaOH solution. The plates were predeveloped twice in chloroform/methanol (1:1). Development was carried out in chloroform/ether (90:10). Then the plates were treated as described previously for phospholipid analysis. (15) Treatment of cholesteryl palmitate with 2 N aqueous NaOH resulted in a complete hydrolysis to cholesterol and palmitic acid (Fig. 7).

Analysis of Phosphohipids by Specific Enzymatic Hydrolysis

1. Phospholipase A_2

Enzymatic hydrolysis was performed directly on the silica layer after separation of the phospholipids in exactly the same manner as for acid hydrolysis.

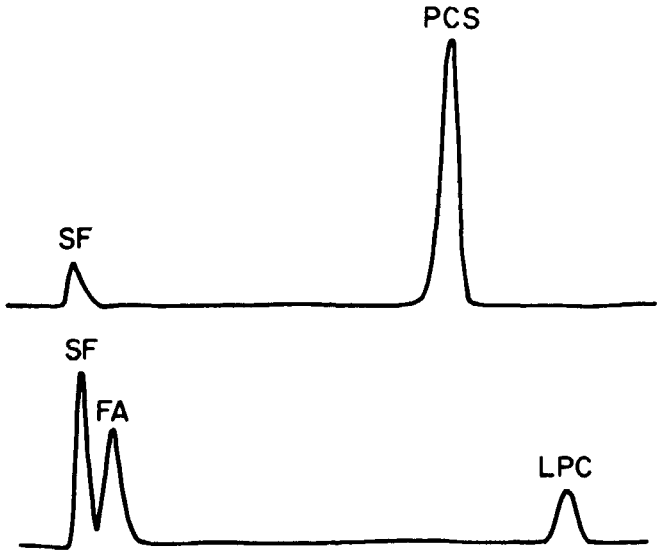

FIGURE 6. Densitometric traces of TLC plates with rabbit epididymal sperm phosphatidylcholine (PCS). The upper trace shows the untreated phospholipid as a single peak (PCS) and the solvent front (SF). The lower trace shows the effect of treatment with 0.8 N methanolic NaOH. Hydrolysis has occurred to give a form of lysophosphatidylcholine (LPCS) and free fatty acid (FA); no detectable parent peak of PCS was observed.

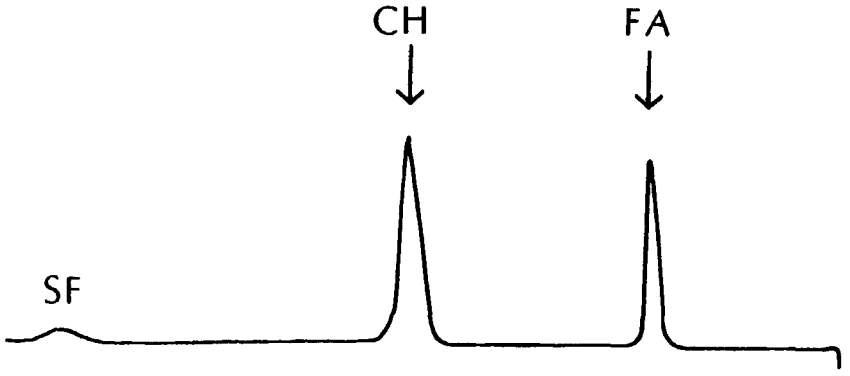

FIGURE 7. Densitometric trace of TLC plates with cholesteryl palimitate after treatment with 2 N aqueous NaOH. Hydrolysis resulted in two peaks: fatty acid (FA) and cholesterol (CH). Solvent front is indicated as SF.

After applying the phospholipids extraced from the cells with chloroform/ methanol (1:1), on the preadsorbent zone, 25 µl of phospholipase A_2 solution (5 mg/ml) in a sodium-tris-phosphate buffer (NTP) with 2.5 mM $CaCl_2$(13) was added to each lane. The plate remained at room temperature for 10 min to allow complete ester hydrolysis. The reaction mixture containing phospholipase A_2 remained at pH of 7.4 ± 0.2, as tested in control lanes, throughout the reaction period. After drying, the chromatograms were developed, and treated with $CuSO_4$ and ninhydrin as described above. (13)

PEB yielded PEB_2 (20%) and $LPEB_2$ (80%) after phospholipase A_2 treatment (Fig. 2d), indicating the presence of an acyl group at the 2 position in 80% of PEB molecules. The PEB fraction that was not hydrolyzed by the enzyme (PEB_2) yielded only GPE and fatty aldehyde after TCA/HCl treatment. The lyso form $LPEB_2$ yielded GPE and fatty aldehyde (65% of total PEB) and a lyso form designated $LPEB_3$ (15% of total PEB) when treated with TCA/HCl. The PEB can, from these results, be resolved tentatively into three fractions: PEB_1 [1,2-di(O-1´-alkenyl)], 20%, and PEB_3 [1-(O-1´-alkenyl)-2-acyl PE], 65%. The lyso PE products also can be assigned from the above distribution: $LPEB_1$, 2-acyl lysoPE; $LPEB_2$, 1-acyl PE and 1-(O-1´-alkenyl) lyso PE, and $LPEB_3$, 1-acyl lyso PE. (13)

2. Phospholipase C

1 to 5 µl aliquots of the synthetic phosphatidylcholine (POPC) were applied to the preadsorbent zone of scored LK5 plates as described previously. Also diacylglycerol (DAG) was added to contiguous lanes for the identification of the products of hydrolysis. Then 25 µl of phospholipase C solution (3mg/ml) in NTP with 2.5 mM $CaCl_2$ was added to each lane. The plates remained at room temperature for 15 min to allow complete phosphoglycerol ester hydrolysis. Similarly to phospholipase A_2 treatment the reaction mixture remained at

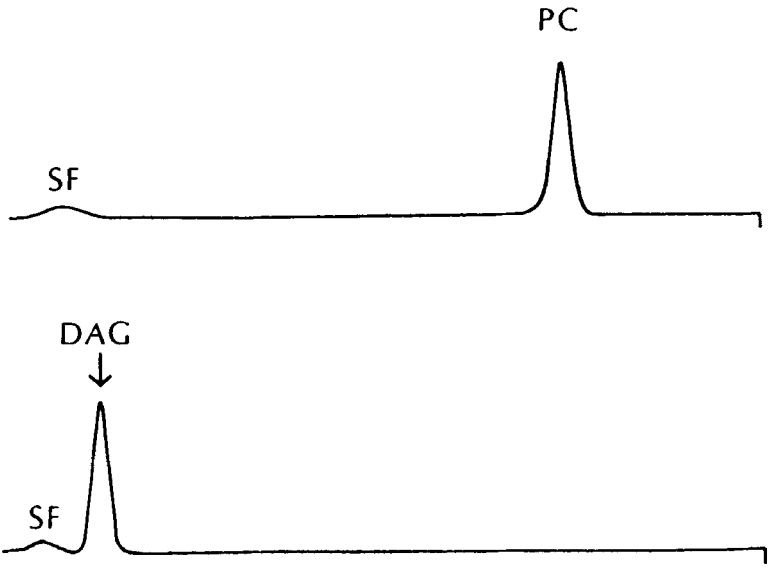

FIGURE 8. Densitometric trace of TLC plates with synthetic phosphatidylcholine after treatment with phospholipase C. Hydrolysis resulted in diacylglycerol (DAG). The phosphocholine moiety could not be detected by $CuSO_4$ staining.

pH 7.4 ± 0.3 as tested in control lanes. The plates were then dried, treated to develop the chromatogram and stained with $CuSO_4$ as described previously.

Treatment of POPC with phospholipase C resulted in complete hydrolysis to DAG (Fig. 8). The phosphocholine moiety was not detected by $CuSO_4$ treatment.

3. Phospholipase D

1 to 5 µl aliquots of the synthetic phosphatidylcholine (POPC) were applied to the preadsorbent zone of scored LK5 plates. Also PA was added to contiguous lanes for the identification of the products of hydrolysis. Then 25 µl of phospholipase D solution (2 mg/ml) in NTP with 2.5 mM $CaCl_2$ was added to the lanes where POPC was applied. The plates remained at room temperature for 15

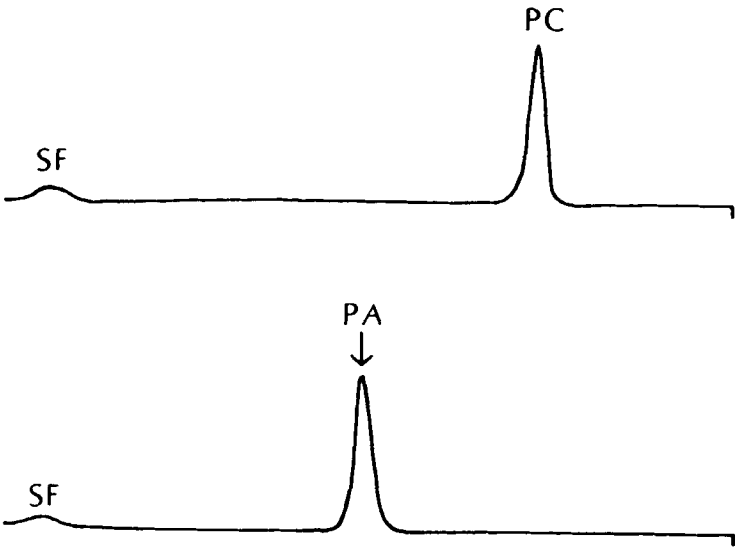

FIGURE 9. Densitometric trace of TLC plates with synthetic phosphatidylcholine after treatment with phospholipase D. Hydrolysis resulted in phosphatidic acid (PA). The choline moiety was not detected by $CuSO_4$ staining.

min to allow complete hydrolysis. The plates were then dried, predeveloped twice and developed as described above.

Treatment of POPC with phospholipase D resulted in complete hydrolysis to PA (Fig. 9). The choline moiety was not detected by $CuSO_4$ staining.

CONCLUSIONS

The procedures described in this paper provide a more rapid and convenient methodology for the analysis and partial characterization of phosphoglycerides from cell suspensions and tissue homogenates. This methodology allows precise detection of these lipids in the nanogram range. Negligible manipulation of the

sample is required since hydrolysis and extraction are performed directly on solid phase with the sample applied on the TLC plates. The acid reagent is highly selective for cleavage of the alkenyl ether bond and so allows accurate determination of plasmalogen content. The alkaline reagent allows accurate determination of acyl group content in a like manner. The use of different lipases makes it possible to characterize the phospholipids with regard to the position of acyl, alkyl, or alkenyl groups on the glycerol moiety. When used in combination, these methods provide a rapid means for classification and quantitation of cell phospholipids. Characterization of the individual types of hydrocarbon chains is not accomplished by these methods as described in this paper, but this could be accomplished by their extension and refinement.

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