FACILE AND HIGH YIELDING SYNTHESES OF PHOSPHATIDYLCHOLINES AND PHOSPHATIDYLETHANOLAMINES CONTAINING ^{2}H-LABELED ACYL CHAINS

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SUMMARY

Improved reliable methods for the partial chemical synthesis of phosphatidylcholines and phosphatidylethanolamines are described. Glycerophosphorylcholine (GPC) as its cadmium chloride complex was acylated rapidly (3 hr) with deuterated acyl anhydrides in dimethylformamide (DMF) to give phosphatidylcholines in > 90% yields. The products were free of sn-1 and sn-2 isomers. 2-Lysophosphatidylcholines were rapidly acylated with acyl anhydrides, used in nearly stoichiometric amounts, in chloroform affording mixed-chain phosphatidylcholines in > 85% yield. Pure phosphatidylethanolamines are prepared routinely in >70% yield by an improved procedure from the corresponding phospatidylcholines using phospholipase-D.

Keywords: Phosphatidylcholines, Phosphatidylethanolamines, Phospholipid synthesis, Deuterium-labeled fatty acid

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INTRODUCTION

Phospholipids with fatty acyl chains that have been labeled with spectroscopic probes, such as ^{2}H, ^{13}C, ^{19}F and spin label, are valuable in physical studies of biological and model membrane systems (1-3). The high cost and frequently low availability of labeled fatty acids necessitate that synthetic routes to phospholipids be consistently high yielding and economical in the amount of fatty acid required. The most widely used procedure for preparing phosphatidylcholines (PC) having the same acyl chain at both sn-1 and sn-2 positions has been the acylation of sn-glycero-3-phosphocholine (GPC) (4-6), or its cadmium chloride complex (7-10). Acyl imidazolides (4, 5, 10) and anhydrides (6-9) are the most widely used acylating agents. The methods have attempted to reduce the amount of acylating agent required as well as decrease reaction times, while conducting the reactions under mild conditions in order to suppress side reactions (5). Although the reported methods can give good yields (60-80%) and pure products, they frequently give variable results. Procedures which give consistently higher yields would be more attractive in the preparation of labeled lipids. We have found that the cadmium chloride complex of GPC can be acylated with nearly stoichimetric amounts of fatty acid anhydride in dimethylformamide and in the presence of N,N-dimethyl-4-aminopyridine (DMAP) within 3 h to give > 90% yields.

Phosphatidylcholines with mixed acyl chains have been prepared most often by acylating 2-lysophosphatidylcholines. Recently lysolecithins have been acylated in yields of approx. 80% at room temperature over a period of 24 to 48 h (9, 10). We have found that a modification of the procedure gives more

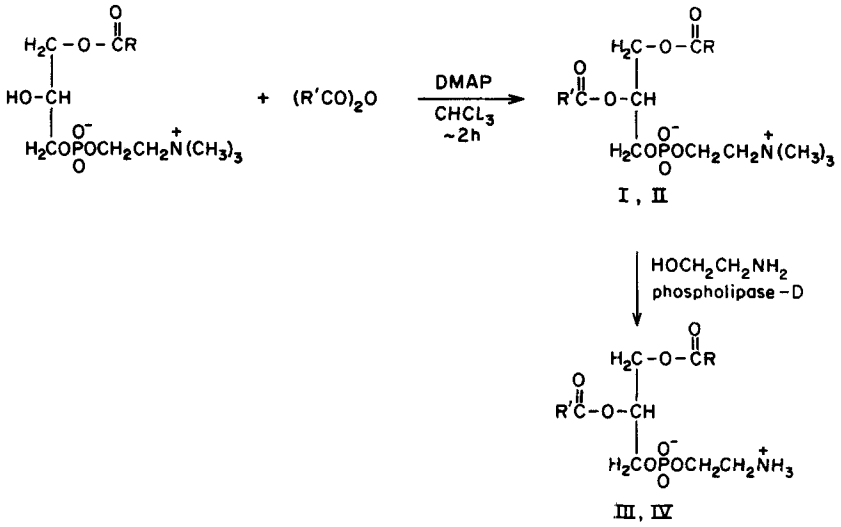
rapid acylation of lysolecithins (2 h) and yields of pure PC's consistently in excess of 85%. In addition unused labeled fatty acid could be recovered nearly quantitatively.

Phosphatidylethanolamines (PE) are of considerable interest because of their ubiquitous presence in biological membranes and their interesting physical properties (12). Several partial synthetic routes to their preparation have been reported (13-15). The most versatile involves introducing the required acyl groups into PC's followed by head-group exchange using phospholipase D (14,15). Yields at the transphosphatidylation step are typically 50-60% after purification. We report a modification of these procedures which gives consistently higher yields (70%) of pure PE.

RESULTS AND DISCUSSION

Acylation of GPC and its cadmium chloride complex in dipolar aprotic solvents has been reported previously (4,7,10). In dimethylsulfoxide (DMSO) at least 2 mole equivalents per OH group of acylating agent were required to achieve yields of 63-88% (4,7,10). DMSO has been noted to be very hydrophilic and difficult to obtain anhydrous (16). Dimethylformamide is easily dried (16) and readily dissolves the cadmium complex of GPC. Acylation of GPC in DMF occurs within 3 h in the presence of 1.2 mole equivalents per hydroxyl group of fatty acid anhydride and DMAP to give consistently pure PC's in yields in excess of 90%. TLC revealed the absence of 1,3-diacyl-glycero-2-phosphocholine. Phospholipase A_2 cleavage of the PC was complete after 2 h to give only fatty acid and lyso-PC. The latter result confirmed the absence of products involving head-group migration (5). The transition temperature as determined by DSC was in agreement with reported values (17). Reproducible yields were obtained on several preparations.

Acylation of 1-acyl-sn-glycerophosphatidylcholine with fatty acid anhydride and DMAP in chloroform-pyridine, 4:1 (v/v) has been reported to give PC's in 80% yields (8,9). The reaction times however were one to two days. When the acylation is conducted with near stoichiometric amounts of acyl anhydride, in chloroform as described in the Experimental Section, PC's are formed rapidly in 2 h and in high yields (Scheme 1). The results for a number of PC's produced by this method are given in Table I. Of particular importance is the consistently high yields shown in Table I. In addition, the washing with 1N HCl effectively removes all of the DMAP from the reaction mixture and allows nearly quantitative recovery of unused fatty acid. No evidence of head-group migration (5) during the acylation was detected by TLC. The products were completely hydrolyzed by phospholipase A_2 to give fatty acid and 2-lyso PC, further



Scheme I.
RCO = 16:0; I, III R'CO = 18:1; II, IV R'CO = 19:cp•

TABLE I

Yields and Analytical Data for Phosphatidylcholines

PC[a]	% Yield[b]	DSC (T_c) (°C)		% Acyl Chain Migration[d]
		Obs.	Lit.[c]	
14:0/[4-$^2H_2$]14:0	90	23.5	23	3 ± 1
16:0/[3-$^2H_2$]16:0	91	41.4	41	4 ± 1
16:0/[14-$^2H_2$]16:0	88	41.3	41	ND
16:0/18:1	92			ND
16:0/[5-$^2H_2$]18:1	94			5 ± 2
16:0/[9,10-$^2H_2$]18:1	93			ND
16:0/[16-$^2H_2$]18:1	89			ND
16:0/19:cp	95			5 ± 1
16:0/[5-$^2H_2$]19:cp	93			ND
16:0/[9,10-$^2H_2$]19:cp	93			ND
16:0/[11-$^2H_2$]19:cp	91			ND
16:0/[16-$^2H_2$]19:cp	93			ND
16:0/[19-$^2H_2$]19:cp	87			7 ± 3

[a]Myristic acid, 14:0; palmitic acid, 16:0; oleic acid, 18:1; dihydrosterculic acid, 19:cp.

[b]Based on lyso-PC; [c]Data from ref. 17; [d]ND - not determined.

confirming that no head-group migration had occurred during the reaction. Analysis of the hydrolysis products established that the amount of sn-1 to sn-2 acyl chain migration was <5% (Table 1). The transition temperatures as determined by DSC given in Table I, are in close agreement with reported values.

The synthesis of PE's from PC's (Scheme 1) was improved in terms of yields and purity. Our experimental procedure allows

TABLE II

Yield of Phosphatidylethanolamines

PE	% Yield[a]
16:0/18:1	73[b]
16:0/[5-$^2H_2$]18:1	76
16:0/[9,10-$^2H_2$]18:1	77
16:0/[16-$^2H_2$]18:1	74
16:0/19:cp	72
16:0/[5-$^2H_2$]19:cp	74
16:0/[9,10-$^2H_2$]19:cp	73
16:0/[16-$^2H_2$]19:cp	73

[a]Yields based on conversion of phosphatidylcholine.

[b]Transition temperature determined, T_c, by DSC to be 26°C.

a fast reaction with low amounts of enzyme. The buffer system must be made fresh since inhibitors seem to develop quickly. No exchange could be obtained in an attempt to use a buffer solution which had been stored at 4°C for 24 hours and no improvement could be achieved by adding more enzyme. Losses of lipid during the chloroform extraction are kept to a minimum by using centrifugation to separate the phases from the fairly stable emulsion. Finally removal of the small amount of ethanolamine impurity is essential to ensure consistent purity and yield. It is noteworthy that this impurity is not removed by the final chromatographic purification since it is eluted from the column together with the PE's. At a concentration as low as 5% relative to the pure lipid (w/w) ethanolamine depresses the bilayer to

hexagonal transition temperature by about 20°C for POPE and PDSPE (B. Perly, I.C.P. Smith, H.C. Jarrell, unpublished results). Moreover, chemical degradation of the lipid is readily observed if impure lipid dispersions in water are heated for short periods of time. This impurity is readily removed by the washing procedure described here, the centrifugation step being essential to ensure efficient removal of ethanolamine and quantitative recovery of the lipids.

EXPERIMENTAL

N,N-Dimethyl-4-aminopyridine was obtained from Aldrich Chem. Co. 1-Myristoyl- and 1-palmitoyl-sn-glycero-3-phosphocholine were purchased from Calbiochem (San Diego, Ca.); these were found to be contaminated with traces of the corresponding PC's and 1,3-diacyl-glycero-2-phosphocholines (<5%). sn-Glycero-3-phosphocholine as its cadmium chloride complex ($GPC \cdot CdCl_2$) was prepared by deacylation of egg phosphatidylcholine (18) or purchased from Sigma Chem. Co. Lyophilized phospholipase A_2 (Crotalus adamanteus) was obtained from Sigma Chem. Co. as a powder. Deuterium labeled myristic (14:0), oleic (18:1), palmitic (16:0) and dihydrosterculic (19:cp) acids were prepared as described previously (19-21). Phospholipase D was isolated from cabbage according to established procedures and stored under a nitrogen atmosphere at -20°C (22).

Thin layer chromatography (TLC) was performed on silica gel 60 plates (E. Merck, Darmstadt, Germany) with chloroform/methanol/water, 65:25:4 (v/v/v) as the eluant. Components were detected with iodine vapour, phosphate reagent and for

phosphatidylethanolamines, ninhydrin (23). Column chromatography was performed on Bio-sil A (100-200 mesh) (Bio-Rad Laboratories).

Chloroform was distilled from phosphorus pentoxide under an argon atmosphere directly into the reaction flask. Dimethylformamide (DMF) was distilled from calcium hydride under an argon atmosphere into the reaction flask.

Quantitative analysis of phospholipase A_2 hydrolysis products consisted of locating the components on the developed TLC plates with iodine, scraping the spots from the plates and directly esterifying with methanolic HCl (1N). The derived methyl esters were analyzed by gas chromatography-mass spectrometry as described previously (19).

Fatty acid anhydrides were obtained by established procedures (24) but after removal of dicyclohexylurea, the solvent was removed and the anhydride was used without further purification. Conversion of fatty acid to the anhydride was estimated by infrared spectroscopy.

Calorimetry was performed on a Microcal MC-1 differential scanning calorimeter (DSC) with a temperature scanning rate of 1.0 degree Celsius/min. The gel to liquid crystal transition of fully hydrated dipalmitoylphosphatidylcholine was used to calibrate the temperature scale. Samples weighed approx. 2 mg and were dispersed in 1.5 ml water.

Preparation of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine

GPC-cadmium chloride (10 mmole) was dispersed in water (50 ml) and lyophilized overnight to give a white powder which was dried further under vacuum (0.5 Torr) at 56°C for 3 h. Myristic anhydride (25 mmole) and DMAP (21 mmole) were added to a solution of dry GPC-cadmium chloride in dry DMF (70 ml) at 45°C. The mixture was stirred at 30°C under an argon atmosphere

for 3 h and protected from light. TLC indicated complete reaction after 3 h and the absence of isomeric side products. Removal of DMF under reduced pressure afforded a residue which was dissolved in methanol-chloroform-water, 5:4:1 (v/v/v) (200 ml). The mixture was passed through Rexyn 102(H^+) (30 g) and the column eluted with the same solvent (400 ml). The eluate was concentrated and the residue chromatographed on silica gel, eluting sequentially with chloroform (100 ml), chloroform-methanol, 1:1 (v/v) (200 ml) and chloroform-methanol, 1:3 (v/v). The eluate was monitored by TLC. Fractions containing the product were combined, filtered and concentrated to give 9.1 m mole of PC (91% yield based upon GPC). The lipid was homogeneous to TLC and had a gel to liquid crystalline phase transition, as determined by DSC, of 23.5°C and a pretransition of 14°C. This is in excellent agreement with the corresponding values of 23-23.9 and 14-15°C reported for dimyristoylphosphatidylcholine (17). Several preparations gave similar results.

Acylation of 2-lysophosphatidylcholine

A dispersion of 2-lysophosphatidylcholine (0.2 mmole) in water (5 ml) was lyophilized overnight to give a powder which was dried at 0.5 Torr and 56°C for 3 h. Fatty acid anhydride (0.24 mmole) and DMAP (0.25 mmole) were added to the lyso-PC and chloroform (5 ml) was distilled from phosphorus pentoxide into the reaction flask under an argon atmosphere. The reaction mixture was protected from light and stirred for 2 h. TLC reveal that in most cases > 90% reaction had occurred after 1.5 h. No isomeric products were detected (see Materials and Methods). The reduction of reaction time is important since it has been shown (25) that over longer periods of time significant acyl chain migration may occur in comparable systems. The reaction mixture

was diluted with chloroform-methanol 1:1 (v/v) (10 ml) and the mixture washed with cold 0.9% KCl in 1N HCl (10 ml) (18). The lower phase was washed with H_2O-MeOH, 1:1 (v/v) (3x5 ml), and chromatographed on silica gel (30 g). The unused fatty acid was quantitatively eluted with chloroform (200 ml). Elution with $CHCl_3$-MeOH, 1:1 (v/v) (200 ml) followed by $CHCl_3$-MeOH, 1:2 (v/v) afforded the product. The filtered eluate was concentrated under reduced pressure. The residue was dispersed in water (10 ml) and freeze-dried overnight to give the PC as a white hydroscopic powder. Yields based upon lysolecithin are given in Table I. Acyl chain migration from the sn-1 to the sn-2 position was monitored by treating the product (5 mg) with phospholipase A_2 according to established procedures (23). The hydrolysis products were separated by TLC and analyzed by g.c.-mass spectrometry of the derived methyl esters (19). Results are given in Table I.

Synthesis of 1,2-diacyl-sn-glycero-3-phosphoethanolamine

A transphosphatidylation buffer was freshly prepared by dissolving $CaCl_2.2H_2O$ (300 mg) and ethanolamine (5 ml) in 20 ml of water, adjusting to pH 5.6 with glacial acetic acid and diluting to 50 ml with water. Crude phospholipase D (20-30 mg) in 20 ml of the buffer was mixed with a solution of 1,2-diacyl-sn-glycero-3-phosphocholine (0.2 mmole) in 20 ml of water washed diethyl ether. The mixture was stirred under reflux in a water bath at 36-38°C. TLC showed that after two hours, only PE was present together with approximately 5% phosphatidic acid (PA) as by-product. Most of the ether was removed under reduced pressure leaving the lipid as a suspension in the aqueous buffer. Any enzymatic activity was destroyed by acidification to pH 1.8 with concentrated hydrochloric acid. The total lipid was extracted at

0°C with 30 ml of cold chloroform with vigorous shaking. Separation of the phases from the resulting emulsion was achieved by centrifugation (5000 rpm, 15 min.) at 0°C. This procedure was repeated and the pooled chloroform extracts were concentrated under reduced pressure to about 20 ml. At this stage ethanolamine hydrochloride is still present at about 5% (w/w) of the amount of lipid. This is removed by diluting the extract with methanol (10 ml) and washing with methanol-water, 1:1 (v/v) (3x10 ml), solvent phases were always separated by centrifugation (15 mm, 7500 rpm) at 0°C. TLC revealed that 95% of the impurity had already been removed by the first washing step. The chloroform phase was then chromatographed on silica gel (10 g). After washing the column with chloroform (100 ml), phosphatidic acid was eluted by chloroform-methanol, 19:1 (v/v) (150 ml). Finally elution with chloroform-methanol, 4:1 (v/v) afforded pure PE which was homogeneous to TLC using iodine, phosphate and ninhydrin detection. All fractions containing PE were combined, filtered and evaporated to dryness. The residue was dispersed at 30°C in water (5 ml) and freeze dried overnight leaving a white powder. Pure PE's were stored under dry nitrogen at -20°C. Yields are reported in Table II.

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