An improved method for the preparation of unsaturated phosphatidylcholines: acylation of *sn*-glycero-3-phosphorylcholine in the presence of sodium methylsulfinylmethide

Thomas G. Warner[1] and A. A. Benson

Marine Biology Research Division, Scripps Institution of Oceanography, La Jolla, CA 92093

Summary An improved method for the partial chemical synthesis of unsaturated and radioactively labeled phosphatidylcholines is described. This procedure offers advantages over conventional acylation methods in that it can be carried out on a millimole or micromole scale under mild conditions and it does not require a large excess of the fatty acid acylating reagent. In this procedure *sn*-glycero-3-phosphorylcholine is reacted with twice the theoretical amount of fatty acid imidazolide and sodium methylsulfinylmethide in dimethylsulfoxide for several minutes at 17°C. Phosphatidylcholine, which was purified by gradient-elution chromatography on silicic acid, was isolated in 60% yield and was estimated to be about 99% pure. The preparations of 1,2-dioleoyl-, 1,2-dilinoleoyl-, and 1,2-dilinolenoyl-*sn*-glycero-3-phosphorylcholine are described. The reaction was also carried out on a small scale for the preparation of high specific activity 1,2-di[1′-^{14}C]oleoyl-*sn*-glycero-3-phosphorylcholine in 38% yield with a specific activity of about 9.7 μCi/μmol.

Supplementary key words phosphatidylcholine synthesis · dimethylsulfoxide · [1-^{14}C]oleic acid

Partial chemical synthesis of phosphatidylcholines has routinely been carried out by the acylation of *sn*-glycero-3-phosphorylcholine or its cadmium chloride complex with fatty acid chlorides or fatty acid anhydrides (1, 2). Although both procedures have been extensively employed for preparing phosphatidylcholines with saturated and unsaturated fatty acids (3, 4), they are not readily adapted to small-scale preparations of phosphatidylcholines containing isotopically labeled fatty acids or other fatty acids that require involved synthesis and are not readily available in large quantities. Other methods of acylation have recently been developed; however, these methods use large excesses of the fatty acid acylating reagent and, unfortunately, also give low yields (5, 6). Furthermore, some procedures (2, 6) require extended reaction times, in some cases several days, at elevated temperatures. These conditions may favor the oxidation and degradation of unsaturated and polyunsaturated fatty acids, although these difficulties have apparently been overcome in small scale preparations (5).

We would like to report here an improved method of synthesis of unsaturated phosphatidylcholine, one which offers the advantages that it can be carried out on a moderate or small scale under mild conditions and uses only twice the theoretical amount of the fatty acid. In this procedure fatty acids are first converted to fatty acid imidazolide with carbonyldiimidazole and then reacted with *sn*-glycero-3-phosphorylcholine and sodium methylsulfinylmethide in dimethylsulfoxide solvent. A reaction scheme for this procedure is shown in **Fig. 1** below. The reaction proceeds to near completion within a few minutes at 17°C. Upon purification by silicic acid chromatography, the phosphatidylcholine is isolated in about 60% yield and it is estimated to be about 99% pure.

The method has been used here for the preparation on a millimole scale of 1,2-dioleoyl-, 1,2-dilinoleoyl- and 1,2-dilinolenoyl-*sn*-glycero-3-phosphorylcholine. The procedure has also been conducted on a micromole scale for the preparation of high specific activity radioactively labeled 1,2-di[1′-^{14}C]oleoyl-*sn*-glycero-3-phosphorylcholine.

Materials and methods

Preparation of sn-glycero-3-phosphorylcholine. *sn*-Glycero-3-phosphorylcholine, GPC, was prepared by deacylation of purified egg phosphatidylcholine (7) with tetrabutylammonium hydroxide (J. T. Baker, Phillipsburg, NJ) in ethyl ether as described by Brockerhoff and Yurkowski (8). A stock solution of the final material was prepared in methanol and stored at −20°C. Prior to utilization of the GPC, samples of the methanolic solution were transferred to the reaction vessel, a 20-ml sample vial, and dried overnight at room temperature over phosphorus pentoxide under vacuum.

Preparation of 1,2-dioleoyl-sn-glycero-3-phosphorylcholine. Fatty acid imidazolide was prepared by adding carbonyldiimidazole (Aldrich Chemical Co., Milwaukee, WI) (1.1 mmol) to oleic acid (Nu-check Prep, Elysian, MN) (1.0 mmole) in 1.0 ml of dry tetrahydrofuran (J. T. Baker Chemical Co.). The reaction was allowed to proceed for 45 min at room temperature under nitrogen with occasional shaking. It should be noted that anhydrous conditions were maintained throughout the preparation by purging the reaction vessels with dry nitrogen. Upon completion of the reaction, the solvent was removed with a stream of nitrogen and the resulting fatty acid imidazolide, in 1.0 ml of freshly distilled dimethylsulfoxide, Me_2SO (Mallinckrodt Chemical Co., St. Louis, MO), was com-

Abbreviations: egg phosphatidylcholine, 1,2-diacyl-*sn*-glycero-3-phosphorylcholine of egg; GPC, *sn*-glycero-3-phosphorylcholine; Me_2SO, dimethylsulfoxide.

[1] To whom correspondence should be addressed.

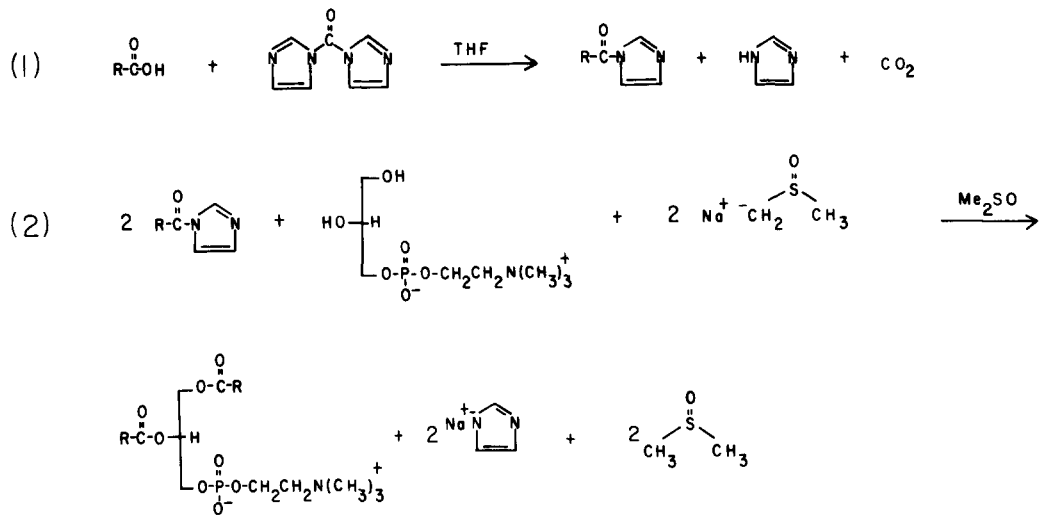

Fig. 1. Reaction scheme for preparation of unsaturated phosphatidylcholines. Reaction (*1*), formation of fatty acid imidazolide from carbonyldiimidazole and fatty acid in tetrahydrofuran, THF. Reaction (*2*), fatty acid imidazolide is reacted, without purification, with *sn*-glycero-3-phosphorylcholine and sodium methylsulfinylmethide in dimethylsulfoxide, Me_2SO. Experimentally, sodium methylsulfinylmethide in excess of the theoretical amount is required due to the presence of the imidazole that is formed in Reaction *1*.

bined with GPC (0.25 mmol) in 6.0 ml of Me_2SO. The vessel containing the fatty acid imidazolide was rinsed twice with 0.5 ml of Me_2SO and this was added to the GPC and fatty acid imidazolide suspension. Prior to adding the fatty acid imidazolide to the GPC–Me_2SO suspension, it was necessary to warm the GPC to about 45°C for several minutes to dislodge material that adhered to the walls of the reaction vessel. After cooling the reaction mixture to 17°C, a 3.6 ml aliquot of sodium methylsulfinylmethide solution (prepared as a stock solution by reacting metallic sodium, 4.4 mmol, with Me_2SO, 7.2 ml, under nitrogen) was slowly added to the rapidly mixing reactants. After 4 min the reactants were placed in an ice bath and the reaction was terminated by the rapid addition of 0.2 N HCl in 4°C, adjusting the pH to about 1.0. The acidified reaction mixture, in about 40 ml, was extracted three times with 20 ml of chloroform–methanol 2:1. The chloroform extracts were pooled and washed twice with 20 ml of methanol–water 1:1 to remove most of the dimethylsulfoxide from the crude lipid extract. The solvent of the washed extract was reduced in volume under vacuum to 2–3 ml and water was removed from the residue by three successive additions and evaporations of benzene.

This material, which contained a small amount of residual dimethylsulfoxide, was suspended in about 5 ml of chloroform and applied to a silicic acid column (Unisil Silicic Acid, 100–200 mesh, Clarkson Chemical Co., Williamsport, PA), in chloroform, with a bed volume of 1.5 × 16 cm. Neutral lipids and the dimethylsulfoxide were eluted with 150 ml of chloroform–methanol 9:1. Phospholipids were removed from the column with a gradient of chloroform–methanol 9:1 (160 ml) with increasing amounts of methanol (200 ml). Fractions of 5 ml were collected and the progress of the column was monitored by thin-layer chromatography. The best fractions were pooled and the solvent was removed under vacuum. The resulting residue was suspended in ethyl ether and cleared of particulate matter by centrifugation (5000 *g*, 4°C, 10 min). The solvent was removed under a stream of nitrogen. About 135 mg (68% yield) of phosphatidylcholine was obtained.

This material was analyzed by thin-layer chromatography on precoated silica gel plates (Brinkman Industries, Westbury, NY), developed in a solvent containing chloroform–methanol–water 65:25:4, using highly purified egg phosphatidylcholine as a standard. Analyses were also conducted by two-dimensional thin-layer chromatography using the solvent system of Rouser, Siakotos and Fleischer (9); first dimension, chloroform–methanol–ammonium hydroxide 13:7:1; second dimension, chloroform–acetone–methanol–acetic acid–water 5:2:1:1:0.5. The lipid areas on the plates were visualized with iodine vapor and lipid phosphorus spray (10) and by radioautography. In some preparations a small amount of [1-^{14}C]-oleic acid was included in the reaction mixture to prepare radioactively labeled phospholipid. This material was analyzed by thin-layer chromatography and the lipid areas were scraped from the plate and quantified as radioactive counts. Counting was carried out in a solution of toluene–Triton X-100 2:1 and diphenyloxazole, PPO, (New England Nuclear, Boston, MA, 5.5 mg/ml).

Preparation of 1,2-dilinoleoyl- and 1,2-dilinolenoyl-sn-glycero-3-phosphorylcholine. 1,2-Dilinoleoyl- and 1,2-dilinolenoyl-*sn*-glycero-3-phosphorylcholine were prepared from their respective fatty acids, linoleic (*cis,cis*-9,12-octadecadienoic acid) and linolenic acid (all *cis*-9,12,15-octadecatrienoic acid) (Nu-chek Prep) essentially as was described above. Yields of 63% for both preparations were obtained. The purity, based on thin-layer chromatographic analysis, was comparable to that of 1,2-dioleoyl-*sn*-glycero-3-phosphorylcholine.

Preparation of high specific activity 1,2-di[1'-^{14}C]oleoyl-sn-glycero-3-phosphorylcholine. The procedure for preparing high specific activity radioactively labeled phosphatidylcholine was similar to that described above except that the reaction was conducted on a greatly reduced scale.

Fatty acid imidazolide was prepared by reacting carbonyldiimidazole (49 μmol) with oleic acid (36.4 μmol) and about 3.6 μmol of [1-^{14}C]oleic acid (Dhom Products, North Hollywood, CA, 58 μCi/μmol) containing 210 μCi in 1.0 ml of tetrahydrofuran for 40 min at room temperature. After removal of the tetrahydrofuran, the residue, in 1.0 ml of Me_2SO, was combined with GPC (10 μmol) in 1.0 ml of Me_2SO, followed by three 0.5-ml washes of Me_2SO.

Reaction was initiated with 1.5 ml of sodium methylsulfinylmethide solution (0.37 mmol of metallic sodium was reacted with 6.9 ml of Me_2SO). After about 5 min at 17°C the reactants were placed on ice and the reaction was terminated with 0.2 N HCl at 4°C. The crude phospholipid was extracted twice with 10 ml of chloroform–methanol 2:1. The chloroform extracts were pooled and washed twice with 10 ml of methanol–water 1:1. After removal of the solvent and water in the crude extract, the residue, in chloroform, was applied to a small column (0.5 × 11.0 cm) containing silicic acid in chloroform. After washing the column with chloroform–methanol 9:1 (50 ml), phospholipid was eluted with a solvent gradient of chloroform–methanol 9:1 (60 ml) and increasing amounts of methanol (75 ml); 2-ml fractions were collected. Progress of the column was monitored by determining the radioactivity present in each fraction. The best fractions were pooled and the solvent was removed under vacuum. A yield of 3.1 mg of 1,2-di-[1'-^{14}C]oleoyl-*sn*-glycero-3-phosphorylcholine containing 40 μCi (38%) was obtained. The specific activity, as determined by phosphorus assay according to a modification of the method of Chen, Toribara, and Warner (11), was about 9.7 μCi/μmol.

Phospholipase treatment of radioactively labeled phosphatidylcholine. The stereochemical purity of the synthetic phosphatidylcholine was assessed by subjecting radioactively labeled material to enzymatic hydrolysis with a commercial preparation of phospholipase A_2, *Crotalus adamanteus* (Worthington Biochemicals, Freehold, NJ; 2500 U/mg). The enzyme was suspended in a buffer containing 10 mM of Na_2BO_4, pH 7.5, and 10 mM of $CaCl_2$ and incubated with labeled phospholipid in ethyl ether at 37°C for 1 hr. The hydrolysis products were analyzed by thin-layer chromatography as described above and each component was quantified in terms of radioactive counts.

Gas–liquid chromatographic analysis. Fatty acids of the synthetic phospholipids were converted to their methyl esters by transmethylation in a 2.0% H_2SO_4–methanol solution containing heptadecanoic acid (Sigma Chemical Co., St. Louis, MO) (175 μg/ml) as an internal standard. The methyl esters were analyzed with a Varian (Palo Alto, CA) Aerograph gas chromatograh, model 1800, fitted with a flame ionization detector and separated on a 6-ft, ⅛-in, stainless steel column packed with 10% DEGS-PS (Supleco, Inc., Bellefonte, PA) at 200°C. Identification of the esters was made using a commercial standard mixture of fatty acid methyl esters (RM-6, Supleco, Inc.) and the amount was quantified relative to heptadecanoic acid.

Results and discussion

When the phosphatidylcholines were analyzed by one-dimensional thin-layer chromatography, a single major spot, R_f 0.34, was evident. Traces of neutral lipid and another minor contaminant, R_f 0.40, were also detected. Utilizing radioactively labeled phosphatidylcholine and thin-layer chromatographic analysis, we have estimated the final material to be at least 99% pure. Further, gas–liquid chromatographic analysis of the fatty acids of each of the phospholipids indicated the presence of only a single component that accounted for nearly all (>98%) of the fatty acid present in the phospholipid sample. In addition, the chromatograms were identical to those of the authentic fatty acid methyl ester. Thus it is apparent that no significant degradation of the unsaturated fatty acids occurs under the reaction conditions.[2]

Nuclear magnetic resonance spectra of the synthetic phospholipids were also consistent with their respec-

[2] Further analysis of the radioactively labeled fatty acid methyl esters on $AgNO_3$-impregnated silica gel thin-layer plates, as described elsewhere (5), revealed that no detectable (less than 1–2%) isomerization occurred under these conditions. Also, ultraviolet spectral analysis of 1,2-dilinolenoyl-*sn*-glycero-3-phosphorylcholine, 1 mM in ethanol, at 233 nm (11), indicated that less than 2% of these fatty acids may contain a conjugated diene system.

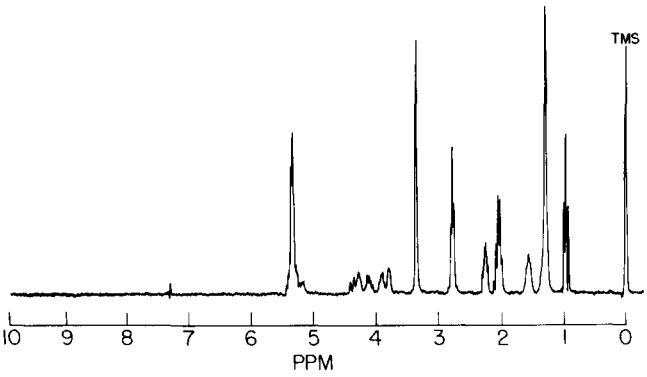

Fig. 2. Nuclear magnetic resonance spectrum of 1,2-dilinolenoyl-*sn*-glycero-3-phosphorylcholine, 117 mg/ml, in C^2HCl_3 with 1% tetramethylsilane, TMS, at room temperature. Assignments of the fatty acid signals are given in the text. Spectra were taken on a Varian HR-220 spectrometer.

tive structures and the chemical shifts were in good agreement with those previously reported for egg phosphatidylcholine (13). A typical spectrum, **Fig. 2**, for 1,2-dilinolenoyl-*sn*-glycero-3-phosphorylcholine gave the following signals for the fatty acid moieties: 0.97δ (terminal methyl, experimental: theoretical protons, 6.3:6 H), 1.30δ (methylene, 16.6:16 H), 2.04δ (allylic, 8.2:8 H), 2.79δ (doubly allylic, 8.3:8 H), 5.34δ (vinyl, 12.6:12 H).

Treatment of 1,2-di-[1′-^{14}C]oleoyl-*sn*-glycero-3-phosphorylcholine with phospholipase A_2 resulted in nearly complete hydrolysis of the synthetic phospholipid with lysophosphatidylcholine and fatty acid accounting for 46% and 54%, respectively, of the total radioactivity present in the hydrolysis products. Trace amounts of phosphatidylcholine were also detected after the enzyme treatment; however, this material contained less than 1% of the total radioactivity present. These results show that the stereochemical integrity of the 2 position of the glycerol moiety is maintained under the acylation conditions and that phospholipids can be prepared by the synthetic method described here without racemization.

This improved method of acylation is possible, in part, because all of the reactants are partially soluble in the dimethylsulfoxide solvent. This is in contrast with conventional procedures where the very polar *sn*-glycero-3-phosphorylcholine is insoluble in the organic solvents employed for the reaction. Also, sodium methylsulfinylmethide provides a strong, yet sterically hindered, base that is required for promoting the acylation process without racemization. In the absence of added base, little reaction occurs even under more vigorous reaction conditions (6). Other organic bases may also promote the reaction; however the optical purity of the final product must be carefully monitored since *sn*-glycero-3-phosphorylcholine has been shown to be extremely labile to racemization (1).

At the present time we have been unable to prepare phosphatidylcholines with long chain saturated fatty acids in good yield under these reaction conditions due to the limited solubility of the fatty acids in dimethylsulfoxide. It is possible that a mixed solvent system may be effective in overcoming these difficulties and experiments toward this goal are currently under way. This method may also be utilized for preparing phosphatidylcholines with short chain fatty acids and perhaps photosensitive fatty acids (14), as well as for preparing other phospholipids.

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