Available online at www.sciencedirect.com
SCIENCE DIRECT®
Chemistry and Physics of Lipids 137 (2005) 94–99
CPL
CHEMISTRY AND PHYSICS OF LIPIDS
www.elsevier.com/locate/chemphyslip

Short communication

Synthesis of phosphatidylcholine: An improved method without using the cadmium chloride complex of *sn*-glycero-3-phosphocholine

Ken'ichi Ichihara*, Hitomi Iwasaki, Kaori Ueda, Ryoko Takizawa, Hideko Naito, Mitsuhiro Tomosugi

Biological Chemistry, Graduate School of Agricultural Science, Kyoto Prefectural University, Shimogamo, Kyoto 606-8522, Japan

Received 11 October 2004; received in revised form 10 June 2005; accepted 10 June 2005
Available online 11 July 2005

Abstract

An improved safe method that does not contaminate the environment with cadmium chloride, a toxic heavy metal salt, was developed for the synthesis of phosphatidylcholine (PC). PC was synthesized from *sn*-glycero-3-phosphocholine (GPC) and fatty acid in one step under mild conditions without the use of cadmium chloride. GPC was prepared from egg yolk PC and adsorbed by kieselguhr in a Teflon vessel. The GPC on kieselguhr was acylated with fatty acid in the presence of two reagents, dicyclohexylcarbodiimide for synthesis of fatty acid anhydride and 4-dimethylaminopyridine as an acylating catalyst, at 30 °C overnight. The PC thus produced was purified by silica gel column chromatography. The yield of dioleoyl PC was 90% based on the starting material, GPC.
© 2005 Elsevier Ireland Ltd. All rights reserved.

Keywords: Cadmium; *sn*-Glycero-3-phosphocholine; Phosphatidylcholine; Synthesis

1. Introduction

The diacyl phosphatidylcholines (PC) are common membrane phospholipids, whose physical, chemical and biological properties have been extensively studied utilizing a range of PCs varying in their acyl chain composition. Many methods have been developed for the chemical synthesis of PC (Jensen and Pitas, 1976; Eibl, 1980). Conventional procedures involve the preparation of *sn*-glycero-3-phosphocholine (GPC) and esterification of the GPC with fatty acid. Most of the methods for synthesis of PC from GPC employ the cadmium chloride complex of GPC, because GPC itself is not soluble in organic solvents except for lower alcohols, such as methanol. Obviously, alcohols cannot be used as solvents for esterification of the hydroxyl groups of GPC. The cadmium chloride complex of GPC, the structure of which is $(GPC)_2(CdCl_2)_3$, can be handled more easily than neat GPC and can be suspended in

* Corresponding author.
E-mail address: ichihara@kpu.ac.jp (K. Ichihara).

0009-3084/$ – see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.chemphyslip.2005.06.001

organic solvents for synthesis (Baer and Kates, 1948; Hanahan, 1962; Chada, 1970). However, the $CdCl_2$ must be removed from the acylation product of the GPC/$CdCl_2$ complex by either ion exchange resin or silica gel column chromatography (Gupta et al., 1977; Patel et al., 1979; Radhakrishnan et al., 1981; Davis and Keough, 1983; Rhodes et al., 1992), followed by disposal of the column material in a licensed landfill according to local regulations in order to avoid polluting the environment.

Heavy metals persist in the environment and bioaccumulation takes place in the food chain (Eisler, 2004). According to the Merck Index and International Labor Organization, cadmium is toxic to aquatic organisms. In humans, cadmium has effects on the kidneys and lungs, resulting in kidney impairment and tissue lesions by long-term exposure. Cadmium is also carcinogenic and may have toxic effects on human reproduction. In Japan, the oral intake of cadmium in food and water is known to cause a skeletal disease associated with a cadmium-induced renal disorder (Shiroishi et al., 1977; Friberg et al., 1985; Aoshima, 1987). This disease, known as Itai-itai disease, is characterized by progressive bone demineralization with painful joints and bones. For this reason, steps need to be taken to prevent cadmium entering the environment.

The present paper deals with a convenient method for the synthesis of diacyl PCs from GPC and a fatty acid, without the use of cadmium or any other heavy metal salt.

2. Experimental

2.1. Reagents

Oleic and linoleic acids, 99% pure, were kindly provided by Nippon Oil & Fats Co. (Amagasaki, Japan). Palmitic and heptadecanoic acids, and glass-distilled chloroform of HPLC grade were obtained from Sigma–Aldrich (St. Louis, MO). Dehydrated, amylene-stabilized chloroform was purchased from Wako Pure Chemicals Industries (Osaka, Japan) and was used as the solvent for synthesis. Methanol, acetone and hexane (Wako Pure Chemicals Industries) were purchased as glass-distilled solvents for analysis of residual pesticides and contained only very small amounts of evaporation residues. Neutral aluminum oxide (70–230 mesh) for column chromatography was obtained from Merck (Darmstadt, Germany) and its water content was adjusted to 2%. Silica gel 60 (0.063–0.200 mm) was also obtained from Merck for column chromatography. The kieselguhr product used was Hyflo Super-Cel (Celite Co., Santa Barbara, CA). Prior to use, the silica gel and kieselguhr were washed several times with methanol and then dried at 120 °C.

2.2. Lipid analysis

The purity of the diacyl PCs was checked by silica gel thin-layer chromatography (TLC) using solvent systems of choloroform/methanol/15 M NH_3 (65:35:5, v/v/v) and chloroform/methanol/acetone/water (65:35:5:4, v/v/v/v). Phospholipids on TLC plates (silica gel 60 F_{254}) were visualized with the molybdenum blue reagent of Dittmer and Lester (1964), and lipid classes were visualized by spraying 50% (w/w) sulfuric acid and then heating at 135 °C. Acylation catalysts on TLC plates were detected under ultraviolet light at 254 nm, and dicyclohexylcarbodiimide (DCC) and dicyclohexylurea (DCU) were detected under ultraviolet light at 352 nm after spraying with 0.001% primuline in ethanol. The acyl chain composition of these PCs was analyzed by gas–liquid chromatography of their fatty acid methyl esters prepared using an alkali-catalyzed transesterification with KOH/methanol in the presence of hexane (Ichihara et al., 1996, 2003). Infrared (IR) spectra were recored on a Hitachi 260-10 spectrophotometer. Analysis by matrix-assisted laser desorption/ionization time of flight mass spectrometry was performed on an AXIMA CFR Plus (Shimadzu, Kyoto, Japan).

2.3. Preparation of GPC from egg yolk PC

Because commercial GPC which had been stored for long time might be contaminated with impurities formed by hydrolysis and migration of the phosphoryl choline group in the molecule and because commercial egg yolk PC may also contain impurities, we prepared GPC from fresh egg yolk by solvent extraction and column chromatography. In brief, egg yolk (80 g) obtained from five hen's eggs was extracted with acetone three times to remove neutral lipids, pigments and water, and the phospholipids were extracted from the precipitate with chloroform/methanol (1:1, v/v). The PC was then

purified using column chromatography on neutral alumina (Renkonen, 1962; Singleton et al., 1965). Crude phospholipids (7.6 g) were applied to a glass column packed with 76 g of aluminum oxide in chloroform. After neutral lipids and pigments were washed away with acetone, PC was eluted with 500 ml of chloroform/methanol (90:10, v/v). Sphingomyelin (SPM) and phosphatidylethanolamine (PE) were retained on the alumina column. The yield of PC was 3.5 g.

GPC was prepared by the method of Brockerhoff and Yurkowski (1965). In brief, a portion (0.817 g, 1.06 mmol) of the pure egg yolk PC was dissolved in 8.2 ml of diethyl ether and deacylated with 0.82 ml of 1.0 M methanolic tetrabutylammonium hydroxide for 1 h at room temperature. The glassy precipitate of GPC was washed with diethyl ether and then dissolved in 0.3 ml of methanol. GPC was reprecipitated from the methanol solution by the addition of 9 ml of diethyl ether, and then the precipitate was washed with diethyl ether. After drying in vacuo over phosphorus pentoxide overnight, GPC was obtained in a yield of 96.8% (0.264 g, 1.03 mmol). Calcd. for $C_8H_{20}O_6NP$: C, 37.36; H, 7.84; N, 5.45; P, 12.04%. Found: C, 36.76; H, 7.88; N, 5.34; P, 11.81%.

2.4. Synthesis of dioleoyl PC from GPC

The GPC (1.03 mmol) was dissolved in 0.88 ml of methanol. The solution was added dropwise to 0.793 g of Hyflo Super-Cel in a Teflon vessel (30 ml centrifuge tube) with vortexing. The amount of kieselguhr must be 3 g or more per 1 g of GPC. Use of glassware for this treatment should be avoided because GPC adheres to the glass surface and is not adsorbed by kieselguhr. The GPC-adsorbed kieselguhr powder was dried over phosphorus pentoxide in vacuo overnight. If GPC-adsorbed kieselguhr hardened into beads, they were broken up with a spatula. The GPC–kieselguhr complex was transferred to a dried, ground-jointed Erlenmeyer flask and suspended in 20 ml of dry, alcohol-free chloroform. To the suspension were added 0.310 g of 4-dimethylaminopyridine (DMAP), 1.38 g of oleic acid and 1.01 g of DCC. The molar ratios of GPC/oleic acid/DCC/DMAP were 1.0:4.8:4.8:2.5. After flushing with N_2, the flask was closed with a ground-glass stopper. The reaction mixture was stirred at 30 °C overnight or more than 14 h in the dark, and then the kieselguhr was removed by filtration under reduced pressure. The kieselguhr residue was washed with a small volume of chloroform, and the washing was combined with the first filtrate. The diacyl PC was the only hydrophobic phosphorus product and no lysoPC was detected on TLC. The combined chloroform solutions were evaporated to dryness and the oily residue was dissolved in 10 ml chloroform. After removal of precipitated DCU by filtration, the solution was applied to a glass column (internal diameter, 2.2 cm) packed with 17.0 g of the methanol-washed silica gel in chloroform. The column was first washed with 120 ml of chloroform and then with 500 ml of chloroform/methanol (95:5, v/v), 500 ml chloroform/methanol (92:8, v/v) and with one 100-ml volume of chloroform/methanol (90:10, v/v) to elute oleic anhydride, free oleic acid, DCC, DCU and DMAP. Dioleoyl PC was then eluted with 1100 ml of chloroform/methanol (80:20, v/v). The dioleoyl PC gave a single spot on TLC plates (Fig. 1), when it was detected with primuline, the molybdenum blue reagent, or 50% sulfuric acid. Although a trace amount of DMAP was detected under UV light of $\lambda_{254\,nm}$, its content was estimated to be less than 0.1%. No contamination with silica gel was found in the PC product. The yield was 0.744 g (0.926 mmol, 90.3%). Calcd. for $C_{44}H_{86}O_9NP$: C, 65.72; H, 10.78; N, 1.74;

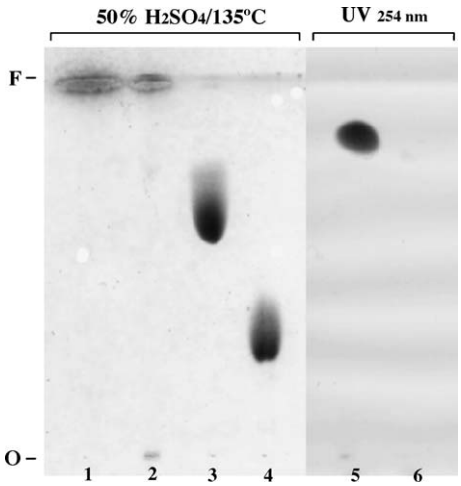

Fig. 1. TLC of the synthesized dioleoyl PC. Lane 1, DCC; lane 2, DCU; lane 3, oleic acid; lanes 4 and 6, PC that was synthesized and then purified by column chromatography; lane 5, DMAP. Spots were detected by spraying 50% H_2SO_4 and then heating at 135 °C for lanes 1–4, or under UV light at 254 nm for lanes 5 and 6. F, solvent front; O, origin. The solvent system was chloroform/methanol/15 M NH_3 (65:35:5, v/v/v).

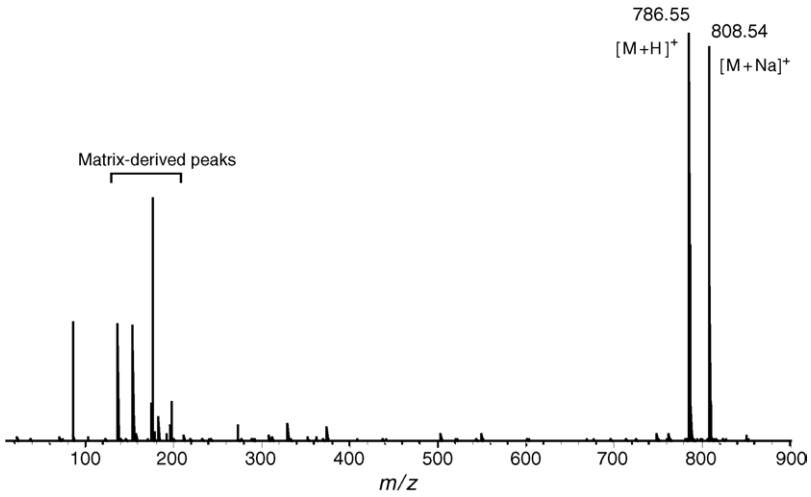

Fig. 2. Mass spectrum of the synthesized dioleoyl PC. The matrix used was 2,5-dihydroxybenzoic acid.

P, 3.85%. Found: C, 66.01; H, 11.05; N, 1.72; P, 3.92%. The structure and purity were confirmed by the IR spectrum (2920, 2850, 1730, 1460, 1240, 1090, 1060 and 970 cm^{-1}) and the mass spectrum (Fig. 2). When removal of the trace amount of DMAP was required, the PC was dissolved in 100 ml chloroform and then the solution was washed with 100 ml of 0.2 M HCl in 50% methanol. After the chloroform layer was washed with 100 ml of 50% methanol twice, the solvent was evaporated to dryness.

3. Results and discussion

3.1. Preparation of pure PC from egg yolk

Egg phospholipids consist of 73% PC, 15% PE, 6% lysoPC and 2.5% SPM (Rhodes and Lea, 1957), and the chromatographic behavior of egg-SPM is similar to that of PC. Thus, PC tends to be contaminated by SPM and lysoPC (Singleton et al., 1965). PC showing only a single spot by TLC can be readily prepared from crude egg yolk phospholipids using alumina column chromatography at atmospheric pressure without resorting to high-performance liquid chromatography (Guerts van Kessel et al., 1981). Using the above-mentioned procedure, lysoPC was eluted from the alumina column with chloroform/methanol (80:20, v/v), and neither sphingomyelin nor lysoPC was eluted even in the last PC fractions with chloroform/methanol (90:10, v/v). Although PC does not decompose on silica gel column chromatography, it takes a longer time to separate PC from PE with silica gel than with aluminum oxide.

3.2. Preparation of the GPC–kieselguhr substrate

GPC, but not its cadmium chloride complex, can be acylated with fatty acid anhydride in the presence of fatty acid potassium salt as a catalyst (Cubero Robles and Van den Berg, 1969; Barton and Gunstone, 1975). Although a large amount of soap can solubilize GPC in fatty acid anhydride, this method has some disadvantages. The reaction requires high temperatures (65–80 °C) and long times (48–72 h) under vacuum and with mechanical stirring. It also requires 2 mol of fatty acid potassium salt and 4 mol of fatty acid anhydride per 1 mol of GPC, i.e., at least 10 mol of fatty acid is needed per 1 mol of GPC to obtain yields of 81% for distearoyl PC and 71% for dioleoyl PC. Borsotti et al. (2001) synthesized PC from cadmium-free GPC, but the yields were 23–65%.

Warner and Benson (1977) developed a method for preparation of unsaturated PC from GPC suspended in dimethylsulfoxide and unsaturated fatty acid imidazolide in the presence of sodium methylsulfinylmethide as a catalyst, but they were not able to synthesize saturated PC by this procedure because of the limited solubility of long-chain saturated fatty acid imidazolides in

dimethylsulfoxide. The starting materials for dioleoyl PC were 1 mmol of oleic acid and 0.25 mmol of GPC, and the yield was 68%. In their reaction system, GPC adhered to the walls of the reaction vessels and it was necessary to warm the GPC suspension at 45 °C for several minutes to dislodge the adhering material prior to addition of acid anhydride. However, we could not successfully suspend GPC in dimethylsulfoxide and could not dislodge GPC from glass vessels using their treatment. These difficulties were overcome by preparing the GPC–kieselguhr complex and by using Teflon instead of glass vessels.

3.3. Catalyst for acylation

4-Dialkylaminopyridines act as nucleophilic acylation catalysts with broad substrate selectivity, and the active acylating reagents are the acyl pyridinium species (Höefle et al., 1978). DMAP is the most widely used dialkylaminopyridine, and the cadmium complex of GPC has been acylated with fatty acid anhydride in the presence of this catalyst under mild conditions (Gupta et al., 1977). 4-Pyrrolidinopyridine is more active than DMAP as an acylation catalyst (Mattai et al., 1987), and hence it has been used more often for synthesis of PC from the cadmium complex of GPC (Patel et al., 1979; Mason et al., 1981). However, when 4-pyrrolidinopyridine was employed in the present synthetic system, small amounts of a by-product were formed. The amount of the by-product was very small, but it could not be separated from PC by silica gel or alumina column chromatography. Patel et al. (1979) removed 4-pyrrolidinopyridine together with cadmium chloride by passing the reaction mixture through an ion exchange column. The catalyst could be removed by washing the chloroform solution of the reaction mixture with 0.5 M HCl in 50% methanol three times, but the by-product impurity could not. This phosphorus-negative impurity migrated close under authentic lysoPC on a TLC plate that was developed with chloroform/methanol/15 M NH_3 (65:35:5, v/v/v), and its amount was estimated to be less than 1% of PC by charring with 50% sulfuric acid on the plate. In contrast to 4-pyrrolidinopyridine, DMAP gave no by-products that were difficult to remove and the catalyst itself could be separated from PC by silica gel column chromatography and by washing with diluted HCl. Therefore, DMAP was selected as the catalyst, although the reaction time required for acylation was more than 14 h, as compared with less than 8 h with 4-pyrrolidinopyridine.

3.4. PC synthesis by acylation

In most methods for synthesis of PC from GPC or its cadmium chloride complex, the acyl donors used were acid anhydrides (Selinger and Lapidot, 1966), acid imidazolide or acid chloride. The donors were prepared prior to mixing with the GPC substrates, except for methods using fatty acid sodium salt as the acylation catalyst. Kodali et al. (1984) synthesized 1,2-dilauroyl-*sn*-glycerol from 1,2-isopropylidene-*sn*-glycerol and free lauric acid in the presence of DCC and DMAP. In a similar method, GPC–cadmium complex was esterified with a C_{18} fatty acid containing conjugated acetylenic bonds by stirring for 60 h (Rhodes et al., 1992). In the present study, PC was synthesized using a one-step method, in which two sequential reactions, the formation of fatty acid anhydride from fatty acid and the acylation of GPC on kieselguhr with the anhydride, are performed in a single reaction vessel. This one-step procedure is superior to the two-step method in its simplicity, efficiency and yield.

Dipalmitoyl PC was prepared using the above procedure with a yield of about 80%, which was lower than that of the corresponding dioleoyl PC. This was probably due to the reduced amount of DMAP (1.5 mol/mol GPC and not 2.5 mol/mol). Some acylating conditions caused the formation of 1,3-diacyl PC (Lammers et al., 1978; Keough and Davis, 1979), but that isomer was not formed in the present procedure.

3.5. Concluding remarks

The present method has a number of advantages as compared with other methods for synthesis of PC: (1) no cadmium compounds are included; (2) the reaction proceeds under mild conditions; (3) the procedure is simple and no sophisticated apparatus is required; (4) the purity and yield are similar to or better than those obtained by the methods reported previously.

Acknowledgement

We thank Shimadzu Corporation for mass analysis.

References

Aoshima, K., 1987. Epidemiology of renal tubular dysfunction in the inhabitants of a cadmium-polluted area in the Jinzu river basin in Toyama prefecture. Tohoku J. Exp. Med. 152, 151–172.

Baer, E., Kates, M., 1948. L-α-Glycerylphosphorylcholine. J. Am. Chem. Soc. 70, 1394–1399.

Barton, P.G., Gunstone, F.D., 1975. Hydrocarbon chain packing and molecular motion in phospholipid bilayers formed from unsaturated lecithins. J. Biol. Chem. 250, 4470–4476.

Borsotti, G., Guglielmetti, G., Spera, S., Battistel, E., 2001. Synthesis of phosphatidylcholine containing ricinoleic acid. Tetrahedron 57, 10219–10227.

Brockerhoff, H., Yurkowski, M., 1965. Simplified preparation of L-α-glyceryl phosphoryl choline. Can. J. Biochem. 43, 1777.

Chada, J.S., 1970. Preparation of crystalline L-α-glycerophosphorylcholine–cadmium chloride adduct from commercial egg lecithin. Chem. Phys. Lipids 4, 104–108.

Cubero Robles, E., Van den Berg, D., 1969. Synthesis of lecithins by acylation of *O*-(*sn*-glycero-3-phosphoryl) choline with fatty acid anhydrides. Biochim. Biophys. Acta 187, 520–526.

Davis, P.J., Keough, K.M.W., 1983. Differential scanning calorimetric studies of aqueous dispersions of mixtures of cholesterol with some mixed-acid and single-acid phosphatidylcholines. Biochemistry 22, 6334–6340.

Dittmer, J.C., Lester, R.L., 1964. A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. J. Lipid Res. 5, 126–127.

Eibl, H., 1980. Synthesis of glycerophospholipids. Chem. Phys. Lipids 26, 405–429.

Eisler, R., 2004. Mercury hazards from gold mining to humans, plants, and animals. Rev. Environ. Contam. Toxicol. 181, 139–198.

Friberg, L., Elinder, C.-G., Kjellstrom, T., Nordberg, G.F., 1985. Cadmium and Health: A Toxicological and Epidemiological Appraisal, vol. III. CRC Press, Boca Raton.

Guerts van Kessel, W.S.M., Tieman, M., Demel, R.A., 1981. Purification of phospholipids by preparative high pressure liquid chromatography. Lipids 16, 58–63.

Gupta, C.M., Radhakrishnan, R., Khorana, H.G., 1977. Glycerophospholipid synthesis: improved general method and new analogs containing photoactivable groups. Proc. Natl. Acad. Sci. U.S.A. 74, 4315–4319.

Hanahan, D.J., 1962. L-α-Glycerylphosphorylcholine. Biochem. Prep. 9, 55–58.

Höefle, G., Steglich, W., Vorbrüeggen, H., 1978. 4-Dialkylaminopyridines as highly active acylation catalysts. Angew. Chem. Int. Ed. Engl. 17, 569–583.

Ichihara, K., Shibahara, A., Yamamoto, K., Nakayama, T., 1996. An improved method for rapid analysis of the fatty acids of glycerolipids. Lipids 31, 535–539.

Ichihara, K., Yamaguchi, C., Nishijima, H., Saito, K., 2003. Preparation of FAME from sterol esters. J. Am. Oil Chem. Soc. 80, 833–834.

Jensen, R.G., Pitas, R.E., 1976. Synthesis of some acylglycerols and phosphoglycerides. Adv. Lipid Res. 14, 213–247.

Keough, K.M.W., Davis, P.J., 1979. Gel to liquid–crystalline phase transitions in water dispersions of saturated mixed-acid phosphatidylcholines. Biochemistry 18, 1453–1459.

Kodali, D.R., Atkinson, D.A., Redgrave, T.G., Small, D.M., 1984. Synthesis and polymorphism of 1, 2-dipalmitoyl-3-acyl-*sn*-glycerol. J. Am. Oil Chem. Soc. 61, 1078–1084.

Lammers, J.G., Liefkens, T.J., Van der Meer, J., 1978. Syntheses and spectroscopic properties of α- and β-phosphatidylcholines and phosphatidylethanolamines. Chem. Phys. Lipids 22, 293–305.

Mason, J.T., Huang, C., Biltonen, R.L., 1981. Calorimetric investigations of saturated mixed-chain phosphatidylcholine bilayer dispersions. Biochemistry 20, 6086–6092.

Mattai, J., Sripada, P.K., Shipley, G.G., 1987. Mixed-chain phosphatidylcholine bilayers: structure and properties. Biochemistry 26, 3287–3297.

Patel, K.M., Morrisett, J.D., Sparrow, J.T., 1979. A convenient synthesis of phosphatidylcholines: acylation of *sn*-glycero-3-phosphocholine with fatty acid anhydride and 4-pyrrolidinopyridine. J. Lipid Res. 20, 674–677.

Radhakrishnan, R., Robson, R.J., Takagaki, Y., Khorana, H.G., 1981. Synthesis of modified fatty acids and glycerophospholipid analogs. Methods Enzymol. 72, 408–433.

Renkonen, O., 1962. Breakdown of lecithin on aluminum oxide columns. J. Lipid Res. 3, 181–183.

Rhodes, D.G., Xu, Z., Bittman, R., 1992. Structure of polymerizable lipid bilayers. V: synthesis, bilayer structure and properties of diacetylenic ether and ester lipids. Biochim. Biophys. Acta 1128, 93–104.

Rhodes, D.N., Lea, D.N., 1957. Phospholipids 4. On the composition of hen's egg phospholipids. Biochem. J. 65, 526–533.

Selinger, Z., Lapidot, Y., 1966. Synthesis of fatty acid anhydrides by reaction with dicyclohexylcarbodiimide. J. Lipid Res. 7, 174–175.

Shiroishi, K., Kjellstrom, T., Kubota, K., Evrin, P.E., Anayama, M., Vesterberg, O., Shimada, T., Piscator, M., Iwata, T., Nishino, H., 1977. Urine analysis for detection of cadmium-induced renal changes, with special reference to β2-microglobulin. A cooperative study between Japan and Sweden. Environ. Res. 13, 407–424.

Singleton, W.S., Gray, M.S., Brown, M.L., White, J.L., 1965. Chromatographically homogeneous lecithin from egg phospholipids. J. Am. Oil Chem. Soc. 42, 53–56.

Warner, T.G., Benson, A.A., 1977. An improved method for the preparation of unsaturated phosphatidylcholines: acylation of *sn*-glycero-3-phosphorylcholine in the presence of sodium methylsulfinylmethide. J. Lipid Res. 18, 548–552.