notes on methodology

A convenient synthesis of phosphatidylcholines: acylation of *sn*-glycero-3-phosphocholine with fatty acid anhydride and 4-pyrrolidinopyridine

Kanu M. Patel, Joel D. Morrisett,[1] and James T. Sparrow[1,2]

Department of Medicine, Baylor College of Medicine and The Methodist Hospital, Houston, TX 77030

Summary A high-yield synthesis of saturated, unsaturated, and short chain phosphatidylcholines from *sn*-glycero-3-phosphocholine is described. The procedure offers advantages over other reported procedures for the synthesis of phosphatidylcholine in that the large-scale synthesis and purification can be achieved in a minimum time. The procedure utilizes 4-pyrrolidinopyridine as a catalyst and moderate amounts of fatty acid anhydride (2 mol eq. of fatty acid anhydride per mol of OH) in a 1:1 mixture of benzene–dimethylsulfoxide (DMSO) at 40°–42°C (oilbath) for 2–5 hr. At the end of the reaction, the phosphatidylcholine can be purified in the usual manner or by using a Waters Prep LC/500 with a radially compressed silica gel column eluted with chloroform–methanol–water 60:30:4. At a flow rate of 200 ml/min, the phospholipid elutes in 10–15 min, depending on the chain length and unsaturation.—**Patel, K. M., J. D. Morrisett, and J. T. Sparrow.** A convenient synthesis of phosphatidylcholines: acylation of *sn*-glycero-3-phosphocholine with fatty acid anhydride and 4-pyrrolidinopyridine. *J. Lipid Res.* 1979. **20:** 674–677.

Supplementary key words phospholipids · preparative liquid chromatography

Various procedures for the synthesis of phosphatidylcholines have been reported. Generally, they have involved the base-catalyzed acylation of *sn*-glycero-3-phosphocholine or its cadmium chloride complex with fatty acid–trifluoroacetic anhydride (1), fatty acid halides (2), anhydrides (3, 4), or anhydrides plus the corresponding fatty acid salt (5). The catalysts most often encountered are pyridine (1), 4-dimethylaminopyridine (3), or sodium oxide (6). More recent methods include acylation with fatty acid acylimidazole (7) alone or in the presence of the sodium salt of dimethylsulfoxide (8). The above procedures have several disadvantages, including phosphoryl migration (9), undesirable by-products, large amounts of fatty acid derivatives, low yields, long reaction times, and elevated temperatures. In addition, several of these methods are not generally applicable to the synthesis of all types of phosphatidylcholines, e.g., the fatty acylimidazole/sodium salt of DMSO procedure cannot be used with saturated fatty acids.

In this report we describe a rapid and simplified synthesis of phosphatidylcholines in high yield from fatty acid anhydrides, 4-pyrrolidinopyridine, and the cadmium chloride complex of *sn*-glycero-3-phosphocholine (**Fig. 1**). With a molar ratio of fatty acid anhydride to hydroxyl group of 2:1, the reaction proceeds to near completion within 2 hr for short chain and unsaturated phosphatidylcholines and within 5 hr for long chain saturated analogs. Using this procedure and the Waters Prep LC/500 for the purification, we have successfully synthesized and isolated on a relatively large scale unsaturated and saturated long chain or short chain phosphatidylcholines in greater than 70% yield in a short period of time.

MATERIALS AND METHODS

Crude egg phospholipids (Sigma Chemical Company, St. Louis, MO) were purified on a Waters Prep LC/500 as described previously (10). Tetrabutylammonium hydroxide (25% in methanol), cadmium chloride (anhydrous), and Rexyn I-300 were purchased from Fisher Scientific, Pittsburgh, PA; DMSO, Amberlite IR 45 and IRC-50 were from Mallinckrodt, St. Louis, MO. Myristic, palmitic, stearic, and arichidic acids were purchased from Nu-Chek Prep, Elysian, MN. Oleic and linoleic acids were from Sigma, St. Louis, MO. Hexanoic, octanoic, and elaidic acids were Aldrich "gold label", St. Louis, MO. All fatty acids were greater than 99% pure as determined by GLC. 4-Pyrrolidinopyridine was synthesized by the procedure of Vorbrüggen (11) and by the simplified procedure of Patel and Sparrow (12). Total phosphorus was determined by the method of Bartlett (13). Chloroform and methanol were Burdick-Jackson "glass distilled", Muskegon, MI. Silica gel plates were from Brinkmann, Westbury, NY. Dicyclohexylcarbodiimide was purchased from Schwarz-Mann, Orangeburg, NY. Benzene was dried and stored over sodium metal; dimethylsulfoxide was dried over activated alumina and stored over 4 Å molecular sieves. Fatty acid anhydrides were prepared according to Selinger and Lapidot (14).

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; DMSO, dimethylsulfoxide; GPC, *sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry.

[1] Established Investigator, American Heart Association.

[2] To whom correspondence should be addressed.

Preparation of cadmium chloride complex of *sn*-glycero-3-phosphocholine

Using 22 g of pure egg yolk phosphatidylcholine, free *sn*-glycero-3-phosphocholine was prepared as described by Brockerhoff and Yurkowski (15). The free *sn*-glycero-3-phosphocholine was dissolved in 90% ethanol and added slowly to a solution of 10 g of cadmium chloride in 10 ml of water to which had been added 90 ml of absolute ethanol; a heavy white precipitate appeared which was stored at 0°C for 4 hr. The precipitate was collected and washed with 200 ml of ether and 200 ml of absolute ethanol. Drying over P_2O_5 for 2 days gave 14.5 g (88%) of $(GPC)_2(CdCl_2)_3$. Phosphorus: calculated for $(GPC)_2(CdCl_2)_3$ (mol wt 1100), 5.63%; found, 5.6%.

General procedure for the synthesis of phosphatidylcholines

The cadmium chloride complex (1.1 g, 1 mmol; dried at 50°C in vacuo for 2 hr over P_2O_5) of *sn*-glycero-3-phosphocholine was dissolved in 5 ml of dry dimethylsulfoxide at 45°C. A solution of the appropriate fatty acid anhydride (8 mmol) in 5 ml of dry benzene containing 0.29 g (2 mmol) of 4-pyrrolidinopyridine was added to the warm dimethylsulfoxide solution. The nearly homogeneous solution was stirred for 2–5 hr at 42–45°C under a N_2 atmosphere. The progress of the reaction was monitored by thin-layer chromatography on silica gel. The silica gel plates were developed in chloroform–methanol–water 60:30:4 and lipid spots were detected with I_2 and/or phospholipid spray (16). After the reaction was judged complete (usually after 3 hr) by TLC, the reaction mixture was diluted with 100 ml of chloroform–methanol–water 5:4:1 and passed through a mix-bed IR 45/IRC 50 column (2.5 × 50 cm) to remove the cadmium chloride and 4-pyrrolidinopyridine; the column was eluted with an additional 200 ml of the solvent mixture. After removing most of the solvent by rotary evaporation, the residue was injected directly onto the Waters Prep LC/500 radially compressed silica gel column pre-equilibrated with chloroform–methanol–water 60:30:4 as previously described (10). (In lieu of the Waters Prep LC/500 purification procedure, standard silicic acid column chromatography can be used at this point to purify the product.) Fractions of ~400 ml were collected and spotted on TLC plates to confirm the location of the phosphatidylcholine. The appropriate fractions were pooled and the solvent was evaporated. The residue was dissolved in benzene, frozen, and lyophilized. The phosphatidylcholines were pure by TLC and were characterized according to their transition temperatures by differential scanning calorimetry and their rotations at the sodium D line; all agreed with the reported values. GLC analysis of the transesterified methyl esters from the unsaturated phospholipids revealed the absence of isomerization of the double bonds.[3] The yields, elution times, rotations, and transition temperatures are given in **Table 1.**

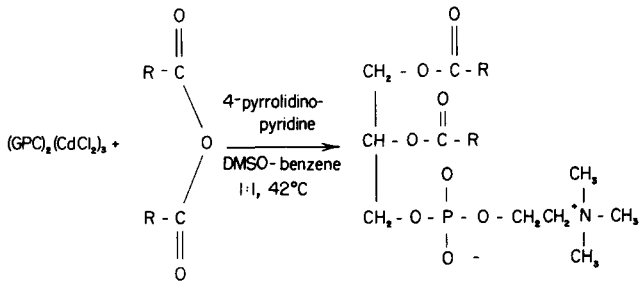

Fig. 1. Synthesis of phosphatidylcholines.

Large-scale preparation of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine

The $(GPC)_2(CdCl_2)_3$ complex (9.6 g, 8.7 mmol) was dissolved in 15 ml of dry dimethylsulfoxide at 42°C. A solution of 29 g (66 mmol) of myristic anhydride and 2.57 g (17.4 mmol) of 4-pyrrolidinopyridine in 15 ml of dry benzene was added and the reaction was stirred at 42°C for 5 hr. The reaction mixture was poured into 200 ml of chloroform–methanol–water 5:4:1 and this solution was passed through a Rexyn I-300 or mix-bed IR 45/IRC 50 column (2.5 × 75 cm). After washing the column with 200 ml of the same solvent, the combined eluants were partially evaporated and the residue was injected onto the Waters Prep LC/500. Fractions 1 to 9 (**Fig. 2**) were collected. TLC indicated that fractions 5 to 7 (combined volume ~1.4 l) contained only phosphatidylcholine. The combined fractions were evaporated and the residue was lyophilized from benzene and dried over P_2O_5 to give 10.5 g (88%) of the title compound. Phosphorus: calculated for $C_{36}H_{72}NO_8P$ (mol wt 678), 4.57%; found 4.57%.

[3] GLC analyses were performed on a Hewlett Packard 5830A with flame ionization detector on a column containing 15.5% OV-275 on 100/120 Chromasorb P AW-DMCS 20 ft × ⅛ in SS column (Supelco, Bellefonte, PA). Temp 220°C; He flow rate, 10 ml/min; sample size, 0.5 μl at a concentration of 10 mg/ml in carbon disulfide. The advantage of this packing material is that *cis* and *trans* isomers of C_{18} fatty acid methyl esters can be separated, i.e., methyl elaidate elutes at 28.91 min, methyl oleate elutes at 30.73 min, methyl linoelaidate elutes at 33.11 min, and methyl linoleate elutes at 38.21 min. In the case of 1,2-dilinoleoyl- and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, only the *cis* isomer was present; no oxidation products were observed.

TABLE 1. Yields and physical properties of synthetic phosphatidylcholines

Phosphatidylcholine	Reaction Time	% Yield	Elution Time[a]	DSC[b]		Rotation[c]	
				Lower T_{m_1}	Upper T_{m_2}		
	hr		*min*	*°C*			
Dihexanoyl	2	72	20			+10.5	c = 6.5
Dioctanoyl	2.5	76	16			+9.7	c = 4.74
Dimyristoyl	5	88	11	14.3	24.1	+7.0	c = 4.2
Dipalmitoyl	5	78	12	35.4	42.4	+6.6	c = 4.3
Distearoyl	5	78	12	51.4	55.5	+6.4	c = 4.59
Diarichidoyl[d]	5	65	13	62.9	66	+6.11	c = 4.5
Dioleoyl	2.5	85.5	10			+6.46	c = 5.56
Dielaidoyl	2.5	79	10			+6.9	c = 5.34
Dilinoleoyl	2.5	75	10				

[a] Prep LC/500 (Waters Associates, Milford, MA) purification was carried out with chloroform–methanol–water 60:30:4 (v/v) at a relative detector response of 2 and a chart speed of 2 min/cm; the flow rate was 200 ml/min. After each injection, the column was flushed with chloroform–methanol–water 60:40:10 and re-equilibrated with a 60:30:4 mixture until a stable baseline was obtained.

[b] Differential scanning calorimetry (Perkin Elmer DSC-2) was performed on hydrated phosphatidylcholine at a heating rate of 1.25 deg/min; range 2 mcal/sec. Lit. (18) values in °C for dimyristoyl: T_{m_1}, 14.2; T_{m_2}, 23.9; dipalmitoyl: T_{m_1}, 35.3; T_{m_2}, 41.4; distearoyl: T_{m_1}, 51.5; T_{m_2}, 54.9.

[c] The optical rotations were determined on a Perkin-Elmer 241 polarimeter in a 10-cm microcell at the Na/D line. All samples were prepared in chloroform–methanol 1:1 (v/v) except that of dihexanoyl, which was in pure chloroform. Lit. values for dimyristoyl: +7.0°; dipalmitoyl: +6.6°; distearoyl: +6.4° (1); dioleoyl: +6.0 (2).

[d] For diarichidoylphosphatidylcholine, the reaction temperature was initially 55°C for 2 hr and then 45°C for 3 hr.

Synthesis of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine

The $(GPC)_2(CdCl_2)_3$ complex (4.75 g, 4.3 mmol) was dissolved in 5 ml of dry dimethylsulfoxide at 42°C. A solution of 18.9 g (34.5 mmol) of oleic anhydride in 5 ml of dry benzene was added, followed by 1.3 g (8.78 mmol) of 4-pyrrolidinopyridine; the reaction mixture was stirred at 42°C for 2 hr. The reaction was complete as judged by TLC and was worked up as for 1,2,dimyristoyl-*sn*-glycero-3-phosphocholine to give 5.8 g (85.8%) of pure 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; however in order to remove $CdCl_2$ and 4-pyrrolidinopyridine, mixed IR 45/IRC 50 resin was used.[4] Phosphorus: calculated for $C_{44}H_{84}NO_8P$ (mol wt 786), 3.94%; found 3.90%.

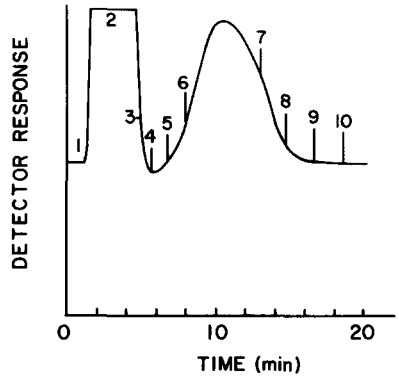

Fig. 2. Elution profile from the Waters Prep LC/500 for dimyristoyl phosphatidylcholine. After ion-exchange chromatography and evaporation to ~50 ml, the reaction mixture was injected onto the silica column equilibrated in $CHCl_3$–MeOH–H_2O 60:30:4. The flow rate was 200 ml/min and the detector response was 2. Fractions 1–3 contained fatty acid, fatty acid anhydride, and dimethylsulfoxide. Fractions 5–7, collected between 7 and 14 min, contained the dimyristoylphosphatidylcholine in ~1.4 l of solvent.

RESULTS AND DISCUSSION

The solubility of the cadmium chloride complex of *sn*-glycero-3-phosphocholine in warm dimethylsulfoxide led us to utilize this solvent for the acylation reaction. A nearly homogeneous reaction mixture resulted by adding to this solution the fatty acid anhydride and 4-pyrrolidinopyridine in an equal volume of dry benzene. Following the progress of the reaction by thin-layer chromatography on silica gel plates indicated that large quantities of phosphatidylcholine had formed in 2–5 hr depending on the chain length of the fatty acid anhydride, i.e., for short chain and unsaturated fatty acids, 2.5 hr and for long chain saturated fatty acids, 5 hr. After purification by ion exchange, IR 45/IRC 50 or Rexyn I-300, and radially compressed silica gel chromatography, the synthetic phosphatidylcholines were obtained in high yield (Table 1) and were homogeneous on TLC in chloroform–methanol–water 95:35:6; this system was shown by Lammers et al (9)

[4] It was found that, at this stage, if the reaction mixture were passed through a Rexyn I-300 resin, 10–15% isomerization to the *trans* isomer occurred, as observed by GLC analysis.

to separate 1,2-diacyl-*sn*-glycero-3-phosphocholine from 1,3-diacyl-*sn*-glycero-2-phosphocholine. The synthetic materials had optical rotations comparable to those reported (2, 17); the transition temperatures by differential scanning calorimetry were in agreement with the reported values (18). Phospholipase A_2 cleavage (6) of the synthetic products was complete after 1.5 hr; only fatty acid and lysophosphatidylcholine were observed by TLC. We feel that the procedures described in this communication will be useful to the synthetic lipid chemist since they provide high yields of pure product in a minimum time.

The authors wish to thank Dr. Henry J. Pownall for assistance in obtaining the DSC thermograms, Ms. Kaye Shewmaker for preparing the figures, and Ms. Saundra Wrye for typing the manuscript. This research material was supported by the Atherosclerosis and Lipoprotein Section of the National Heart and Blood Vessel Research and Demonstration Center, Baylor College of Medicine, a grant-supported research project of the National Heart, Lung, and Blood Institute, National Institutes of Health, grant no. 17269.

Manuscript received 8 January 1978; accepted 26 January 1979.

REFERENCES

1. Pugh, E. L., and M. Kates. 1975. A simplified procedure for synthesis of di[^{14}C]acyl-labeled lecithins. *J. Lipid Res.* **16:** 392–394.
2. Baer, E., and D. Buchnea. 1959. Synthesis of saturated and unsaturated 1-α-lecithins. Acylation of the cadmium chloride compound of L-α-glyceryl-phosphorylcholine. *Can. J. Biochem. Physiol.* **37:** 953–959.
3. Gupta, C. M., R. Radhakrishnan, and H. G. Khorana. 1977. Glycerophospholipid synthesis: Improved general method and new analogs containing photoactivable groups. *Proc. Natl. Acad. Sci. USA.* **74:** 4315–4319.
4. Yabusaki, K. K., and M. A. Wells. 1976. 1-Hydroxy-2-*tert*-butyldimethylsilyl-glycero-3-phosphorylcholine. A useful intermediate in the synthesis of short acyl chain 1-acyl-*sn*-glycero-3-phosphorylcholines. *Chem. Phys. Lipids.* **17:** 120–127.
5. Cubero Robles, E., and D. van den Berg. 1969. Synthesis of lecithins by acylation of *O*-(*sn*-glycero-3-phosphoryl)choline with fatty acid anhydrides. *Biochim. Biophys. Acta.* **187:** 520–526.
6. Hubbell, W. L., and H. M. McConnell. 1971. Molecular motion in spin-labeled phospholipids and membranes. *J. Am. Chem. Soc.* **93:** 314–326.
7. Boss, W. F., C. J. Kelley, and F. R. Landsberger. 1975. A novel synthesis of spin-label derivatives of phosphatidylcholine. *Anal. Biochem.* **64:** 289–292.
8. Warner, T. G., and A. A. Benson. 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.
9. Lammers, J. G., T. J. Liefkens, J. Bus, and J. van der Meer. 1978. Synthesis and spectroscopic properties of α- and β-phosphatidylcholines and phosphatidylethanolamines. *Chem. Phys. Lipids.* **22:** 293–305.
10. Patel, K. M., and J. T. Sparrow. 1978. Rapid, large-scale purification of crude egg phospholipids using radially compressed silica gel columns. *J. Chromatogr.* **150:** 542–543.
11. Vorbruggen, H. 1972. Simple amination of aromatic hydroxy-nitrogen heterocycles. *Angew. Chem. Int. Ed. Engl.* **11:** 305–306.
12. Patel, K. M., and J. T. Sparrow. 1978. Phase transfer catalysis: Reaction of 4-bromopyridine with cyclic secondary amines. *Synth. Commun.* **9:** 251–253.
13. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
14. Selinger, Z., and Y. Lapidot. 1966. Synthesis of fatty acid anhydrides by reaction with dicyclohexylcarbodiimide. *J. Lipid Res.* **7:** 174.
15. Brockerhoff, H., and M. Yurkowski. 1965. Simplified preparation of L-α-glyceryl phosphoryl choline. *Can. J. Biochem.* **43:** 1777.
16. Dittmer, J. C., and R. L. Lester. 1964. A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. *J. Lipid Res.* **5:** 126–127.
17. Baer, E., and M. Kates. 1950. Synthesis of enantiomeric-α-lecithins. *J. Am. Chem. Soc.* **72:** 942–949.
18. Mabrey, S., and J. M. Sturtevant. 1976. Investigation of phase transitions of lipids and lipid mixtures by high sensitivity differential scanning calorimetry. *Proc. Natl. Acad. Sci. USA.* **73:** 3862–3866.