notes on methodology

A simplified procedure for synthesis of di-[^{14}C]acyl-labeled lecithins

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Summary A simplified procedure for synthesis of 1,2-di-[1′-^{14}C]oleoyl-, 1,2-di-[1′-^{14}C]linoleoyl-, and 1,2-di-[1′-^{14}C]eicosatrienoyl-*sn*-glycero-3-phosphorylcholine is described. The method involves acylation of the $CdCl_2$ complex of glycerophosphorylcholine with a ^{14}C-labeled fatty acid in the presence of trifluoracetic anhydride and pyridine. The ^{14}C-labeled lecithin is isolated in pure form by preparative thin-layer chromatography and alumina column chromatography in an overall yield of 12–24%. No isomerization or peroxidation of the unsaturated acids was detected.

Supplementary key words ^{14}C-labeled dioleoyl-, dilinoleoyl-, and dieicosatrienoyl lecithins · trifluoroacetic anhydride · $CdCl_2$ complex of glycerophosphorylcholine · silicic acid–$AgNO_3$ thin-layer chromatography

Studies on the metabolism or enzymatic hydrolysis of phospholipids often require pure radioisotopically labeled phospholipids of known structure and with known or specified fatty acyl components that can only be supplied by chemical synthesis. However, the available synthetic procedures (e.g., for lecithin [1–3]) are not readily adaptable to small-scale preparation of labeled material and usually involve the use of fatty acid chlorides or anhydrides, which are difficult to prepare on a small scale.

During investigations of a phospholipid desaturase in the yeast *Candida lipolytica* (4, 5), we required some di-[^{14}C]oleoyl lecithin as substrate. Attempts to synthesize this material by acylation of the $CdCl_2$ complex of glycerophosphorylcholine (GPC) with [^{14}C]oleoyl chloride (prepared from [^{14}C]oleic acid by reaction with oxalyl chloride) following the method of Baer and Buchnea (1) gave only poor yields of ^{14}C-labeled lecithin in which the oleoyl chains were partially isomerized and peroxidized. We have therefore modified this procedure (1), so as to be able to use the ^{14}C-labeled free fatty acid rather than the acid chloride, by utilizing trifluoroacetic anhydride (6) as a catalyst. This compound has been shown to act by rapidly forming a mixed anhydride with a free carboxylic acid, which then acts as the acylating agent (6).

We report here the synthesis, on a micromole scale, of 1,2-di-[^{14}C]oleoyl-, 1,2-di-[^{14}C]linoleoyl-, and 1,2-di-[^{14}C]eicosatrienoyl-*sn*-glycero-3-phosphorylcholine.

*Synthesis of 1,2-di-[^{14}C]oleoyl-*sn*-glycero-3-phosphorylcholine.* A solution of [1-^{14}C]oleic acid (Amersham/Searle Corp.; 162 μmoles of unlabeled oleic acid plus 1 μmole of [^{14}C]oleic acid containing 50 μCi) in benzene was added to 6.8 μmoles (7.5 mg) of the $CdCl_2$ complex of 3-*sn*-glycerophosphorylcholine (7) ($[C_8H_{22}O_8NP]_2[CdCl_2]_3$, equivalent to 13.6 μmoles of GPC), which had been dried in vacuo at 56°C for 5 hr in a glass-stoppered tube (2 ml). The solvent was removed under a stream of nitrogen, and 4 mmoles (0.3 ml) of anhydrous pyridine or triethylamine[1] was added, followed by 325 μmoles (0.07 ml) of trifluoroacetic anhydride. A small stirring bar was introduced, the tube was flushed with nitrogen, and the reaction mixture was stirred for 2 days at room temperature. The reaction was terminated by the addition of 0.1–0.2 ml of methanol, and the mixture was brought to dryness under a stream of nitrogen. Lipids were extracted by addition of 2 ml of methanol and 2 ml of chloroform followed by 1.8 ml of 0.01 N HCl. After centrifugation, the chloroform phase was removed, diluted with an equal volume of benzene (to remove traces of water), and brought to dryness on a rotary evaporator. The residual lipids were subjected to preparative TLC (4) on silica gel H (0.75 mm thick; Brinkmann Instruments Ltd.) using the solvent system chloroform–methanol–water 65:25:4 (v/v). The [^{14}C]lecithin (R_f 0.31), eluted from the plate with chloroform–methanol–water 1:2:0.8 (v/v) as described elsewhere (8), was chromatographically pure (**Fig. 1**) and was obtained in a yield of 1.6 μmoles (12% from GPC) with a specific activity of 0.6 μCi/μmole of phosphorus. Only traces of [^{14}C]lysolecithin were detected in the reaction mixture when the above conditions were followed. The unreacted [^{14}C]oleate was recovered by elution of the band moving with the solvent front, using chloroform–benzene 1:1 (v/v).

*Synthesis of 1,2-di-[^{14}C]linoleoyl-*sn*-glycero-3-phosphorylcholine and 1,2-di-[^{14}C]eicosatrienoyl-*sn*-glycero-3-phosphorylcholine.* [1-^{14}C]Linoleic acid (*cis-cis*-9,12-octadecadienoic acid; New England Nuclear) or [1-^{14}C]homo-γ-linolenic acid (all-*cis*-8,11,14-eicosatrienoic acid; New England Nuclear) (81 μmoles of the unlabeled

Abbreviations: GPC, glycerophosphorylcholine; TLC, thin-layer chromatography.

[1] Use of this base avoids formation of brown pigments formed when pyridine is used, but it does not change the yield or purity of the product.

fatty acid plus 1 μmole of the respective ^{14}C-labeled fatty acid containing 50 μCi) was reacted with 3.4 μmoles of the complex (3-*sn*-GPC)$_2$($CdCl_3$)$_3$ (containing 6.8 μmoles of GPC) in the presence of 4 mmoles (0.3 ml) of anhydrous pyridine or triethylamine and 162 μmoles of trifluoroacetic anhydride as described above for dioleoyl lecithin. The reaction mixture was processed as described above, and the lipid products were chromatographed on a column of 1 g of silicic acid (100–200 mesh; Bio-Rad Laboratories, Richmond, Calif.). The column was eluted successively with 10 ml each of chloroform–methanol 9:1 (v/v), 1:1 (v/v), and 1:4 (v/v); the eluates were monitored by thin-layer chromatography. The unreacted ^{14}C-labeled fatty acids (as methyl esters) were quantitatively recovered in the chloroform–methanol 9:1 eluates. The ^{14}C-labeled lecithin was obtained in the chloroform–methanol 1:1 eluate and was further purified by chromatography on 1 g of aluminum oxide (neutral, activity grade 1 for chromatography; M. Woelm, Eschwege, Germany). The alumina column was eluted successively with chloroform, chloroform–methanol 1:1 (v/v), and chloroform–ethanol–water 2:5:2 (v/v) according to the method of Hanahan, Turner, and Jayko (9). Each of the ^{14}C-labeled lecithins was recovered in the chloroform–methanol 1:1 eluate and was chromatographically pure (Fig. 1); R_f values were 0.31 in chloroform–methanol–water 65:25:4 (v/v) and 0.28 in chloroform–methanol–acetic acid–water 25:15:4:2 (v/v). The 1,2-di-[^{14}C]linoleoyl-*sn*-glycero-3-phosphorylcholine was obtained in a yield of 24% with a specific activity of 1.2 μCi/μmole of phosphorus, and the 1,2-di-[^{14}C]eicosatrienoyl-*sn*-glycero-3-phosphorylcholine in a yield of 12% with a specific activity of 1.1 μCi/μmole of phosphorus.

Fatty acid composition of synthetic lecithins. The three ^{14}C-labeled lecithins prepared as described above were deacylated by mild alkaline hydrolysis according to the procedure of Dawson (10), modified as described elsewhere (11). The resulting fatty acid methyl esters were separated into saturated, monoenoic, dienoic, etc., fractions on silica gel G plates impregnated with 10% $AgNO_3$ in the solvents given in **Table 1**; radioactive bands were located by radioautography and scraped into vials, and ^{14}C was determined by scintillation counting as described elsewhere (4).

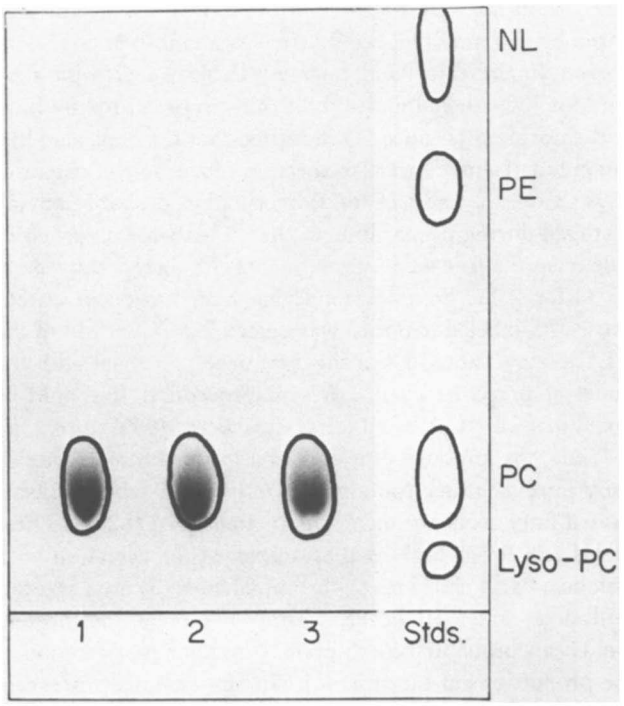

Fig. 1. Thin-layer chromatogram and radioautogram of: *1*, di-[^{14}C]-oleoyl lecithin; *2*, di-[^{14}C]linoleoyl lecithin; and *3*, di-[^{14}C]eicosatrienoyl lecithin. Standards (Stds.): NL, neutral lipids (fatty acid methyl esters + free fatty acids); PE, phosphatidylethanolamine; PC, phosphatidylcholine; and Lyso-PC, lysophosphatidylcholine. Solvent system, chloroform–methanol–water 65:25:4 (v/v). Spots were detected with iodine vapor. All standard phospholipid samples were obtained from Serdary Research Laboratories, Inc., London, Canada.

TABLE 1. Fatty acid composition of synthetic ^{14}C-labeled lecithins

[^{14}C] Lecithin	^{14}C in Methyl Esters Analyzed[a]	Distribution of ^{14}C in Fatty Acid Fractions[b]				
		trans-Monoenes	*cis*-Monoenes	*cis*-Dienes	*cis*-Trienes	*cis*-Tetraenes
	dpm × 10^{-3}			*% of total*		
1,2-Di-[^{14}C] oleoyl-GPC[c]	9.8	0	99	1		
1,2-Di-[^{14}C] oleoyl-GPC[d]	25.0	16	77	1		
1,2-Di-[^{14}C] linoleoyl-GPC[c]	9.6			98.8	1.2	0
1,2-Di-[^{14}C] eicosatrienoyl-GPC[c]	9.6			0	98.5	1.5

[a] Fatty acid methyl esters were prepared by mild alkaline deacylation (10, 11) of the ^{14}C-labeled lecithins.

[b] Determined by chromatography of fatty acid methyl esters on $AgNO_3$-impregnated silica gel G plates in the following solvents: chloroform–ethanol 200:1.5 (v/v) for *cis*-monoenes (R_f 0.60), *trans*-monoenes (elaidic acid, R_f 0.68), and saturated species (R_f 0.75), and chloroform–methanol 98:2 (v/v) for *cis*-dienes (R_f 0.53), *cis*-trienes (R_f 0.32), and *cis*-tetraenes (R_f 0.17).

[c] Prepared by procedure described in this paper. Only traces of ^{14}C could be detected at the origin of the TLC plates of the fatty acid methyl esters. No ^{14}C-labeled saturated species were present.

[d] Prepared by procedure using [^{14}C]oleoyl chloride (1). ^{14}C (5–6%) was present at the origin of the TLC plate of the fatty acid methyl esters.

The results showed that each of the synthetic lecithins prepared by our modified procedure was about 99% pure with respect to the fatty acid moiety (Table 1). Comparative data for ^{14}C-labeled dioleoyl lecithin prepared by the fatty acyl chloride procedure (1) indicated that the oleic acid had undergone isomerization to the *trans* form to the extent of 16% (Table 1), most of the isomerization probably having occurred during preparation of the ^{14}C-labeled oleoyl chloride from the free acid by treatment with oxalyl chloride at 63°C for 2 hr. Some peroxidation had also occurred because ^{14}C-labeled material was detected at the origin of the TLC plate (Table 1), but this was never observed with the material prepared by the present procedure. It should be noted that all ^{14}C-labeled fatty acids were >99% pure.

Thus, the procedure described affords chromatographically pure lecithins, containing two like ^{14}C-labeled unsaturated fatty acids per molecule, in yields of 12–24%. These yields are not as high as those reported for acylation with unlabeled acyl chlorides (1) or anhydrides (3) on a several-millimole scale. However, improvements of the present yields can probably be achieved by further modification of the proportions of the reactants. In any case, the unreacted ^{14}C-labeled fatty acids can readily be recovered and recycled. Finally, it should be noted that isomerization or peroxidation of the unsaturated ^{14}C-labeled fatty acids did not occur under the conditions employed in the present procedure.

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