PREPARATION OF LABELLED PHOSPHOLIPIDS:

Acylation of 2-lysophosphatidylcholine with [9,10-^{3}H]oleic acid.

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SUMMARY

1-Acyl-2-[9,10-^{3}H]oleoyl-sn-glycero-3-phosphorylcholine was prepared by acylating 1-acyl-sn-glycero-3-phosphorylcholine with labelled oleic acid, in the presence of dicyclohexylcarbodiimide (DCC), as condensing agent. A simple column chromatographic procedure allowed both purification of the labelled phospholipid and recovery of the unreacted oleic acid, at the end of the reaction. In standard conditions, yields of 35-40 % were obtained.

The major feature of this procedure is that it is a "one pot" reaction not requiring the isolation of an activated acid derivative prior to esterification.

This method is also suitable for preparing labelled phospholipids other than phosphatidylcholine, provided that free or unmasked amino or hydroxyl groups are absent in the molecule.

INTRODUCTION

Specific labelling of phospholipids in the acyl moiety can be carried out in a convenient way according to many published procedures. However, the enzymatic acylation of lyso-phospholipids, catalyzed by microsomal enzymes (1-5), yields

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phospholipids of low and unpredictable specific activity, due to the presence of consistent amounts of free fatty acids in the microsomal preparations. On the other hand, the acylation of lysophospholipids with acylchlorides or other fatty acid derivatives (6-10) does not seem suitable for small scale laboratory preparations. In fact, strictly anhydrous conditions are to be kept during reaction and, in addition, reagents have to be handled in very small reaction volumes.

The aim of the present work was to develope a rapid and easy procedure for introducing a labelled fatty acid into phospholipid molecules. The acylation of lysophosphatidylcholine is described in this paper; however, the same method is also suitable for labelling other phospholipids.

EXPERIMENTAL

Preparation of lysophosphatidylcholine

2-Lysophosphatidylcholine (Lyso-PC) was prepared by hydrolysis of ox brain phosphatidylcholine (PC) with phospholipase A_2 from porcine pancreas (1). 750 mg (1 meq) of PC were solubilized in 500 ml of peroxide-free and water-saturated diethyl ether. 46 ml of an aqueous solution containing 10 mM Tricine buffer (pH 8.0 - Sigma) and 1 mM $CaCl_2$ were added to the ether solution together with 0.23 ml (2.3 mg) of a phospholipase A_2 suspension from porcine pancreas (600 I.U./mg - Sigma /P 9139) and 32 ml of methanol. The mixture was incubated at 36-37°C for three hours with magnetic stirring.

PC hydrolysis was monitored during the incubation by analyzing small samples of the reaction mixture by TLC on Silica

gel G plates (Merck) developed with chloroform/methanol/acetic acid/water; 25:15:4:2 (by vol).

At the end of the reaction, the ether phase was removed and the lower phase was acidified to pH 4-5 with 0.1 N HCl. The acid suspension was extracted three times with petroleum ether (40-70°C) to remove free fatty acids, then neutralized with NaOH and dried under vacuum. Lyso-PC was extracted from the solid residue with Chloroform/Methanol; 2:1 (by vol).

Alternatively, the lower phase was evaporated under vacuum, the residue dissolved in Chloroform/Methanol; 2:1 (by vol.) and lyso-PC was isolated by preparative TLC on Silica Gel G plates with the same developing system described above. Lyso-PC was identified on the plates by light exposure to iodine vapours and eluted from the silica scrapes with chloroform/methanol/water; 65:25:4 (by vol).

The purity of the lyso-PC isolated by both methods was tested by TLC.

Acylation of 2-lysophosphatidylcholine

In a standard preparation, 5 mCi of [9,10-^{3}H]oleic acid (Amersham - TRK 140), added to unlabelled oleic acid (Merck) in order to obtain a specific activity of 5 mCi/mmol, were dissolved in freshly-distilled ethyl ether (5 ml). Lysophosphatidylcholine (500 mg, 1 mmol), DCC (226 mg, 1 mmol) and dimethylaminopyridine (12 mg, 0.1 mmol) were added to the ether solution. The reaction was allowed to proceed for 20 hours at room temperature with magnetic stirring. The reaction mixture was centrifuged and the ether phase was decanted. The reaction vessel and the

dicyclohexylurea precipitate were washed three times with diethyl ether. The ether phases were pooled, filtered and evaporated under vacuum. The residue was dissolved in a small volume of chloroform.

1-Acyl-2-[9,10-^{3}H]oleoyl-sn-glycero-3-phosphorylcholine of higher specific activity (2 Ci/mmol) was synthesized on a micro-scale by reacting 100 mCi of [9,10-^{3}H]oleic acid (S.A. 2 Ci/mmol) in a final reaction volume of 0.25 ml, taking constant the reagent concentrations.

The acylation reaction has been carried out in acetonitrile or dichloromethane, in some preparation, to study the solvent-effect on the yield of the reaction.

Characterization of the product.

Labelled PC was purified by column chromatography (1.2x25 cm) on Silica gel H (Merck), equilibrated in chloroform. Unreacted oleic acid was eluted first with chloroform, then PC and lyso-PC were separately eluted with chloroform/methanol/water; 65:25:4 (by vol.)(11). Fractions (3 ml) were collected and radioactivity and phosphorus quantitated in each fraction (12).

The radiochemical purity of the labelled PC was checked by HPTLC on Silica Gel plates (Merck n.5641), using chloroform/methanol/acetic acid/water; 25:15:4:2 (by volume), as developing system.

The stereochemical purity of the isolated product was assessed by enzymatic hydrolysis with phospholipase A_2 from bee venom. An aliquot of the chloroform solution of PC (about 1 microcurie) was evaporated and the residue was dissolved in

diethyl ether (1 ml). 0.1 mg of phospholipase A_2 from bee venom (1500 I.U./mg protein, Sigma) in 0.01 ml of 0.2 M borate buffer (pH 7.4) were added to the ether solution and the mixture was incubated 30 min at 20°C. Solvent was evaporated and the residue was chromatographed on HPTLC Silica Gel plates, using Chloroform/methanol/acetic acid/water; 25:15:4:2 (by vol), as developing system. Complete hydrolysis of PC was taken as a proof that all the product had L-configuration. The formation of the unlabelled lyso-PC demonstrated that [^{3}H]oleic acid was specifically esterified in the position two of the glycerol moiety.

RESULTS

The yield of the reaction depends on the molar ratio of the reagents (Table 1).

TABLE 1

Lyso-PC (mmols)	1	1	1
Oleic Acid (mmols)	0.5	1	2
PC synthetized (mmols)	0.202	0.365	0.381

By increasing the concentration of labelled oleic acid, the amount of the label in PC increases; but the yield of the reaction, calculated on the base of the labelled precursor reacted, decreases. Therefore, the molar ratio 1:1 was preferred for standard preparations. A yield of about 36% was obtained both in the standard and in the micro-scale preparation.

The nature of the solvent used in the condensation reaction is critical, as shown in Fig.1. The highest incorporation of the label into PC was obtained when the reaction was carried out in

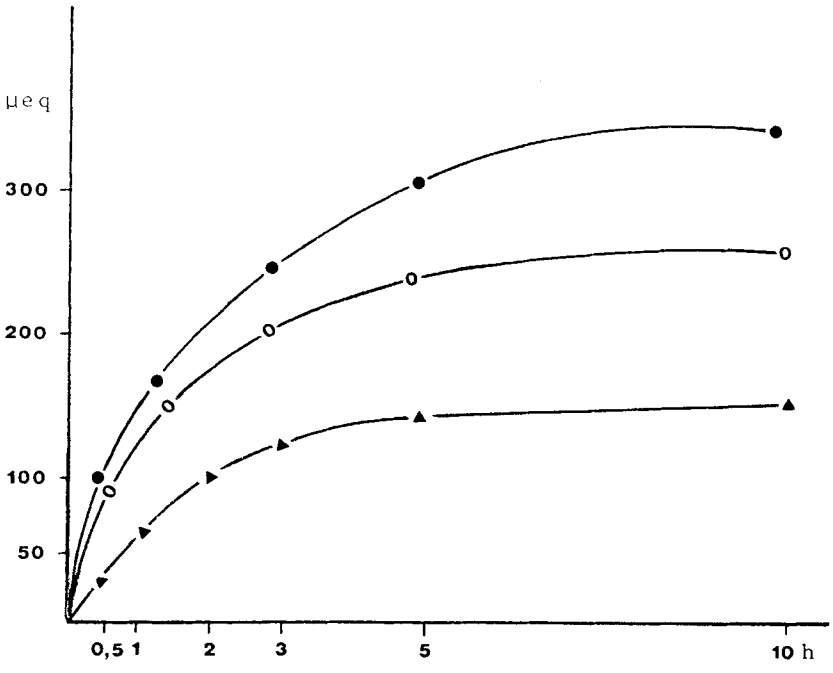

FIG 1 - Microequivalents of synthetized labelled phosphatidyl-choline versus time by using different solvents.
● = diethyl ether, o = acetonitrile, ▲ = dichloromethane

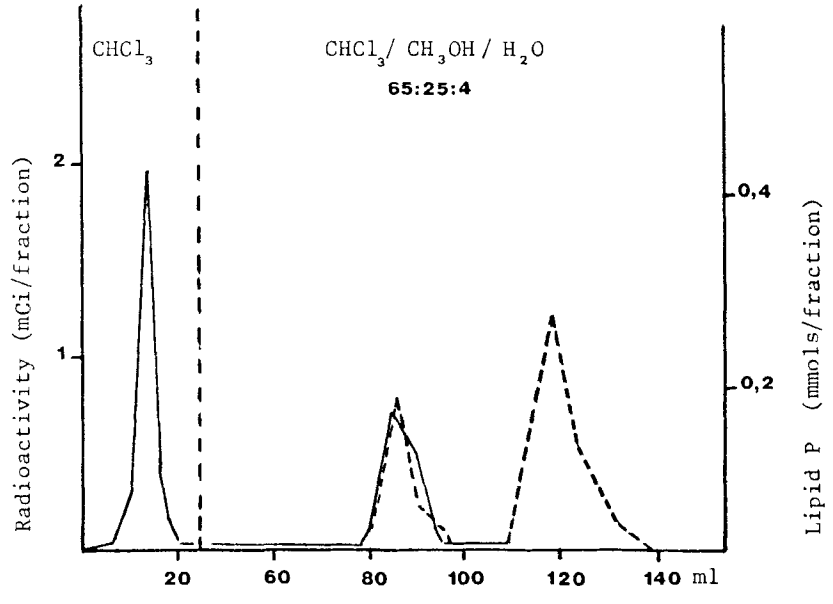

FIG 2 - Chromatographic pattern of reaction products.
Continuous line = radioactivity measurements
Dotted line = phosphorus determinations

diethyl ether, whereas acetonitrile and dichloromethane seem to be inferior solvents.

Isolation of the product was carried on by column chromatography, on preparative scale. Fig.2 shows a typical elution pattern obtained after a standard preparation. With this chromatographic system, a rapid and complete separation of labelled PC, unreacted oleic acid and lyso-PC was achieved.

Labelled PC was analyzed by TLC (see Experimental). Chromatoscanning of the plates confirmed that the labelled lipid co-chromatographes with an authentic PC standard and that its radiochemical purity was greater than 94% (Fig.3).

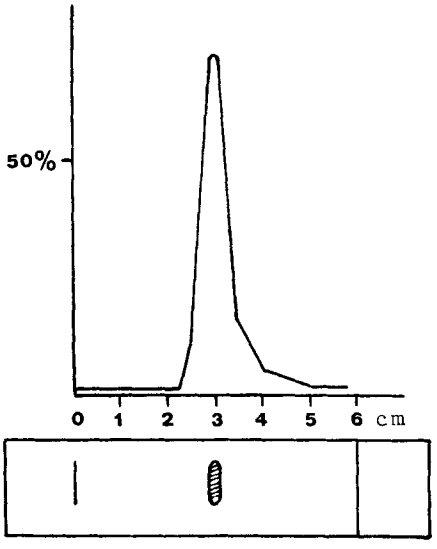

FIG 3 - HPTLC of prepared [^{3}H]oleoyl-phosphatidylcholine

In our experiments, no isomerization of the lyso-PC seems to occur during purification of the product by the extractive method

described. In fact incubating the labelled PC with Phospholipase A_2(14), practically all the lipid (99%) was converted into lyso-PC and labelled oleic acid. No label was found in lyso-PC, after hydrolysis, this demonstrating that [9,10-^{3}H]oleic acid is specifically esterified in the position two of the glycerol backbone (14).

Lyso-PC purification by preparative TLC could result into a partial isomerization of the product (13) which is, in fact, hydrolized for only 95% by phospholipase A_2.

DISCUSSION

The method described in this paper has an advantage over the methods previously used for preparing phosphatidylcholine labelled in the fatty acid moieties as it is very simple to reproduce in any conventionally equipped laboratory. All operations, including the condensation reaction, are carried out in relatively large volumes, in the standard procedure, while, in the micro-scale procedure, high specific activities can be achieved by using relatively small amounts of radioactivity.

The yield of the reaction is not 100%, but total recovery of the unreacted fatty acid is obtained in the purification step, so that an almost complete utilization of the labelled fatty acid can be reached by recycling it in subsequent acylation reactions. The method can be applied to acylate any other lysophospholipid, provided that the hydroxylic group of the glycerol is the only reactive group unmasked.

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