An improved procedure for the synthesis of ^{14}C-labeled phosphatidylserine from cerebral phosphatidic acid

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Abstract A complete procedure to prepare a highly labeled phosphatidyl-L-[U-^{14}C]serine possessing the same fatty acid composition of brain phospholipids is reported. CDP-diglyceride was synthesized by reaction between phosphatidic acid and CMP-morpholidate as the dicyclohexylcarboxamidium salt. The reaction between CDP-diglyceride and L-[U-^{14}C]serine to produce the labeled phosphatidylserine was catalyzed by the CDP-diglyceride: L-serine phosphatidyl transferase (EC 2.7.8.8) from *E.coli*. A selective inhibition of phosphatidylserine decarboxylase activity, present as contaminant in the enzyme extract, was introduced in order to avoid a low yield of product. Traces of phosphatidylethanolamine (about 1%) were easily removed by preparative thin-layer chromatography. The yield of the labeled product was as high as 87% and its specific radioactivity was 170 mCi/mmol.—**Orlando, P., G. Ippolito, L. Binaglia, C. Giordano, and G. Porcellati.** An improved procedure for the synthesis of ^{14}C-labeled phosphatidylserine from cerebral phosphatidic acid. *J. Lipid Res.* 1980. **21:** 1053–1057.

Supplementary key words CDP-diglyceride · L-serine phosphatidyltransferase · phosphatidylserine decarboxylase

Experimental evidence has recently been presented on the pharmacological effects of phosphatidylserine (PS) upon the central nervous system in vivo (1, 2). It has been shown, in this connection, that the administration of PS liposomes produces in the mouse and rat cerebral cortex an accumulation of free glucose (1) and an increase of acetylcholine output (2), respectively.

Among the brain cortex phospholipids, PS is the most active component; it induces an increased turnover of cerebral catecholamines (3), an activation of tyrosine hydroxylase (EC 1.14.16.2) (4), and a stimulation of the adenylate cyclase (EC 4.6.1.1) activity followed by increased levels of cyclic AMP (5).

These findings have also indicated that only PS extracted from brain is active, whereas PS obtained from different sources was found to be inactive. Probably, the composition and/or the degree of unsaturation of the constituent fatty acids play a role for the activity in the nervous system.

The purpose of the present work is to prepare a labeled PS with high specific radioactivity and with the same fatty acid pattern of the brain component, in order to study the molecular mechanisms which underlie the observed pharmacological effects.

Various chemical methods have been published on the synthesis of PS (6, 7), but the only one that produces an unsaturated labeled molecule is based on the condensation of native phosphatidic acid with *N-ter*butoxy-carbonyl-L-[^{14}C]serine-*ter*butyl ester, by using tri*iso*propyl-benzene-sulphonylchloride as the condensing agent (7). Masking groups are easily removed by mild acidification without damaging the unsaturated PS molecule. The procedure, however, to obtain the masked [^{14}C]serine is troublesome and rather expensive. Unlabeled phospholipids, including PS, have been synthesized by Eibl (8) via the formation of cyclic phosphate esters of diglycerides with phospholipid bases or base-derivatives; however, the labeling of the serine moiety of PS seems to be, in this case also rather expensive.

Phospholipid base-exchange reactions have been shown to be effective in obtaining labeled PS (9). By incubating microsomal membranes with labeled serine in the presence of Ca^{2+} ions, it is possible to obtain labeled PS. However, the yield is very low and the method is not suitable for preparative purposes. Another technique has recently been reported by Comfurius and Zwaal (10), employing the reversal of phospholipase D (EC 3.1.4.4) reaction. However, the method utilizes too high a serine concentration in order to obtain a reasonable specific activity.

Abbreviations: PS, phosphatidylserine; TLC, thin-layer chromatography; PE, phosphatidylethanolamine.

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The use of the CDP-diglyceride:L-serine phosphatidyltransferase from *E.coli* (11) seems to represent the best choice to synthesize ^{14}C-labeled phosphatidylserine. With the present work, a complete procedure is reported to obtain good yields of [^{14}C]-serine-labeled PS by using the transferase from *E. coli* and CDP-diglyceride prepared from ox brain phospholipids.

EXPERIMENTAL

Products

Pure phosphatidic acid from bovine brain, prepared by the action of phospholipase D (EC 3.1.4.4) on ox brain phosphatidylcholine, following the Wurster and Copenhaver procedure (12), was obtained from Fidia Research Labs. (Abano Terme, Padova, Italy).

Pure lipid moieties, used as reference standards, were prepared in our laboratory by Florisil column chromatography. Cytidine-5′-monophospho-morpholidate as the dicyclohexyl-carboxamidine salt and cytidine-5′-monophosphoric acid were from Sigma Chemical Co. (St. Louis, MO). Hydroxylamine sulfate and 4-deoxypyridoxine hydrochloride were obtained from Koch-Light Labs. (Buckingamshire, England). L-[U-^{14}C]Serine (sp act 170 Ci/mol) was purchased from the Radiochemical Center (Amersham, England).

Silica gel G-coated plates (0.25 mm of thickness) were obtained from Macheray-Nagel (516 Düren, West Germany). *E.coli* cell suspensions were kindly supplied by the Istituto Superiore di Sanità (Rome, Italy). All the other chemicals were from the Merck Company (Darmstadt, West Germany). Solvents were all distilled before use and stored under nitrogen when necessary.

The purity of CDP-diglyceride was checked by thin-layer chromatography (TLC) on precoated plates, using a pure reference standard for comparison.

The solvent system used (di*iso*butylketone–acetic acid–water 40:30:7 (v/v/v)) was found to give a complete separation of CDP-diglyceride from contaminating phosphatidic acid. Lipid spots were detected with iodine vapors and/or fluorescence at 254 nm excitation wavelength.

Assay of CDP-diglyceride

CDP-Diglyceride was spectrophotometrically assayed after saponification for 2 hr at room temperature with 0.3 M NaOH. The solution was evaporated and the residue dissolved in 2 M HCl (3 ml). Fatty acids were then removed by extraction with 3 ml of petroleum ether (40–60°C). The difference in optical density at 280 and 310 nm was measured in a model M4 QIII Zeiss spectrophotometer against a calibrated CMP solution.

Assay of CDP-diglyceride: L-serine phosphatidyl transferase

Activity was assayed according to Kanfer and Kennedy (11). Protein was determined following the procedure of Lowry et al. (13); crystallized bovine serum albumin was used as a standard.

Determination of radioactivity and fatty acid analysis

The radioactivity content of the labeled phospholipid was estimated by liquid scintillation counting in a model 3385 Packard spectrometer. Quenching correction was carried out by the external standard ratio method.

The quantitative scanning of the TLC plates was done by liquid scintillation counting of scraped spots. Recovery was nearly 100%. Gas–liquid chromatographic analysis of lipid fatty acids was carried out on a Pye Unicam GCD chromatograph according to Porcellati and Binaglia (14).

RESULTS

CDP-diglyceride preparation

Ox brain phosphatidic acid in the free form and cytidine-5′-monophospho-morpholidate were allowed to react for 65 hr at room temperature in pyridine (15); moisture and oxygen were strictly avoided. CDP-Diglyceride was extracted with chloroform–methanol 1:1 (v/v), washed with 10^{-4} M HCl and again extracted with methanol–2 M NH_4OH 2:1 (v/v). The product was found to be pure by TLC analysis and no unreacted phosphatidic acid was detected. The yield was 54% by weight after lyophilization, but the product seemed to retain water in an appreciable amount. By assaying the CMP content, a yield of 34.5% was observed, which was very close to the reported maximal values (15). The product was stored under benzene at 0°C; moisture and oxygen were strictly avoided. By this means, CDP-diglyceride, even when esterified with unsaturated fatty acids as in this case, was found to be stable for several weeks.

Preparation of CDP-diglyceride: L-serine phosphatidyltransferase from *E.coli* B

E.coli cell suspension, incubated at room temperature for a few minutes with lysozyme (0.5 mg/g), was

dropped into liquid nitrogen and allowed to melt at room temperature.

Ten volumes of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA–5 mM β-mercaptoethanol buffer were added and the resulting suspension was stirred for 45 min at 0°C. A complete cellular lysis was thus achieved. After centrifugation for 45 min at 10,000 *g*, the crude enzyme was recovered in the protein fraction precipitating between 30 and 50% of ammonium sulfate saturation (11).

Further purification (11) of the crude enzyme was not found necessary. An appreciable PS decarboxylase activity was in fact still present after $MnCl_2$ precipitation (**Table 1**), while a recently published purification method (16) appeared to be unreliable for our purpose. For these reasons, our efforts have been directed toward inhibiting the contaminating PS decarboxylase activity which occurs in the crude enzyme preparation.

Inhibition of phosphatidylserine decarboxylase

Table 1 shows the effect of 4-deoxypyridine and hydroxylamine when added in different concentrations to the enzyme incubation mixture. Both inhibitors, when added at concentration values as high as those reported to produce a 50% inhibition of the PS decarboxylase activity (17), i.e., 5×10^{-3} M 4-deoxypyridoxine and 1×10^{-4} M hydroxylamine, were found to act noticeably also on the incorporation of L-serine into lipid (99 and 90% of inhibition, respectively). However, by decreasing the concentration of inhibitors, 1×10^{-6} M hydroxylamine was found to represent the concentration which inhibited the decarboxylase activity almost completely without affecting PS synthesis by the transferase enzyme (Table 1).

TABLE 1. The inhibition of phosphatidylserine decarboxylase of *E. coli* extracts[a]

Enzyme Preparation	Inhibitor Added	Radioactivity Incorporated into		
		Total Lipids	PS	PE
Crude		100	60	40
$MnCl_2$-precipitated		100	70	30
Crude	4-deoxypyridoxine (5×10^{-3} M)	1	1	
Crude	4-deoxypyridoxine (1×10^{-5} M)	8	8	
Crude	Hydroxylamine (1×10^{-4} M)	10	10	
Crude	Hydroxylamine (1×10^{-5} M)	93	92	4
Crude	Hydroxylamine (1×10^{-6} M)	98	97	1

[a] Values are represented as the percentages of radioactivity incorporated into lipids when no inhibitor was added. Experimental conditions as described in the text.

Synthesis of phosphatidyl-L-[U-^{14}C]serine

The synthesis of phosphatidyl-L-[U-^{14}C]serine was performed by modifying the procedure reported by Kanfer and Kennedy (11). More precisely, the incubation system (1 ml of final volume) contained 0.1 M Na_2SO_4, 10 mM β-mercaptoethanol, 40 mM Tris-HCl buffer (pH 8.0), 2 mM EDTA, 1 mM CDP-diglyceride, 1 mM L-[U-^{14}C]serine (170 μCi) and 1×10^{-6} M hydroxylamine. The incubation was started by adding 2.5 mg of enzyme protein and 20 μl of octanol, and was performed at 37°C for 1 hr in a shaking incubator (60 strokes/min).

The reaction was terminated by the addition of 14 ml of chloroform–methanol 2:1 (v/v) with the further addition of 100 μmoles of unlabeled L-serine. The precipitate was removed through filtration on a sintered glass filter. The extract was washed with 30 ml of 2M KCl and twice with water, and then evaporated to dryness under nitrogen.

Lipids were purified by preparative TLC on silica gel-G plates by using chloroform–methanol–1 M NH_4OH 65:25:4 (v/v/v) as the solvent system. The ^{14}C-labeled PS spot, identified under ultraviolet light by co-chromatography with an authentic sample, was scraped from plates and the phospholipid was extracted twice with chloroform–methanol–water 60:30:4 (v/v/v). The extract was filtered on glass wool in a small column and taken to dryness under nitrogen (contaminating [^{14}C]phosphatidylethanolamine was easily removed due to the sharply different migration on the TLC plate).

Phosphatidyl-L-[U-^{14}C]serine was stored in a benzene solution at 0°C. Moisture and oxygen were strictly avoided. Under these conditions, the product was found to be stable for at least 3 weeks. **Fig. 1** shows the rate of conversion of free L-serine into lipid-serine as a function of enzyme concentration. A maximum value of synthesis was reached at a concentration of about 2.5 mg protein/ml.

Successive experiments were carried out by varying the incubation time, in order to obtain the best yield of product formation. **Fig. 2** indicates that a yield of 87% for PS is obtained after 1 hr of incubation; longer incubation times increased this yield up to 90%, but PE production also slightly increased under these conditions (2%).

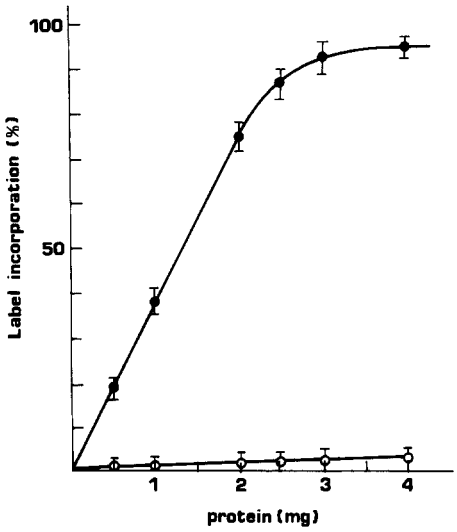

Fig. 1. The effect of enzyme concentration upon the rate of phospholipid synthesis. PS, ● — ● — ●; PE, ○ — ○ — ○. Enzyme concentration (mg crude protein/ml) is plotted against the percentage of L-[U-^{14}C]serine incorporated into lipid. See the text for the experimental conditions.

Fatty acid composition of the starting phosphatidic acid and of the final product is shown in **Table 2**.

TABLE 2. Fatty acid composition of reagent and product lipids

Fatty Acid	Phosphatidic Acid	Phosphatidylserine
14:0	1.0 ± 0.4	1.1 ± 0.5
16:0	33.3 ± 0.7	32.3 ± 1.0
16:1	1.4 ± 0.2	1.6 ± 0.4
18:0	10.0 ± 0.3	10.2 ± 0.2
18:1	41.5 ± 1.2	39.9 ± 2.0
18:2	0.6 ± 0.2	0.7 ± 0.2
20:0	0.3 ± 0.2	0.4 ± 0.2
18:3 (n-3)	2.3 ± 0.3	2.2 ± 0.3
20:2	0.2 ± 0.1	0.2 ± 0.1
20:3	0.2 ± 0.1	0.3 ± 0.1
20:4	3.5 ± 0.6	3.6 ± 0.6
20:5	0.2 ± 0.1	0.3 ± 0.1
22:0	0.6 ± 0.2	0.7 ± 0.2
22:4	1.4 ± 0.2	1.5 ± 0.2
22:5	1.1 ± 0.2	1.4 ± 0.4
22:6	3.0 ± 0.4	2.8 ± 0.2

Fatty acid methyl esters were obtained by transesterification and analyzed by gas–liquid chromatography. The data are from one single experiment in which phosphatidic acid was converted into PS, through intermediate CDP-diglyceride. Data are expressed as percent weight ± standard deviation.

DISCUSSION

The results reported in the present work describe a complete procedure to prepare a highly labeled phosphatidyl-L-[U-^{14}C]serine (170 Ci/mol) with a molecular species composition similar to that of the native brain lipid. In this respect, the labeled product may be employed to investigate the molecular mechanisms which underlie the pharmacological effects of the ox brain phosphatidylserine on the central nervous system (1–5). The long and tedious procedures usually adopted to purify the CDP-diglyceride: L-serine phosphatidyltransferase have been avoided in this work; the contaminating phosphatidylserine decarboxylase, in fact, has been found to be almost completely inhibited by 1×10^{-6} M hydroxylamine. Under these experimental conditions, a crude enzyme preparation from *E.coli* was found to yield 90% lipid-bound radioactivity, almost completely represented by labeled phosphatidylserine.

The overall yield of the procedure is sufficiently high and for this reason the method may be employed also to prepare a doubly-labeled phosphatidylserine, starting from [^{3}H]glycerol-labeled phosphatidic acid.

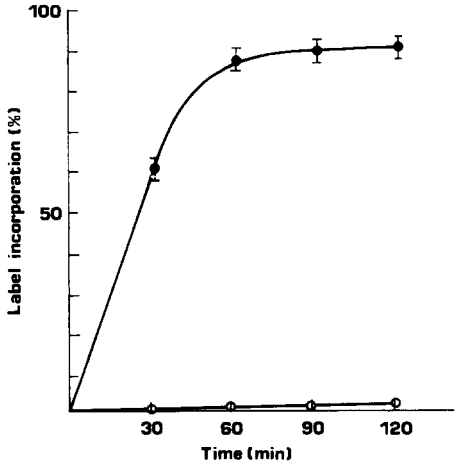

Fig. 2. Time course of L-[U-^{14}C]serine incorporation into PS (● — ● — ●) and PE (○ — ○ — ○) under the experimental conditions reported in the text. Enzyme added: 2.5 mg crude protein/ml. Time of incubation (min) is plotted against the percentage of L-[U-^{14}C]serine incorporated into lipid. See the text for the experimental conditions. The final product was found to possess a specific activity of 170 Ci/mol.

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