notes on methodology

Preparation of labeled alkyl acyl glycerophosphorylcholine

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SUMMARY A method for the preparation of 1-alkyl 2-acyl glycero-3-phosphorylcholine, labeled in the 2-position with oleic acid-1-^{14}C is described. This method, based on the acylation of a 1-alkyl glycero-3-phosphorylcholine-$CdCl_2$ complex which is, in turn, formed from 1-alk-1-enyl glycero-3-phosphorylcholine by hydrogenation and subsequent reaction with cadmium chloride, offers a relatively simple means of preparing a labeled saturated ether phospholipid suitable for use in metabolic studies.

SUPPLEMENTARY KEY WORDS phosphatidalcholine · 1-alkyl 3-glycero-3-phosphorylcholine–$CdCl_2$ complex · acylation · saturated ether phospholipid · lecithinase C hydrolysis

The alkyl ether phospholipids have attracted considerable interest over the last few years, especially in view of their possible role as plasmalogen precursors (1, 2.) However, as yet little is known of their metabolism. This may be due to the fact that methods involved in the preparation of suitably labeled derivatives require the use of lengthy organic preparative techniques with intermediates which are not easily obtainable (3, 4). It is the purpose of this paper to describe a method for the preparation of a radioactive alkyl acyl phospholipid which is convenient, rapid, and does not require the use of unfamiliar intermediates. The method is based on the acylation of the cadmium chloride complex of 1-alkyl glycero-3-phosphorylcholine which is in turn derived from the alkaline hydrolysis of ox heart lecithin (mixture of phosphatidyl- and phosphatidalcholine).

Bovine heart muscle was supplied by the British Beef Co., Ltd., London, England. Silicic acid (Mallinckrodt 100 mesh) was first sieved, and the 100–200 mesh size was washed with methanol and diethyl ether. Neutral silica gel (Mallinckrodt SilicAR TLC-7) was used for all thin-layer chromatographic analyses. Oleic acid-1-^{14}C was obtained from the Radiochemical Centre, Amersham, England. Aldehydogenic lipid was assayed by the Schiff procedure (5) after hydrolysis with 5 mM mercuric chloride in 90% acetic acid. Acyl esters were assayed as described by Rapport and Alonzo (6) using dipalmitin and distearin (Sigma Chemical Co., St. Louis, Mo.) as standards. Phosphorus estimations were made using the method of McArdle and Zilkha (7). Radioactivity was measured in 10 ml of a 0.3% solution of 2,5-diphenyloxazole in toluene using a Packard Tri-Carb liquid scintillation spectrometer.

Ox heart lecithin was isolated by silicic acid chromatography of a chloroform–methanol 2:1 extract of the heart muscle. Further purification of the phospholipid fraction was achieved by streaking samples (55 μg-atoms of lipid phosphorus) on washed, activated plates of neutral silica gel (20 × 20 cm, 500 μ thick) and by developing chromatograms in chloroform–methanol–35% ammonia 70:30:5(v/v/v). The lecithin zones were detected by streaking ovolecithin markers on to the edges of the plate, and the choline plasmalogen–lecithin fraction was eluted from the silica gel with chloroform–methanol 1:2. The phospholipid fraction had an aldehyde:phosphorus molar ratio of 0.6:1, while the average yield of phosphorus recovered per plate was 40 μg-atoms.

Preparation of 1-Alkenyl Glycerophosphorylcholine. Lysophosphatidalcholine was prepared by the alkaline hydrolysis of the purified ox heart lecithin as described by Dawson (8), but with some modifications. The phospholipid fraction (101 μmoles of aldehyde, 175 μg-atoms of phosphorus) was dissolved in carbon tetrachloride (5.6 ml) and ethanol (52 ml). Water (4.6 ml) and an aqueous 1 N solution of sodium hydroxide (1.75 ml) were added, and the mixture was shaken at 37°C for 30 min. Ethyl formate (0.8 ml) was then added, and the mixture was shaken for another 5 min. The solvent was evaporated off under reduced pressure at 47°C, the residue was shaken with the two-phase system described by Dawson (5 ml of solvent phase, 2.5 ml of aqueous phase), and the mixture was then centrifuged. Difficulty was experienced in completely separating the two phases, but the addition of a few drops of saturated potassium chloride solution aided the breakdown of the emulsion which had formed. The upper phase was reextracted with lower phase, and the combined lower phases were evaporated to dryness under reduced pressure at 40°C. Traces of water were removed as the chloroform azeotrope. The yield of phosphorus was 90 μg-atoms which indicated that some of the plasmalogen had broken down under these conditions (9). Thin-layer chromatographic analysis (chloroform–methanol–water 60:35:8) of the lipid remaining after hydrolysis showed the presence of a substance which migrated near the lysolecithin area of the chromatogram, and which reacted with both fuchsin and Dragendorff reagents.

Purification of the lysophosphatidalcholine was achieved by chromatography on silicic acid at 4°C.

Abbreviations: TLC, thin-layer chromatography.

The alkali-stable lipid (89 μmoles of aldehyde) in 10 ml of chloroform–methanol 1:1 was applied to a column of silicic acid (8 g, 24 × 1 cm) in the same solvent mixture. 150 ml of chloroform–methanol 1:1 and 250 ml of chloroform–methanol 2:5 were then passed through the column. 10-ml fractions were collected automatically at a rate of 1.5 ml/min, and were monitored by TLC in chloroform–methanol–35% ammonia 70:30:5. The lysophosphatidalcholine (72 μg-atoms of phosphorus) was eluted by the latter solvent mixture, and probably contained small amounts of 1-alkyl glycero-3-phosphorylcholine (9).

The product (71 μmoles of aldehyde), which had a phosphorus:aldehyde molar ratio of 0.95:1.0, was dissolved in 10 ml of methanol. A freshly prepared palladium-charcoal catalyst (4 mg, 5% w/w) was added, and the mixture was shaken at 37°C for 2 hr in the presence of a slight positive pressure of hydrogen (10). A further 5 mg of catalyst was added, and the hydrogenation was continued for another 30 min period at the same temperature. Under these conditions the alk-1-enyl ether linkage takes up 1 mole of hydrogen for every atom of phosphorus and is converted to the acid-stable alkyl ether bond (9, 11). Less than 1% of aldehydogenic material remained after this treatment. The resulting substance was stable to 90% aqueous acetic acid (45 min at 55°C) and 0.1 N aqueous sodium hydroxide solution (15 min at 37°C).

The 1-alkyl glycero-3-phosphorylcholine was converted to the cadmium chloride complex in the following manner. An alcoholic solution of cadmium chloride dihydrate (0.7 ml), prepared by dissolving the cadmium salt (145 mg) in water (0.1 ml) and then adding 99% ethanol (1.6 ml), was mixed with 0.8 ml of an alcoholic solution of 1-alkyl glycerophosphorylcholine (81 μg-atoms of phosphorus). The mixture was allowed to stand at 0°C overnight and was then filtered through sintered glass. The precipitate was washed successively with ice-cold ethanol and ether, and then dried over phosphorus pentoxide at room temperature. The yield of the cadmium chloride adduct was 62.5 mg.

Acylation of Cadmium Chloride Addition Complex. The addition complex was reacted with oleoyl chloride. Oleoyl chloride-1-^{14}C was prepared by reacting oleic acid-1-^{14}C (0.68 mmoles, 300 μCi) with freshly distilled thionyl chloride in a 1:2 molar ratio. The thionyl chloride was removed by heating to 90°C under vacuum, and a portion of the reaction mixture, equivalent to 0.34 mmoles of oleic acid, was dissolved in dry, ethanol-free, chloroform (0.28 ml). This solution was then added to a small test tube containing the cadmium chloride addition complex (60 mg) and 2 ml of chloroform-washed glass beads. A mixture of dry pyridine (0.17 ml) and dry, ethanol-free chloroform (1.7 ml) was slowly added. The tube was stoppered and shaken, under anhydrous conditions, at 37°C for 2 hr. The reaction mixture was filtered through sintered glass, and the precipitate was washed with chloroform. The filtrate and washings were evaporated to dryness under vacuum, dissolved in 20 ml of chloroform–methanol–water 5:4:1, and then passed through a column (11.5 × 2.5 cm) containing the mixed resins IR-45(OH^-) and IRC-50(H^+). The eluate was evaporated to dryness under reduced pressure at 40°C and dissolved in 20 ml of chloroform–methanol 4:1. Purification of the radioactive ether phospholipid was achieved by chromatography of this solution on a column of silicic acid (15 × 2.5 cm) made up in chloroform–methanol 4:1. Unreacted oleic acid-1-^{14}C was eluted with 250 ml of chloroform–methanol 4:1, and the labeled phospholipid was eluted with 250 ml of chloroform–methanol 1:1. 10-ml fractions were collected at a rate of 2 ml/min, and were monitored by TLC using chloroform–methanol–water 60:35:8. The ether phospholipid was located by exposure of the plates to iodine vapor. Fractions containing the labeled phospholipid were pooled, and samples of the pooled eluate were chromatographed in two different solvent systems (chloroform–methanol–water 60:35:8, and chloroform–methanol–water–glacial acetic acid 65:43:3:1). Radioautograms were prepared by exposing the dried chromatograms to Agfa-Gevaert Curix X-ray film for 5 days. More than 98% of the radioactivity moved with the same chromatographic mobility as ovolecithin. In order to demonstrate the absence of phosphatidalcholine, a sample of the purified lipid was subjected to the two-dimensional chromatographic technique used for the detection of aldehydogenic phospholipids (12). An examination of the radioautograph prepared from the chromatogram showed the absence of radioactive lysophosphatidalcholine which would have been formed if any radioactive plasmalogen were present. The phosphorus:acyl ester molar ratio was 1:0.95, while the yield of product was 31 μg-atoms of phospholipid phosphorus, which represented a 40% conversion from 1-alkyl glycero-3-phosphorylcholine. The main products of lithium aluminum hydride hydrolysis behaved like stearyl alcohol and batyl alcohol on thin-layer chromatograms (petroleum ether–diethyl ether–acetic acid 30:70:1). A sample of the product (8.3 μg-atoms of phosphorus) was further tested for purity by hydrolysis with lecithinase C (*Clostridium welchii*) as described by Kiyasu and Kennedy (13). Under these conditions more than 95% of the radioactivity was recovered in the ether phase. The main radioactive product of enzymic hydrolysis moved with the same chromatographic mobility as 1-alkyl 2-acyl glycerol in hexane–diethyl ether 60:40, and hexane–diethyl ether–methanol 80:20:5. The latter substance was prepared from a specimen of alkyl diacyl glycerol (generously supplied by Dr. F. Snyder, Oak Ridge, Tenn.) by pancreatic lipase hydrolysis as described by Snyder and Piantadosi (14). Small amounts (6% of the total radioactivity) of what was assumed to be the 1,3-isomer (R_F 0.40 in hexane–diethyl-ether–methanol 80:20:5) was also present but was probably due to acyl migration either during the hydrolytic procedure or during chromatography. This substance was formed from the 1,2-isomer under slightly acid conditions: preparative TLC of the 1,2-isomer on Silica Gel G was sufficient to cause extensive migration of the radioactive oleic acid group. No such effect was noted when TLC was carried out on SilicAR TLC-7. Traces (3%) of a substance which had the chromatographic characteristics of diacyl glycerol were also present which indicated that the isolated lysophosphatidalcholine may have contained small amounts of lysolecithin. The absolute radiochemical purity of the phospholipid was 96%.

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