Isolation of disaturated phosphatidylcholine with osmium tetroxide

Robert J. Mason[1], Jean Nellenbogen, and John A. Clements[2]

Cardiovascular Research Institute, University of California Medical Center, San Francisco, California 94143

Summary A simple, rapid, inexpensive method for isolating disaturated phosphatidylcholine from adult rat lung has been devised. Total lipids are reacted with osmium tetroxide dissolved in carbon tetrachloride, and the disaturated phosphatidylcholine is isolated on a column of neutral alumina. More than 99% of the fatty acids in the phosphatidylcholine fraction are saturated and 94% of this material migrates as phosphatidylcholine on subsequent thin-layer chromatography.

Supplementary key words lung lipids · phosphatidylcholine

For investigators interested in phospholipid metabolism in the lung, there is a need for a rapid method for isolating disaturated phosphatidylcholine. This species of phosphatidylcholine, which has only saturated fatty acids, is found in high concentration in purified surface active material and is mainly responsible for the ability of the latter substance to produce low surface tensions (1–3). Ever since dipalmitoylphosphatidylcholine was first isolated from lung by Thannhauser, Benotti, and Boncoddo (4), many means of isolating disaturated phosphatidylcholine have been devised. These methods include precipitation in cold ethanol (1) or acetone (4, 5), oxidation with ozone (6) or permanganate and periodate (7), formation of mercuric acetate adducts (8–10), and argentation thin-layer chromatography (11). Species of phosphatidylcholines can also be separated by diglycerides after treatment with phospholipase C (12, 13). Most of these methods require isolation of phosphatidylcholines before the specific separation procedures. Exceptions are the method of Young and Tierney (14), in which mercuric acetate adducts are formed with total phospholipids and the disaturated phosphatidylcholine is isolated by thin-layer chromatography, and the method of Brown (1) that uses solvent fractionation for analysis of material obtained by endobronchial lavage.

A rapid, simple, quantitative method for isolating disaturated phosphatidylcholine would be useful for analyzing amniotic fluid to assess fetal maturity, for studying secretion of surface active material by type II alveolar cells, and for performing turnover studies in intact lung. We have developed a method which does not require prior isolation of phosphatidylcholine from lipid extracts.

Osmium tetroxide is a powerful oxidant that reacts with double bonds to form complexes (15–18). One molecule of osmium tetroxide reacts with each double bond in egg phosphatidylcholine, and the reaction proceeds rapidly to completion (15, 17).

Our method is simply to react total lipids with osmium tetroxide in carbon tetrachloride and to isolate the disaturated phosphatidylcholine by column chromatography on neutral alumina.

Methods

Lipids are prepared by extracting cells or homogenized tissue with chloroform–methanol 2:1 (v/v),

[1] Established Investigator of the American Heart Association.
[2] Career Investigator of the American Heart Association.

TABLE 1. Recovery of disaturated phosphatidylcholine

Method	Preparation	N	Disaturated PC[b] % of Total PL	Recovery (%)
Osmium tetroxide	A[a]	10	23.1 ± 0.4	90.7 ± 2.8
Osmium tetroxide	B[a]	6	21.6 ± 0.4	92.5 ± 1.2
Mercuric acetate adducts	A	3	21.6 ± 1.8	87.3 ± 1.0

[a] Lipids extracted from lungs of two different rats (A and B).
[b] The disaturated phosphatidylcholine (PC) is expressed as a percentage of total phospholipid (PL) as determined by measurement of total lipid phosphorus (20). The recoveries are determined from the added ^{14}C-labeled dipalmitoylphosphatidylcholine (21). The numbers are the mean ± standard deviation.

filtering, separating the solvents into two phases by adding 0.1 M KCl, and washing according to the procedure of Folch, Lees, and Sloane Stanley (19). Osmium tetroxide (Electron Microscopy Sciences, Port Washington, Pa.) is dissolved in carbon tetrachloride (100 mg/ml) and stored in the dark at 4°C. This stock solution is stable for at least two years. An aliquot of lipid (1 mg) is evaporated and the residue is redissolved in 0.5 ml of carbon tetrachloride that contains 3.1 mg of osmium tetroxide. The reacting solution darkens, presumably as osmium dioxide is formed (15). After waiting 15 min for completion of the reaction, the solution is evaporated and the residue is redissolved in chloroform–methanol 20:1 (v/v). This material is applied to a column formed by placing 0.8 g of aluminum oxide (neutral alumina, 100–200 mesh, Biorad Laboratories, Richmond, California, lots #10461 and #7579, activated for 1 hr at 110°C) on a plug of glass wool in the neck of a disposable 9-in Pasteur pipette. Neutral alumina was chosen as the adsorbent for chromatography because it binds acidic lipids under the conditions we use in this method. Neutral lipids not altered by the osmium tetroxide are first eluted with 10 ml of chloroform–methanol 20:1 (v/v). The disaturated phosphatidylcholine is then eluted with 5 ml of chloroform–methanol–7 M ammonium hydroxide 70:30:2 (v/v). A dark residue remains at the top of the column.

The reaction of unsaturated lipid and osmium tetroxide proceeds rapidly and is complete in the time necessary to evaporate the sample, but we have arbitrarily decided to wait 15 min for the reaction to go to completion. When the columns are eluted, the black band should stay at the top and will do so unless the rate of elution is too fast. The rate of elution (approximately 0.8 ml/min) is controlled by the packing of the glass wool in the neck of the Pasteur pipette. This detail is easily learned by experience. Up to 12 columns have been run simultaneously and they can stand for as long as 15 min between eluting solutions without affecting the separation or recovery.

The methods used in the analyses have been reported previously (10, 20–22). Phosphorus was determined by the method of Bartlett (20). Radioactivity was measured by scintillation spectroscopy with samples prepared with Aquasol (New England Nuclear Corp., Boston, Mass.)—water 15:4 (v/v). Methyl esters of fatty acids of total lipids and isolated phosphatidylcholines were prepared with methanol and sulfuric acid, separated by gas–liquid chromatography on a column of ethylene glycol succinate, and quantitated by integration of the areas of the individual peaks (21). The purity of the fraction that contained the isolated disaturated phosphatidylcholine was determined by thin-layer chromatography and phosphorus measured in the sample lanes and in blank lanes (22).

Isolation of disaturated phosphatidylcholine by the mercuric acetate adduct method, for comparison with the method in this report, required the isolation of phospholipids by silicic acid column chromatography, isolation of mixed phosphatidylcholines by thin-layer chromatography, and isolation of disaturated phosphatidylcholine by silicic acid column chromatography after formation of mercuric acetate adducts of the unsaturated species (21).

Results and discussion

We used total lipids extracted from rat lung to develop the method. The results are shown in **Tables 1** and **2.** ^{14}C-labeled dipalmitoylphosphatidylcholine (synthesized chemically in our laboratory) was added to the total lipids for quantitation of recovery (21). Two batches of rat lung lipids were tested and gave similar results. The values agreed with those obtained by a mercuric acetate adduct method (21). The recoveries were 90% with 16 samples of rat lung and 90.3 ± 3.1% (mean ± standard deviation) with a total of 71 samples of various tissue extracts. All the fatty acids (>99%) from phosphatidylcholines isolated from rat and rabbit lungs by this method have been saturated (N = 30). As shown in **Fig. 1,** the material isolated by this method appears as a single spot in the phosphatidylcholine area on thin-layer chromatography, and this spot contains 93.9 ± 1.5% (N = 3) of the phosphorus recovered from the plate (22). The limits of the resolution of the thin-layer chromatographic separation is 98%, as judged by the distribution of purified, chemically synthesized [^{14}C]dipalmitoylphosphatidylcholine. Other disaturated phospholipids could be present in trace amounts.

TABLE 2. Fatty acid composition[a]

Preparation	14:0[b]	15:0	16:0	16:1	18:0	18:1	18:2	20:4	Unidentified
A	1.9	tr[c]	35.8	4.6	11.8	14.6	18.5	10.4	2.5
B	2.5	tr	39.5	5.2	10.8	12.4	10.8	14.4	4.1
Disaturated phosphatidylcholine	6.3	1.9	87.9		3.9				

[a] The mean of triplicate analyses of methyl esters of total lipids and the mean of 16 determinations of the isolated disaturated phosphatidylcholines are presented. There was no difference in the fatty acid composition of the disaturated phosphatidylcholine of the two preparations (A and B). The percent composition is given as mole %.

[b] Number of carbon atoms: number of double bonds.

[c] tr stands for trace (0.5%).

Under different conditions of chromatography and with different solvents, acidic phospholipids can be eluted from columns of neutral alumina and some phosphatidylcholine can be converted to lysophosphatidylcholine (23, 24). With our procedure and lipids from adult rat lung, no acidic lipids or lysolecithin were eluted as determined by inspection of the thin-layer plate (Fig. 1) or measuring the phosphorus on the plate.

We have shown that for adult rat lung 94% of the phosphorus found in the chloroform–methanol–ammonium hydroxide eluate is disaturated phosphatidylcholine. Similar results have been found with fluid obtained by endobronchial lavage of lungs of adult rabbits.[3] For use with other biologic materials, the method will have to be validated. We have not tested this method with amniotic fluid samples. Our method is less satisfactory with extracts that contain very little disaturated phosphatidylcholine. With rabbit liver and heart, 3.9% and 4.2% of the total phospholipid elutes as disaturated phosphatidylcholine. It is likely that some of the phosphorus eluted with chloroform–methanol–ammonium hydroxide is not disaturated phosphatidylcholine. Previously, with different samples of these tissues, we found that ≤2% of the phospholipids were disaturated phosphatidylcholine. The method used required initial isolation of mixed phosphatidylcholines and then separation of the disaturated species after mercuric acetate adduction (25). For samples containing a low percentage of disaturated phosphatidylcholine (<10% of lipid phosphorus), this species can be separated from the unsaturated phosphatidylcholines by thin-layer chromatography on silica H plates with chloroform–methanol–7 M ammonium hydroxide 70:30:3.5 (v/v) after reaction of isolated phosphatidylcholines (but not total lipids) with osmium tetroxide.

The method as described in this report will be valuable for multiple analyses of similar samples for comparative purposes (i.e. secretion of surface active material) and for simple analysis of lipids containing a moderate amount of disaturated phosphatidylcholine (>10% of lipid phosphorus). The composition of the eluate from the alumina column should be checked with different biologic materials, and the product may have to be further purified for materials that contain a low amount of disaturated phosphatidylcholine.

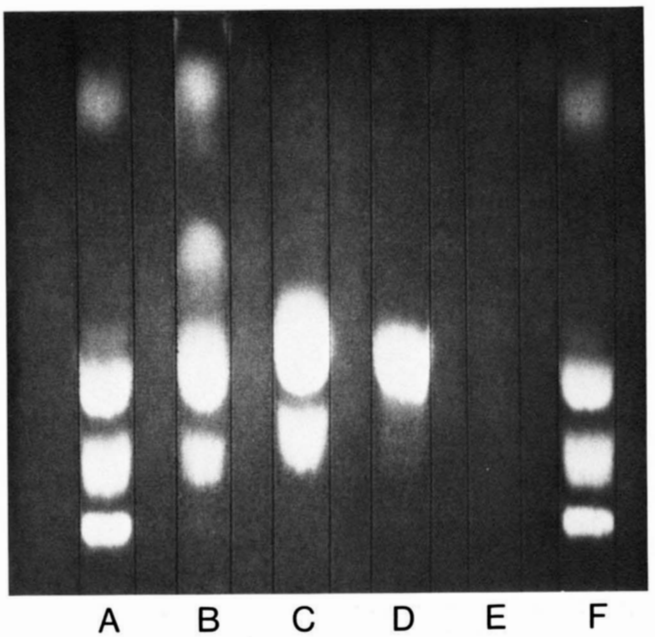

Fig. 1. A thin-layer plate of lipid standards, rat lung lipids, and eluates from the alumina column. Different lipids were applied to the designated lanes: (A) 375 μg of a phospholipid standard (phosphatidylethanolamine, phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine); (B) 340 μg of total lipids from rat lung; (C) 310 μg of rat lung phospholipids eluted with chloroform–methanol–ammonium hydroxide (not reacted with osmium tetroxide); (D) 200 μg of the same phospholipids after reaction with osmium tetroxide, (E) blank lane for background phosphorus measurement, (F) same as A. 94.9% of the phosphorus in lane D is in the phosphatidylcholine area, 3.7% in the sphingomyelin area, 0% in the lysophosphatidylcholine area and the remainder at the origin or above the phosphatidylcholine area. The silica H thin-layer plate was developed with chloroform–methanol–acetic acid–water 100:50:14:7 (v/v), sprayed with 0.25% 8-anilino-1-naphthalenesulfonic acid in water and illuminated with ultraviolet light.

[3] Oyarzun, M., and J. A. Clements, unpublished observations.

During the development of this method, several other means of separation and refinements of the technique were evaluated. With an eluting solution of chloroform–methanol 1:1 (v/v), the recovery decreased to 75%. Addition of acetic acid to the solution elutes excess phosphorus, some of the black material, and fatty acids. Separation on columns of diethylaminoethyl cellulose or thin-layer plates of silica gel or alumina were less satisfactory in terms of both purity of the final product and of total recovery.

This method is similar in principle to a recently published procedure by Shimojo, Abe, and Ohta (7) in which mixed phosphatidylcholines were oxidized with permanganate and periodate and the unaffected disaturated phosphatidylcholine was isolated by thin-layer chromatography. The major differences are that the permanganate and periodate method apparently requires an initial isolation of phosphatidylcholines from mixed lipids, an extraction procedure after the reaction, thin-layer chromatography for isolation of the disaturated phosphatidylcholine, and elution from the thin-layer plate for recovery of the disaturated phosphatidylcholine. Our method is quicker, simpler, and just as reliable for processing whole lung lipids.

In conclusion, a rapid method for isolating disaturated phosphatidylcholine from mixed lipids from adult rat lung has been devised. A drawback of the method is that unsaturated lipids are probably destroyed and cannot be analyzed further.

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