notes on methodology

A method for determination of saturated phosphatidylcholine

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Summary Phosphatidylcholine preparations containing saturated and unsaturated molecular species were subjected to $KMnO_4$–$NaIO_4$ oxidation in aqueous acetic acid, which left only disaturated species intact. After the oxidation, the remaining intact phosphatidylcholine was separated by thin-layer chromatography. The procedure could be used as a simple and rapid method for microdetermination of the saturated species in phosphatidylcholine preparations containing more than 0.1 μmole of the saturated species. The contents of the saturated species in native phosphatidylcholines obtained from rat lung tissue and washings by this procedure were 35.7% and 58.3%, respectively.

Supplementary key words dipalmitoyl lecithin · $KMnO_4$–$NaIO_4$ oxidation · thin-layer chromatography

Disaturated 1,2-acyl-*sn*-glycerol-3-phosphorylcholine, saturated lecithin, has been reported to be a constituent of various biological materials (1) and has attracted considerable interest in view of the possible role of this compound as a surface-active material in the lung alveolar lining layer. The procedures hitherto reported for estimating saturated lecithin depend on (*a*) hydrolysis of the lecithins with phospholipase C followed by argentation thin-layer chromatography of resulting diacylglycerols (2, 3), (*b*) chromatographic separation of mercuric acetate adducts of lecithins on a Sephadex LH-20 column (4), or (*c*) reductive ozonolysis followed by thin-layer chromatography of the resulting lipids (5).

It is the purpose of this paper to describe a simple and rapid method for analysis of saturated lecithin. The present method is based on oxidative cleavage of the unsaturated species at the ethylenic linkage followed by isolation of the remaining saturated lecithin by thin-layer chromatography. The method takes advantage of the large difference between R_F values of saturated and partially oxidized unsaturated lecithins on thin-layer plates.

Isolation of lecithin. Lecithin fractions were isolated from various rat tissues by DEAE-cellulose column chromatography as described by Rouser et al. (6) and were further purified by preparative thin-layer chromatography using Kieselgel G and a solvent mixture of chloroform–methanol–water 70:30:5. Synthetic 1,2-dipalmitoyl-*sn*-glycerol-3-phosphorylcholine (β,γ-dipalmitoyl-$_{DL}$-α-lecithin, purchased from Sigma Chemical Co., St. Louis, Mo.) was purified by thin-layer chromatography before use.

Procedure. An appropriate amount of lecithin containing 0.1–0.5 μmole of the saturated species (0.2–1.0 mg or 0.3–1.5 μmoles for lecithins from rat lung) was dissolved in 0.1 ml of 90% acetic acid to which was added 0.1 ml of an aqueous oxidant solution containing 0.024 M potassium permanganate and 0.02 M sodium periodate. The reaction mixture was shaken for 45 min at room temperature. When the color of the permanganate ion disappeared during this period, additional oxidant solution was added until the color remained. The mixture was then decolorized by addition of 0.02 ml of 20% sodium bisulfite solution. After addition of 5 ml of chloroform–methanol 2:1 and 3 ml of water, the mixture was thoroughly shaken and centrifuged. The saturated and partially oxidized unsaturated lecithins were recovered in the lower layer. After evaporating the solvents, the lipids thus obtained were redissolved in a small amount of chloroform–methanol 2:1 and spotted quantitatively on a thin-layer plate of Kieselgel G. The plate was developed with chloroform–methanol–water 70:30:5. Lipid spots were visualized under UV light after spraying with 0.2% 2,7-dichlorofluorescein in ethanol. The spot corresponding to that of authentic lecithin was scraped off, and the lipid was eluted from the gel according to the method of Arvidson (7). The phosphorus content was determined by the method of Bartlett (8). The amount of saturated lecithin was corrected with a recovery value of 92% obtained with authentic lecithin using the same procedure. The content of saturated lecithin was expressed as a percentage of the original lecithin.

Fatty acid composition of the remaining lecithins. Lecithins from liver and lung were oxidized as described above, and the remaining lipids were extracted and separated by thin-layer chromatography. The thin-layer chromatogram is shown in **Fig. 1**. Fatty acid methyl esters were prepared from the remaining intact lecithin with the use of BF_3 (9). The esters were analyzed by gas–liquid chromatography, using a 1-m column packed with Chromosorb W coated with polyethylene glycol succinate polyester. Only palmitate (96.1%), stearate (3.9%), and a trace of myristate were detected with lecithins from rat lung and palmitate (69%) and stearate (31%) with lecithins from rat liver, showing that no unsaturated lecithins remained after the oxidation.

Effect of oxidation time. The effect of time of oxidation was also studied. With lecithin from rat lung, the amount decreased rapidly during the first 30 min and then reached a constant level (**Fig. 2**). With dipalmitoyl leci-

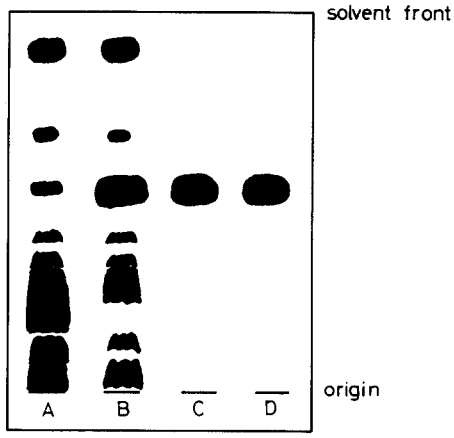

Fig. 1. Thin-layer chromatography of the lipids after oxidation with $KMnO_4$–$NaIO_4$. 0.75 μmole of lecithin from rat lung, 2 μmoles of lecithin from rat liver, and 0.2 μmole of dipalmitoyl lecithin were oxidized. The lipids extracted from the total reaction mixtures were spotted on a thin-layer plate. *A*, lecithin from rat liver; *B*, lecithin from rat lung; *C*, dipalmitoyl lecithin; *D*, dipalmitoyl lecithin without oxidation.

thin, the initial amount was unchanged during 60 min of oxidation, showing the resistance of saturated lecithin to the oxidation procedure.

Recovery. As shown in **Table 1**, the recovery was 92 ± 2.4% using dipalmitoyl lecithin. The 8% loss was due to a deficiency in the recovery of material from the plate. A good recovery was also observed using a mixed sample of dipalmitoyl lecithin and lecithins from rat lung.

Saturated lecithins in various tissues. The contents of saturated species in native lecithins of various rat tissues and of rat lung washings are given in **Table 2**; literature values are also shown (10).

Discussion. Argentation thin-layer chromatography (7) has been used for the resolution of molecular species of lecithins according to the degree of unsaturation. However, this method has been found to be less suitable (11) than other methods described below in resolving the intact saturated and monoenoic lecithins. This resolution has been achieved by argentation thin-layer chromatography after conversion of lecithins into diacylglycerols by phospholipase C (2, 3) or into diacylglycerol acetates (12, 13).

A nondestructive method has been described by King and Clements (4). In this procedure, lecithins were separated into the molecular species by column chromatography on Sephadex LH-20 after their conversion to the mercuric acetate adducts. Using this method, Tierney and Young (10) measured the amount of saturated lecithin in various rat tissues. Their results are in agreement with

TABLE 1. Recoveries of saturated lecithins

Lecithin	Initial Amount	Unoxidized Amount	Recovery
	μmoles	*μmoles*	%
Dipalmitoyl lecithin	0.5	0.46 ± 0.012[a]	92 ± 2.4[a]
Lecithins from rat lung	0.45	0.14 ± 0.004	
Lecithins from rat lung + dipalmitoyl lecithin	0.45 0.5	0.595 ± 0.014	99 ± 2.3

Experimental conditions are presented in the text. Values are means of two experiments ± SD.

[a] Values obtained from eight experiments.

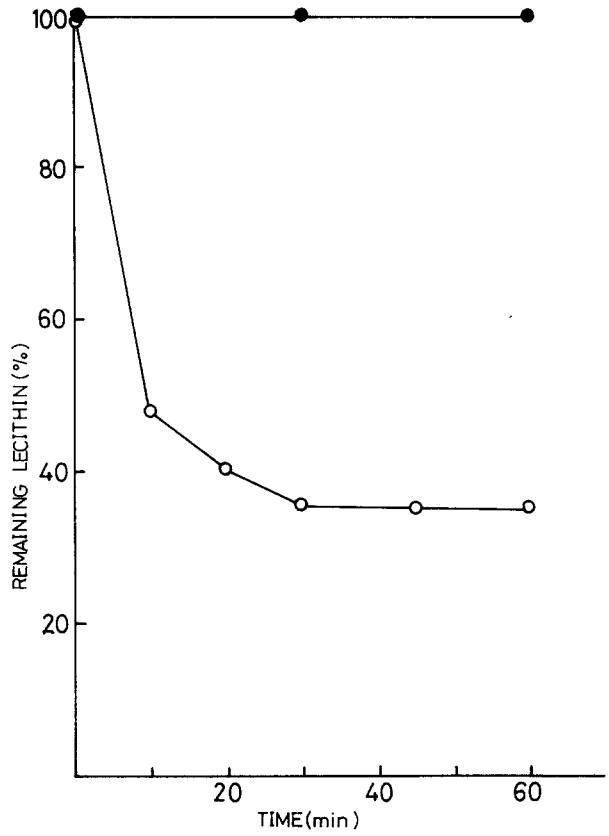

Fig. 2. Effects of oxidation time on the amount of remaining lecithin. ○, lecithins from rat lung; ●, dipalmitoyl lecithin.

TABLE 2. Contents of saturated lecithins in various rat tissues[a]

	Present Method	Tierney and Young (Ref. 10)
	%	%
Lung		
Whole tissue	35.7 ± 0.4	36
Washings[b]	58.3 ± 0.6	57
Spleen	22.4 ± 0.9	33
Brain	20.2 ± 0.7	26
Kidney	15.6 ± 0.3	16
Whole blood	14.9 ± 1.1	32
Intestine	12.6 ± 0.2	11
Heart	6.0 ± 0.1	
Muscle	4.1 ± 0.1	
Liver	3.7 ± 0.1	5

[a] Values are means of two experiments ± SD and are expressed as percentages of total lecithin.

[b] The lungs of three rats were washed with 0.9% saline solution according to the method reported by Toshima, Akino, and Ohno (16).

those reported in this paper with the exception of considerably higher values in spleen and in whole blood (Table 2).

An excellent method using reductive ozonolysis was reported by Privett and Blank (5). Saturated lecithin is left intact, but the procedure requires special equipment for ozonization.

$KMnO_4$–$NaIO_4$ oxidation has been used for analysis of double bond position in fatty acids (14, 15), and we have used this type of oxidation for the analysis of saturated lecithin. Acyl ester bonds were shown to be stable, and unsaturated lecithins were converted quantitatively to oxidized forms, which could be easily separated from the intact saturated species by thin-layer chromatography. The spot of the remaining intact lecithin obtained from rat heart showed no reactivity with dinitrophenylhydrazine–HCl on a thin-layer plate, showing that the plasmalogenic double bond could be oxidized in the same manner as regular double bonds. The procedure may be useful for rapid microanalysis and for preparation of saturated lecithins, including saturated alkylacylglycerylphosphorylcholine, which can be estimated by the determination of alkali-stable lipid phosphorus. Although the unsaturated species are lost by the oxidation, the present method will be very useful in the study of the disaturated constituents of lecithin preparations. The method may also be suitable for tracer experiments with saturated lecithin, especially lecithin with labeled phosphorus or choline.

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