Separation by gel chromatography of naturally occurring phosphatidylcholine mixtures according to number of ethylenic linkages

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SUMMARY This paper describes a procedure for the separation of lecithins according to the number of ethylenic bonds in their fatty acid residues. The procedure uses a column of alkylated dextran (Sephadex LH-20) eluted with an organic solvent system, the unsaturated lipids being separated as their mercuric acetate addition compounds. The system is capable of resolving at least four species of lecithin, and the intact lecithin molecules can be recovered for further study. The chromatographic system has been tested with lecithin derived from dog lung, rat liver, and hen's egg.

SUPPLEMENTARY KEY WORDS alveolar surface active material · Sephadex LH-20 · phosphatidylcholine chromatography

SEVERAL methods are available for separating phospholipids according to their polar groups (1), but the further separation of these phospholipids according to their esterified fatty acids is still a problem of current interest. The study of lipid metabolism of mammalian lung is an area of research in which this separation problem is particularly relevant, since the lung rapidly incorporates suitable precursors into a lecithin fraction of distinctive composition (2). This lecithin has a rapid turnover (2), contains a large amount of DPC, and plays an important part in the maintenance of the surface-active coating of the alveolar surface (3). The fractionation problem facing biochemists working with lung lipids is that of separating a lecithin fraction containing a large amount of a species with two saturated fatty acids esterified in the 1- and 2-positions, without destroying the alcoholic or fatty acid functions, and of resolving at least the four following fractions: anenoic (the molecular species containing no ethylenic linkages in the two esterified fatty acids), monoenoic, dienoic, and polyenoic.

Existing methods which can resolve molecular species of lecithin are discussed in a review article by Renkonen (4), and the pioneering work in this field will not be reiterated in detail. Several methods have been used to obtain elegant separations of phospholipid species after modifying the polar group (5–9) or the ethylenic linkages of the esterified fatty acids (10), but with all of these methods some portion of the lecithin molecule is lost for further study. Several methods have been described which separate intact lecithin species (11–13), but these methods require further chromatography to resolve adequately at least four fractions. The method of Arvidson (14), which is excellent for fractionating unsaturated lecithin mixtures, does not clearly resolve saturated lecithins from the monoenoic species.

This paper describes an alternate method for the separation of both intact lecithin species and fatty acid methyl esters according to their degree of unsaturation, and for the application of the procedure to several different naturally occurring lecithins. The separation is achieved on a column of partially methylated dextran (Sephadex LH-20; Pharmacia Fine Chemicals Inc.,

Abbreviations: DPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; B–C–M, benzene–chloroform–methanol; C–M, chloroform–methanol.

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Piscataway, N.J.), and the unsaturated lipids are fractionated as their mercury acetate addition compounds.

Preparation of Lipids. Lecithin fractions were isolated from several natural sources by either silicic acid or alumina gel chromatography as described by Hanahan (15). Since the objective of the separation was to obtain homogeneous phospholipid classes, fractions containing more than one phospholipid, as determined by TLC, were discarded.

Egg lecithin was separated on an alumina gel column which was eluted with chloroform–methanol mixtures. Rat (Sprague–Dawley) liver lecithins were obtained by a sequential elution of silicic acid columns. Preparations of surface-active material were obtained from mongrel dog lungs by an extension of the method of Abrams (16), using isopycnic centrifugation in NaBr density gradients. Total lipids were extracted with 20 volumes of chloroform–methanol, and mixed lecithins were obtained by silicic acid chromatography.

Fatty acid methyl esters were purchased from Applied Science Laboratories Inc., State College, Pa.

All lecithin fractions were analyzed by TLC Silica Gel G plates prior to use, and each fraction migrated as a single spot with the R_f of a lecithin standard (synthetic DPC). 80–120 μg of the phospholipid in each fraction was spotted on prepared plates of Silica Gel G, and developed 14–15 cm with chloroform–methanol–H_2O 60:35:5. Fractions were assayed for phosphorus, using the method of Bartlett (17).

Synthetic DPC was purchased from Fluka AG, Buchs SG, Switzerland (Purissima grade), and was repurified by silicic acid chromatography before use.

Preparation of the Mercuric Acetate–Lipid Adducts. The mercuric acetate reagent was prepared according to Mangold (13). 1.4 g of mercuric acetate was dissolved in a mixture of 25 ml of methanol, 0.25 ml of H_2O, and 0.1 ml of glacial acetic acid. The reagent was stored in a dark bottle and was discarded after 1 week. For the egg and lung lecithins, 1 ml of the mercuric acetate reagent was added to 1–5 mg of the lipid which had been dried under nitrogen, and the solution was stored under nitrogen in the dark for 18–22 hr at room temperature. For the rat liver lecithin separations, as much as 15 ml of mercuric acetate reagent was used for each 4–5 mg of lecithin. Prior to applying the lecithin derivatives to the Sephadex column, the excess mercuric acetate reagent was removed by solvent partition as follows. The methanol solution of the mercuric acetate-addition compounds was concentrated to 1 or 2 ml, and twice this volume of chloroform was added. This was followed by addition of 0.5 ml or 1 ml of H_2O, respectively, and four to five drops of glacial acetic acid. The solution was mixed, centrifuged, and the water-rich top phase was removed by aspiration. The lower chloroform-rich phase was washed with about 2 ml of a water-rich top phase of the same solvent composition that was removed, together with two additional drops of glacial acetic acid. This washing was followed by two more washings without added acetic acid, two washings with five to six drops of added 0.5 M Tris–maleate buffer, pH 7.38, followed by three washings with the chloroform–methanol–H_2O top phase. The initial addition of the acetic acid prevented formation of yellow mercury oxide precipitates, and the nine washings removed most of the unreacted mercuric acetate. In three experiments, the recoveries of phosphorus after these washings were 103, 100.2, and 99.0%. The lower chloroform-rich phase containing the lipid adducts was dried under N_2 at room temperature in a tube wrapped in aluminum foil. The lipids were dissolved in a small volume of the solvent used to equilibrate the Sephadex column, and applied quantitatively to the column in a volume of 1–2 ml.

Solvent Systems and Column Procedure. The results reported in this study were obtained by using a sequential elution system, which varied only by the addition of increasing amounts of glacial acetic acid to the solvent mixture. The Sephadex was swollen from the dried state in the solvent mixture that was to be used for the experiments, or a previously used column was reequilibrated with a new solvent mixture. The column was 2.8 cm in diameter and 27 cm long, with an approximate bed volume of 166 ml. The void volume was determined with polyvinyl pyrrolidone (mol wt 40,000) dissolved in the solvent mixture, and was about 60 ml. Flow rates of 40–80 ml/hr were used without any notable differences in elution patterns in this range.

4-ml fractions were collected, and 0.4-ml aliquots were taken from each tube. The aliquots were analyzed for total lipid by a modification of the charring method of Marsh and Weinstein (18), in which volumes were reduced to increase sensitivity. The method was capable of detecting less than 5 μg of lipid per aliquot.

The remainder of the material in the fractions was combined according to the elution pattern, concentrated under vacuum at 37°C, and taken up in 4 ml of $CHCl_3$. 1 ml of 5% HCl-methanol was added to each combined fraction to reverse the mercury adduction, and this was followed by the addition of 1 ml of methanol and 1 ml of H_2O. Each combined fraction was washed as previously described, and the free lipids were recovered in the lower chloroform-rich phase.

Two solvent systems were used to obtain the results described in this paper. These systems are shown in Tables 1 and 3, respectively. In both systems, the column was equilibrated with the initial solvent mixture.

Upon completion of an experiment, the column was washed with 200 ml of the solvent mixture with 1% added acetic acid, followed by 1000–1400 ml of the solvent

mixture without acetic acid. The same gel was used for all the experiments reported, as well as for most of the work used in the development of these procedures, and there was no evidence of deterioration over a 6 month period.

Analysis of the Recovered Fractions. GLC of the fatty acid methyl esters from each fraction was done using a Perkin–Elmer Model F 11 GLC with a $^1/_8$ in. × 11 ft column packed with 12% ethylene glycol succinate (Hi-EEF-2BP) (Applied Science Laboratories Inc.) on Chromosorb W (80–100 mesh). The following chromatographic conditions were used: injection temperature 250° C; isothermal column temperature 186°C; nitrogen carrier gas at 54 lb/sq in. The amount of material was calculated from the response of a flame ionization detector. Fatty acid methyl esters were obtained from aliquots of the recovered lecithin fractions by a transesterification reaction in 1 ml of 1% H_2SO_4 in methanol for 60 min at 70°C; they were recovered in the heptane phase after a heptane–water partition. Relative weight percentage of each methyl ester was calculated from the product of peak height times retention time, or by computer integration of the areas under each curve using an automated system devised by Dr. R. J. Goerke, Cardiovascular Research Institute, University of California, San Francisco, Calif. Molar percentages were calculated by dividing the weight percentage of each methyl ester by its molecular weight.

The average lipid recovery of each fraction was based on the phosphorus content, and the average molecular weight was calculated from the GLC analysis. Fatty acid compositions of the initial mixtures were arithmetically reconstituted (6) from the fatty acid composition of each fraction and its percentage of the total recovered, for comparison with the experimentally determined composition of the initial mixture.

TLC of the recovered fraction showed that all the recovered lipids migrated as single spots with the R_f of lecithin.

In some of the experiments, DPC, labeled with ^{14}C in both fatty acid substituents, was added to the lipid mixture in order to investigate the separation of the anenoic species from the remainder of the lecithin. Liquid scintillation counting was done with a Packard Model 3003 scintillation spectrometer with an internal standard, and counts were corrected for background by statistical averaging. The scintillation fluid used was a mixture of 2,5-diphenyloxazole (15 g/liter) and 1,4-bis(2-[5-phenyloxazoyl])benzene (0.3 g/liter) in toluene, and was diluted with ethanol (4 volumes of ethanol added to 10 volumes of scintillation fluid) to increase the solubility of lipid.

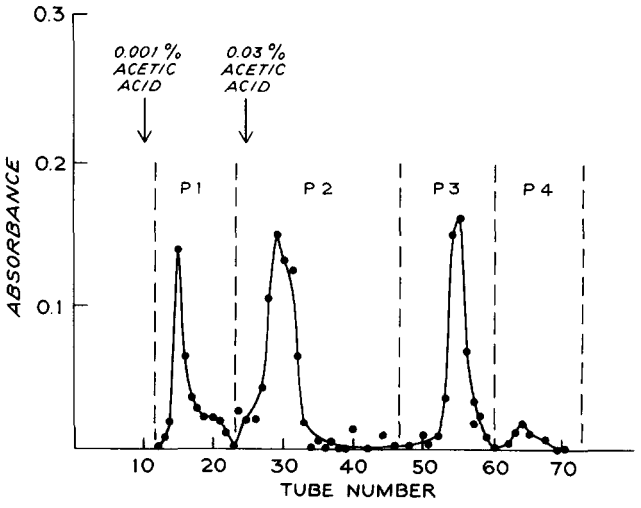

FIG. 1. The elution pattern obtained from a mixture of synthetic DPC and the total lecithin derived from egg. 0.45 mg of DPC and 3.3 mg of egg lecithin were applied to the column. Solvent system: B–C–M 30:30:40 (v/v/v) with added amounts of glacial acetic acid as shown. The dashed lines show the tubes combined into the respective fractions. Experimental details are described in the text.

Results. Three separate lecithin mixtures and a mixture of fatty acid methyl esters were used to test the separation procedure. Typical elution patterns of two of the lecithin mixtures (egg lecithin with added DPC and rat liver lecithin) are shown in Figs. 1 and 2, and the analytical results are given in Tables 1–3.

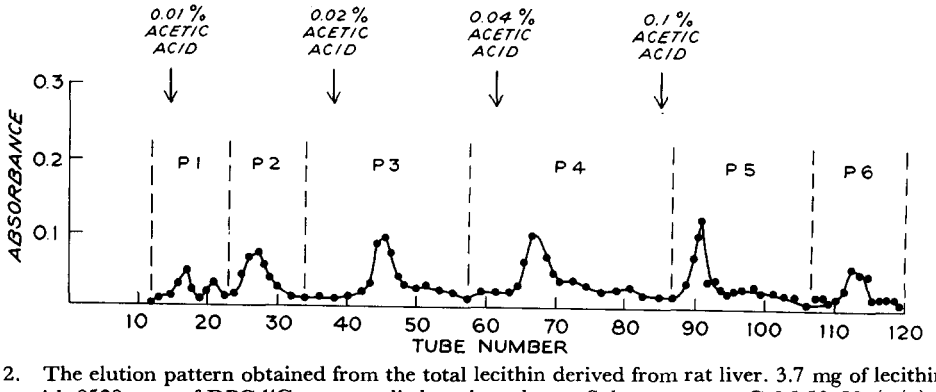

FIG. 2. The elution pattern obtained from the total lecithin derived from rat liver. 3.7 mg of lecithin together with 9520 cpm of DPC-^{14}C were applied to the column. Solvent system: C–M 50:50 (v/v) with added amounts of glacial acetic acid as shown. The dashed lines show the tubes combined into the respective fractions. Experimental details are described in the text.

TABLE 1 FATTY ACID COMPOSITION AND RECOVERY DATA AFTER CHROMATOGRAPHY* OF LECITHIN FROM SURFACE-ACTIVE MATERIAL OF DOG LUNG

	P1	P2	P3	P4	Original Total	Reconstituted Total
			moles %			
14:0†	11.1	0.85	3.8	—	7.5	6.7
16:0	82.9	48.6	42.1	23.0	60.7	64.2
16:1	—	19.7	33.4	13.1	9.6	9.7
18:0	6.0	2.4	—	5.4	3.0	4.7
18:1	—	28.3	11.6	28.3	7.2	9.2
18:2	—	—	—	16.6	2.6	2.3
20:4	—	—	—	—	6.9	—
Unidentified	—	—	9.0	13.5	2.5	2.4
% Recovery					Total	
Phosphorus	56.9	23.6	5.7	13.7	88.8	
% Recovery DPC-^{14}C	99.4	0.2	0	0.4	99.0	

* 0–40 ml B–C–M 30:30:40 (v/v/v); 40–100 ml B–C–M 30:30:40 with 0.001% (v/v) glacial acetic acid; 100–300 ml B–C–M 30:30:40 with 0.03% (v/v) glacial acetic acid.

† Number of carbon atoms: number of double bonds.

TABLE 2 FATTY ACID COMPOSITION AND RECOVERY DATA AFTER CHROMATOGRAPHY* OF A MIXTURE OF DIPALMITOYL AND HEN'S EGG LECITHIN

	P1	P2	P3	P4	Original Total	Reconstituted Total
			moles %			
14:0	0.275	0.25	0.1	1.8	0.05	0.2
16:0	95.9	42.5	24.4	23.7	35.7	36.1
16:1	—	0.3	2.3	1.8	0.8	1.2
18:0	3.9	7.0	9.5	20.3	12.1	8.2
18:1	—	50.0	17.9	7.8	31.6	34.3
18:2	—	—	45.2	11.5	17.2	19.1
20:4	—	—	0.8	33.9	2.55	1.1
% Recovery					Total	
Phosphorus	2.8	53.4	41.8	2.1	73.7	

* Solvent system shown in Table 1.

Lecithin from Pulmonary Surface-Active Material. In these experiments we added DPC-^{14}C to the total lecithin before Sephadex chromatography in order to test rigorously the separation of the anenoic and monoenoic species. As seen in Table 1, more than 99% of the recovered label was found in the anenoic fraction, demonstrating a good separation of these lecithin species which are of particular importance in the analysis of lung lipids.

Mixture of Egg Lecithin and Synthetic DPC. The mean recovery of phosphorus in these experiments was 73.7%, and a comparison of the reconstituted fatty acid composition with the original (Table 2) suggests that some of this loss may have occurred selectively in the lecithin containing 18:0 and 20:4. Antioxidants were not used in these experiments, but their use might be warranted when this procedure is applied to lecithin mixtures with substantial amounts of polyunsaturated acids.

Lecithin from Rat Liver. We experienced some difficulty in obtaining complete mercuric acetate adduct formation of this highly unsaturated lecithin mixture. Several experiments were attempted using about a 10-fold excess of mercuric acetate to lecithin ethylenic bonds (on a molar basis), but apparently the adduct formation was not stoichiometric. The composition data shown in Table 3 represent the means of the results obtained by using a 50-

TABLE 3 FATTY ACID COMPOSITION AND RECOVERY DATA AFTER CHROMATOGRAPHY* OF LECITHIN FROM RAT LIVER

	P1	P2	P3	P4	P5	P6	Original Total	Reconstituted Total
				moles %				
12:0	0.5	0.8	0.3	—	0.5	0.5	Trace (0.1%)	0.3
14:0	2.3	1.4	2.0	1.4	1.3	2.1	Trace	1.6
14:1	—	—	—	—	—	—	—	—
16:0	33.4	23.9	18.0	16.9	19.3	19.7	18.3	19.7
16:1	4.1	3.6	4.3	3.0	3.1	4.1	Trace	3.5
18:0	55.4	26.0	17.5	29.4	25.8	23.0	32.7	26.3
18:1	4.0	14.5	7.2	1.8	4.8	4.4	3.9	6.0
18:2	0.6	28.3	44.2	6.8	2.8	3.5	16.0	18.9
18:3	—	—	2.1	—	—	—	Trace	0.6
20:4	—	1.7	4.5	40.9	34.4	27.9	22.4	21.2
22:6	—	—	—	—	8.2	14.8	6.7	1.9
							Total	
% Recovery Phosphorus	5.5	17.5	25.5	30.6	18.9	4.2	83.7	
% Recovery DPC-^{14}C	94.9	5.2	0	0	0	0	58.2	

Mean values obtained from two experiments.

* 0–60 ml C–M 50:50 (v/v); 60–160 ml C–M 50:50 with 0.01% (v/v) glacial acetic acid; 160–260 ml C–M 50:50 with 0.02% (v/v) glacial acetic acid; 260–360 ml C–M 50:50 with 0.04% (v/v) glacial acetic acid; 360–540 ml C–M 50:50 with 0.1% (v/v) glacial acetic acid.

fold and 100-fold excess of mercuric acetate, respectively. Fraction P2 contains a significant amount of 18:2 plus small amounts of 20:4, probably because of an incomplete adduct formation of the lecithin. The reason for the low recovery of DPC-^{14}C was not apparent.

Fatty Acid Methyl Esters. This lipid mixture was chosen because of the certainty of characterizing from the GLC results the recovered lipid fractions as monoenoic, dienoic, etc. and because it might be used to investigate whether a specific polar group (such as phosphorylcholine) affects the results of the separation. The solvent system described in Table 3 was used to elute the column. The following six elution fractions were obtained from this mixture: fraction 1 contained only anenoic fatty acid methyl esters; fractions 2 and 3 both contained over 90% monoenoic esters; fraction 4 contained 95% dienoic esters; fraction 5 was a mixture of dienoic and trienoic esters; and fraction 6 was a mixture of trienoic and tetraenoic. On a qualitative basis, the separation of the anenoic, monoenoic, dienoic and tetraenoic fractions was good, but there was some overlap of the trienoic with the di- and tetraenoic esters. In the monoenoic fractions 14:1 was eluted after 18:1 giving two peaks principally composed of monoenoic fatty acids, and this may represent a partial separation based upon molecular exclusion by size.

Discussion. The method described here has been successful in resolving four species of intact lecithin, including the anenoic, with one chromatographic procedure. The separations obtained by this method are generally reproducible to within 10%, the over-all recoveries are from 65 to 90%, and the procedure is capable of handling lipid samples of magnitude frequently encountered in biological studies. A similar system can be used to separate fatty acid methyl esters, suggesting that the primary basis of the separation of the lecithins depends upon the methoxy mercuric addition to the ethylenic linkages in the fatty acids. Thus, the method has promise of being applicable to a variety of complex lipids. With the solvent systems used thus far, we have not been able to resolve our lecithin mixture into as many unsaturated species as has been reported by workers using enzymatic methods (5, 6, 7), diazomethanolysis (8, 9), and argentation (14). The method, as developed so far, represents a compromise between the advantages of being relatively easy to use and of separating the intact molecules, and the disadvantage of poorer resolution between the dienoic and trienoic species.

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References

1. Rouser, G., G. Kritchevsky, and A. Yamamota. 1967. *In* Lipid Chromatographic Analysis. G. V. Marinetti, editor. Marcel Dekker, Inc., New York. **1:** 99–161.
2. Tierney, D. F., J. A. Clements, and H. J. Trahan. 1967. *Amer. J. Physiol.* **213:** 671.
3. Clements, J. A., and D. F. Tierney. 1965. Respiration. *In* Handbook of Physiology. W. O. Fenn and H. Rahn, editors. American Physiological Society, Washington, D.C. **2:** 1565–1583.
4. Renkonen, O. 1967. *Advan. Lipid Res.* **5:** 329.
5. Renkonen, O. 1965. *J. Amer. Oil Chem. Soc.* **42:** 298.
6. Lands, W. E. M., and P. Hart. 1966. *J. Amer. Oil Chem. Soc.* **43:** 290.
7. Privett, O. S., and L. J. Nutter. 1967. *Lipids.* **2:** 149.
8. Wurster, C. F., Jr., and J. H. Copenhaver, Jr. 1966. *Lipids.* **1:** 422.
9. Renkonen, O. 1968. *Biochim. Biophys. Acta.* **152:** 114.
10. Privett, O. S., and M. L. Blank. 1963. *J. Amer. Oil Chem. Soc.* **40:** 70.
11. Inouye, Y., and M. Noda. 1958. *Arch. Biochem. Biophys.* **76:** 271.
12. Blank, M. L., L. J. Nutter, and O. S. Privett. 1966. *Lipids.* **1:** 132.
13. Mangold, H. K. 1961. *J. Amer. Oil Chem. Soc.* **38:** 708.
14. Arvidson, G. A. E. 1965. *J. Lipid Res.* **6:** 574.
15. Hanahan, D. J. 1960. Lipid Chemistry. John Wiley & Sons, Inc., New York. 11–42.
16. Abrams, M. E. 1966. *J. Appl. Physiol.* **21:** 718.
17. Bartlett, G. R. 1959. *J. Biol. Chem.* **234:** 466.
18. Marsh, J. B., and D. B. Weinstein. 1966. *J. Lipid Res.* **7:** 574.