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Rapid quantitative measurement of lung tissue phospholipids

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SUMMARY A rapid procedure for the separation of phospholipids of lung tissue into acidic and nonacidic fractions by means of diethylaminoethyl cellulose acetate microcolumns is described. The fractions are then resolved into individual phospholipids by thin-layer chromatography and quantified by transmission densitometry.

KEY WORDS lung · phospholipids · acidic · nonacidic · DEAE cellulose columns · thin-layer chromatography · *quantification* · *charring* · *transmission densitometry*

STUDIES IN THIS laboratory of the synthesis and metabolism of phospholipids in developing fetal lungs necessitated a rapid, reliable method for quantifying phospholipids in scores of samples of extracted lipids. Recent reports (1–4) emphasize the advantages of quantitative analysis of lipids by TLC and transmission densitometry. This analysis is complicated by the tendency of nonacidic and acidic phospholipids to overlap on TLC. The nonacidic phospholipids (principally lecithin, sphingomyelin and phosphatidyl ethanolamine) can be separated from acidic phospholipids (mainly phosphatidyl serine and phosphatidyl inositol) on diethylaminoethyl cellulose in the acetate form (DEAE cellulose acetate), introduced by

Abbreviations: DEAE, diethylaminoethyl; TLC, thin-layer chromatography.

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Rouser and coworkers (5, 6) for column chromatography of lipid mixtures.

This report describes the use of single microcolumns of DEAE cellulose acetate to separate quantitatively the phospholipids from total lipid extracts of tissues into nonacidic and acidic phospholipid groups and the resolution and quantification of individual phospholipids by TLC and transmission densitometry.

MATERIALS AND METHODS

In this report methods are described as employed for extracts of adult and fetal rabbit lungs. However, they can also be used on a variety of other mammalian tissues.

Extraction of Lipids. Tissues are homogenized at 0–2°C under nitrogen for 2 min at 30,000 rpm in a Virtis "45" homogenizer, in 20 volumes of deaerated chloroform–methanol 3:1 (v/v). The lipids are extracted, dried, and reextracted under nitrogen in a way similar to that described by Rouser, Bauman, Kritchevsky, Heller, and O'Brien (5). Extracted lipids were not washed to remove nonlipids. Extracted samples dried to constant weight may be sealed under nitrogen and stored in the freezer. A sample to be applied to a column is dissolved in benzene–acetone 9:1 to a concentration of 250–500 μg/μl with the use of Hamilton syringes, 10–50 μl.

Preparation of DEAE Cellulose Acetate. DEAE cellulose (Cellulose *N*,*N*-diethylaminoethyl ether, Eastman Organic Chemicals, Rochester, N.Y.) is soaked in 1 N HCl (15 g in 400 ml), filtered, washed, stirred in 1 N KOH, and filtered. It is resuspended with vigorous stirring for about $^1/_2$ hr in glacial acetic acid, filtered, and resuspended in absolute methanol. The suspension is filtered and washed repeatedly with methanol until the filtrate is free from acetic acid (as indicated by nitrazine paper). The tendency for DEAE cellulose acetate to form uneven clumps is overcome by homogenizing it in methanol briefly at about 20,000 rpm in a Virtis "45" homogenizer; the uniform suspension produced is deaerated and stored in methanol in a closed glass container.

Preparing the Columns. The microcolumns used are Pasteur pipettes (7 mm I.D. × 95 mm Kontes Glass Co., Vineland, N.J.). A small plug of glass wool is placed at the tapering portion and the pipette packed with DEAE cellulose acetate to a height of 5 cm (250 mg of dry powder). The packing is tight enough if the flow rate is not greater than 0.5–0.6 ml/min with the first solvent combination.

Elution. The column is rinsed with 10–20 ml of benzene–acetone 9:1 and the sample, containing 5–10 mg of total lipid, is applied to the top of the column by means of a Hamilton syringe. The elution scheme is shown in Table 1.

TABLE 1 Elution of Lipids from DEAE Cellulose Acetate Columns

Fraction	Eluent	Substances Eluted
I	Benzene–acetone 9:1	Cholesterol, cholesterol esters, free fatty acids, mono-, di-, and triglycerides
II	Ether–benzene 8:2	
III	Chloroform–methanol 7:3	Phosphatidyl ethanolamine, sphingomyelin, lecithin, lysolecithin, lysophosphatidyl ethanolamine
IV	Ethyl acetate–ether 1:1	
V	Ethyl acetate–methanol 1:1, containing 0.05% NH_4OH	Phosphatidyl serine, phosphatidyl inositol, poly(glycerol phosphate)

For samples containing 5–10 mg total lipid, 10 ml of each solvent combination is adequate for complete recovery.

Fractions I and II are combined as the total nonphospholipid fraction. Fractions III and IV, containing the nonacidic phospholipids, are combined; like fraction V, which contains the acidic phospholipids, they are evaporated under reduced pressure under nitrogen. Several small portions of chloroform are added to the mixture as evaporation of fraction V nears completion to assist the evaporation of ammonium acetate eluted from the column. The lipids are rinsed from the evaporating flask with a small amount of chloroform, filtered under nitrogen through a membrane filter (0.45 μ pore diameter, Millipore Filter Corp., Bedford, Mass.), and collected in weighed tubes in the vacuum flask. The collected fractions are dried to constant weight under a stream of nitrogen.

Resolution of the Individual Phospholipids. Thin layers of Silica Gel H, 250 μ, were prepared from a slurry of 30 g/70 ml of water. The sample, 25–50 μg in 5–10 μl of chloroform, is applied with an automatic delivery syringe (Hamilton), 2.5 cm from the bottom of the plate to give a spot not more than 3–4 mm in diameter. Chloroform–methanol–water 65:25:4 is kept in the glass tank for $^1/_2$–1 hr before the plates are run, and then allowed to ascend 10 cm. The plate is dried in air, sprayed with 50% sulfuric acid (7) and charred for 15 min on a hot plate at 280°C in a fume hood.

Densitometric Measurement. TLC plates are scanned when freshly charred, since the background darkens after prolonged standing, with a transmission density unit (Photovolt Corp., New York, N.Y., Model 52-C), equipped with a synchronous motor-driven chromatogram stage and a 6.0 × 0.1 mm collimating slit. Readings are made with a photometer (Model 501-A) driving a variable response recorder (Model 42-B) set at logarithmic response and equipped with an integrator

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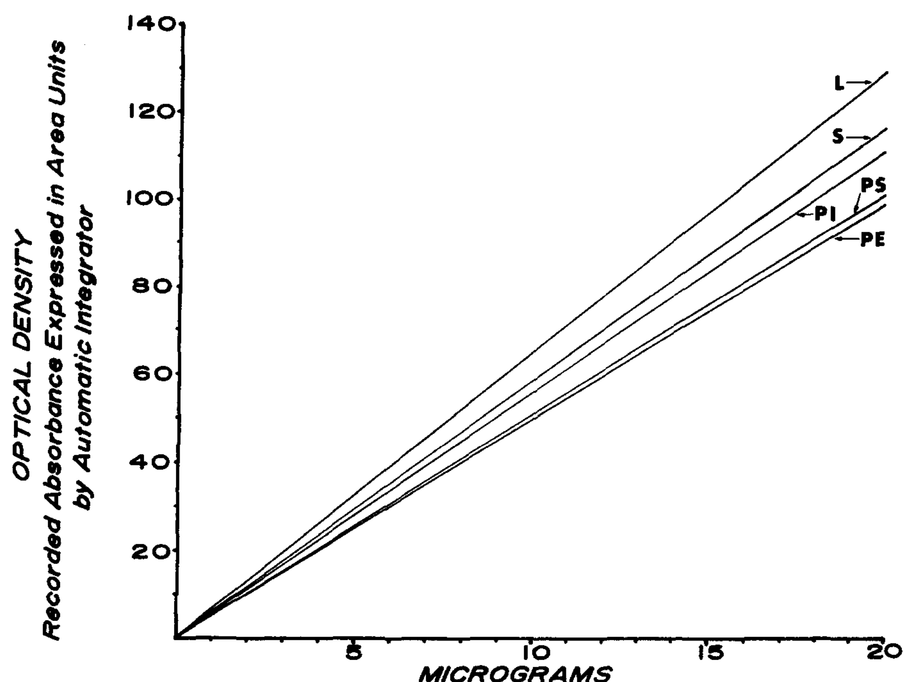

FIG. 1. Standard curves for densitometry of individual major phospholipids on thin-layer plates. The standard error of the reading of ±5% is not shown. Abbreviations as in Fig. 2.

(Model 49). The number of pips registered by the integrator represents the reciprocal of the transmission through any area and is read as a concentration from a standard curve for the corresponding phospholipid.

Construction of Standard Curves for Densitometry. Standard curves (Fig. 1) were prepared from phospholipids isolated from adult rabbit lung both on silicic acid columns (8, 9) and on DEAE cellulose acetate columns by the present method, and purified by TLC. Commercially available standards also were used for comparisons.

The curve for each phospholipid had a different slope; Beer's law was obeyed only for low concentrations. Repeated measurements on a large number and variety of individual and mixed purified phospholipids established that the most reliable readings, with the smallest standard deviations, could be obtained between 2 and 20 μg of any pure phospholipid.

RESULTS

Fig. 2 shows a typical thin-layer chromatogram after separation of fractions from fetal lung on a column of DEAE cellulose acetate.

It was previously noted (6) that there may be reversal on TLC of the positions of phosphatidyl inositol and phosphatidyl serine, which is dependent on heat activation of plates and on equilibration conditions in the solvent chamber. We also have found that a particularly high concentration of phosphatidyl inositol may result in a greater R_f value than that of phosphatidyl serine. A companion TLC plate of the acidic phospholipid fraction should be developed and sprayed with ninhydrin reagent to assure identification of phosphatidyl serine. The other phospholipids maintain a constant relative R_f.

Comparison of Charring Methods for Densitometry. Table 2 is a comparison of TLC densitometric readings after charring with 70% (by volume) H_2SO_4–$K_2Cr_2O_7$ at 180°C (1, 10) and with 50% H_2SO_4 at 280°C.

Although on silica gel with $CaSO_4$ binder (Silica Gel G, AG Merck, Darmstadt, Germany), the two sets of readings are significantly different, they are not different on silica gel without $CaSO_4$ binder (Silica Gel H or HR, AG Merck, Darmstadt, Germany). It is possible that $CaSO_4$ binder is responsible for the reported (1, 10) variations in carbon density. Sulfuric–chromic charring produces appreciably more background darkening of TLC plates than does charring with 50% H_2SO_4.

Recovery from Columns. The recovery from microcolumns of known amounts of purified phospholipids applied individually or in mixtures ranged between 97 and 102%. Phosphatidyl inositol consistently gave recoveries about 3% less than the amount applied but the other phospholipids were recovered quantitatively. Total lipid extract from lung gave 95–98% recovery, the losses presumably being nonlipid impurity, soluble in organic solvents, which is retained on the column. This was verified by

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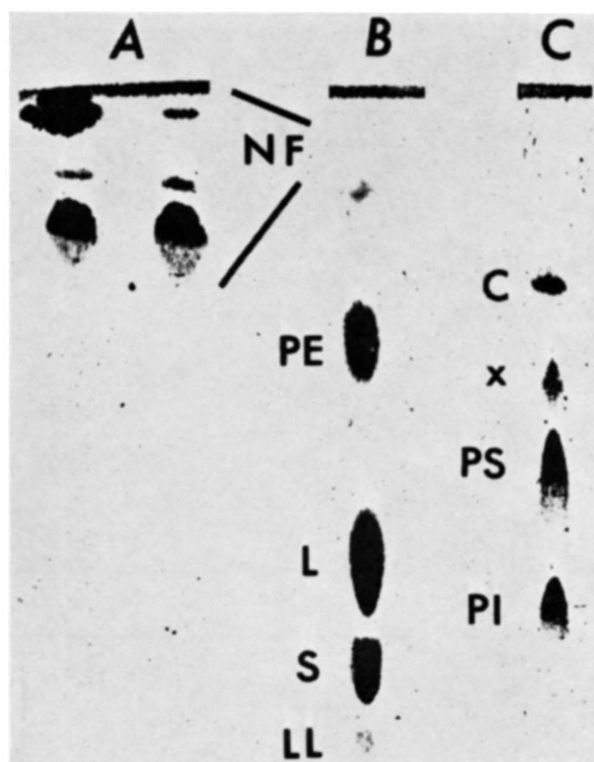

FIG. 2. TLC (on Silica Gel H, chloroform–methanol–water 65:25:4) of fetal lung phospholipids eluted from DEAE acetate columns. *A*, neutral lipids eluted with solvent combinations I and II (Table 1). *B*, nonacidic phospholipids (solvents III and IV combined). *C*, acidic phospholipids (solvent V). Abbreviations: *NF*, neutral lipids; *PE*, phosphatidyl ethanolamine; *L*, lecithin; *S*, sphingomyelin; *LL*, lysolecithin; *PS*, phosphatidyl serine; *PI*, phosphatidyl inositol; *C*, poly(glycerol phosphate); *x*, incompletely identified fraction.

the consistent recoveries of a variety of mixtures of pure phospholipids added to various amounts of previously analyzed extracts from lungs.

Effects of Procedure on Fatty Acid Composition. One portion of egg lecithin was eluted once from the DEAE cellulose acetate column and another portion twice. Methylated hydrolysates of both eluates and of the original sample had identical composition according to gas–liquid chromatography.

Determination of the Component Phospholipids of Lung. Examples of individual analyses obtained by the method described are illustrated in Table 3. The figures for adult lung are in general agreement with values described by others (11–13).

TABLE 2 CARBON DENSITIES ON DIFFERENT TLC ADSORBENTS AFTER CHARRING BY TWO DIFFERENT METHODS

	Density Readings (Area of Recorder Curve by Integrator)			
	Silica Gel G ($CaSO_4$ Binder)		Silica Gel H and Silica Gel HR (no $CaSO_4$ Binder)	
Standard Mixtures Applied for TLC	70% Sulfuric–Chromic and Charring 180°C	50% H_2SO_4 Charring 280°C	70% Sulfuric–Chromic and Charring 180°C	50% H_2SO_4 Charring 280°C
Sphingomyelin 3 μg,	22	27	19	18
lecithin 4 μg,	19	22	28	27
phosphatidyl ethanolamine 3 μg	19	12	14	15
Sphingomyelin 6 μg,	46	44	35	34
lecithin 8 μg,	41	47	55	53
phosphatidyl ethanolamine 6 μg	40	24	29	31

TABLE 3 ANALYSES OF LUNG LIPIDS BY CHROMATOGRAPHY ON DEAE CELLULOSE ACETATE, TLC, AND DENSITOMETRY
Total lipid extracts are corrected for nonlipid contaminants.

	% of Total Lipids		% of Phospholipids
Fetal Rabbit Lung, 30 days gestation (term)			
Total lipid extract (5.4 mg)			
Neutral lipids	20.4		
Nonacidic phospholipids	72.2	79.6	90.7
Acidic phospholipids	7.4		9.3
Nonacidic phospholipids			
Lecithin	52.0		65.1
PE	8.7		10.9
Sphingomyelin	11.5		14.6
Acid phospholipids			
PS	3.7		4.6
PI	2.2		2.8
Unknown + PGP	1.5		1.9
Adult Rabbit Lung (pregnant)			
Total lipid extract (4.7 mg)			
Neutral lipids	34.0		
Nonacidic phospholipids	59.6	65.9	90.3
Acidic phospholipids	6.3		9.7
Nonacidic phospholipids			
Lecithin	33.0		50.1
PE	14.4		21.9
Sphingomyelin	11.8		17.9
Acidic phospholipids			
PS	3.0		4.0
PI	3.0		4.0
Unknown + PGP	1.0		1.3

PGP, poly(glycerol phosphate). For other abbreviations, see Fig. 2.

DISCUSSION

Transmission densitometry requires uniform TLC plates, clean separation of components, and charred spots less wide than the collimating slit (usually 6.0 mm) for the photometer tube. The commercially available densitometer used in these studies is satisfactory, although the custom designed black TLC stage and assortment of collimating slits used by Blank, Schmit, and Privett (1) seems much more versatile. From the slopes of their

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standard curves these authors found little variation among many compounds of the same class in extent of conversion to carbon and constructed a single TLC standard curve relating area of absorption curve to micrograms of carbon. Although this relationship held true for cholesterol palmitate, many fatty acids, mono-, di-, and triglycerides for example, it did not for phosphatidyl ethanolamine, sphingomyelin, or methyl esters of long-chain fatty acids. We use an individual standard curve for each phospholipid, checked frequently with stock known phospholipid mixtures.

Silica Gel H, without $CaSO_4$, gives better TLC separation of individual phospholipids than Silica Gel G; it is more translucent, which favors its use in densitometry; and the absence of $CaSO_4$, seems to avoid the introduction of discrepancies in carbon density following charring with 50% H_2SO_4.

Evaluation of the plasmalogen component of tissues is not possible with DEAE cellulose acetate columns since plasmalogens are hydrolyzed under acidic conditions.

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