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Quantitative two-dimensional thin-layer chromatography of naturally occurring phospholipids

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SUMMARY A method for the complete separation and essentially quantitative isolation of individual components of phospholipid mixtures is described. The mixture is chromatographed in two dimensions on thin layers of Silica Gel G and the phospholipids are extracted from the adsorbent with a solvent mixture containing formic acid.

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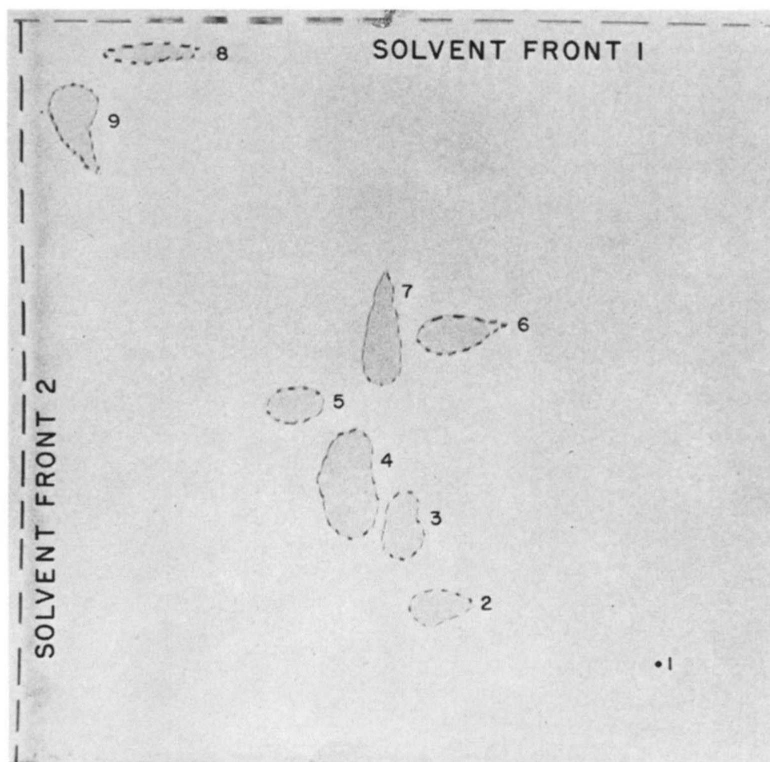

FIG. 1. Two-dimensional TLC of a synthetic mixture of reference phospholipids. The spots were detected with iodine vapor, outlined, and immediately photographed. *1*, origin; *2*, lysolecithin; *3*, sphingomyelin; *4*, phosphatidyl choline; *5*, phosphatidyl inositol; *6*, phosphatidyl serine; *7*, phosphatidyl ethanolamine; *8*, phosphatidic acid; and *9*, cardiolipin.

AMONG THE CHROMATOGRAPHIC techniques proposed for the separation of phospholipids, silicic acid columns and thin-layer plates of silica gel have been used by Phillips (1), Skipski et al. (2), and Skidmore and Entenman (3), while Marinetti (4) has employed silicic acid-impregnated paper; these techniques yield separations with overlap among a number of phospholipids. We propose a method, which incorporates certain features of previously described techniques (2, 3), for the complete separation and quantification of phospholipids utilizing two-dimensional thin-layer chromatography (TLC) on Silica Gel G.

Monophosphatidyl inositol, phosphatidyl serine, lysophosphatidyl choline, and sphingomyelin were from L. Light Co., Colnbrook, England; beef heart phosphatidyl choline, sphingomyelin, and cardiolipin from Sylvana Chemical Co., Milburn, N.J.; phosphatidyl choline from Nutritional Biochemicals, Inc., Cleveland, Ohio; brain phosphatidyl inositol from Dr. Roscoe O. Brady, National Institutes of Health, Bethesda, Md.; and synthetic phosphatidyl (dipalmitoyl) ethanolamine from Mann Research Laboratories, Inc., New York, N.Y. All phospholipids were purified before use by unidimensional preparative TLC on Silica Gel G using chloroform–methanol–glacial acetic acid–water 260:100:32:14 (v/v); the same system was used to isolate phosphatidic acid for use as a reference compound. Phospholipids were detected by means of the reagents described by Skidmore and Entenman (3), and identified by comparing their R_F values with those of purified reference compounds. Organic solvents were high quality commercial preparations (chromatoquality, Matheson, Coleman, and Bell) and were used without additional purification.

Chromatoplates (20 × 20 cm) of 250 μ thickness were prepared as described by Mangold and Malins (5), using a suspension of 30 g of Silica Gel G (according to Stahl, E. G. Merck, A. G. Darmstadt, Germany) in 63 ml of 0.01 M Na_2CO_3 (2). Only those plates that appeared to be uniform in both transmitted and reflected light were used; they were activated at 110° for 30 min immediately prior to use. Samples (5–100 μl) were applied as a spot of less than 5 mm diameter on the lower right corner of the plates under a stream of warm air.

Plates were first developed, in a standard Brinkmann developing chamber previously saturated with vapors of the solvent mixture, with chloroform–methanol–glacial acetic acid–water 250:74:19:3 (v/v). When

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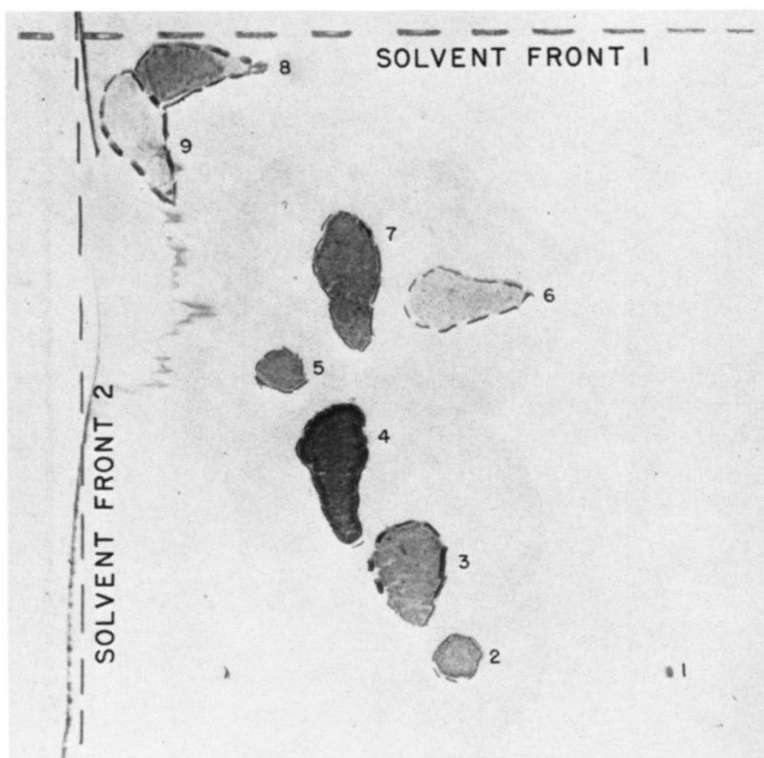

Fig. 2. Two-dimensional TLC of a mixture of phospholipids isolated from rat thymus. The spots were made visible by charring with H_2SO_4 at 110°. See Fig. 1 for identification of spots.

the solvent front had migrated about 15 cm, plates were dried in air for 15 min and developed in the second dimension (90 degree rotation clockwise) with chloroform–methanol–7 M ammonium hydroxide 230:90:15 (v/v); the solvent front was again allowed to move about 15 cm.

Developed plates were dried in air for 5 min and exposed to iodine vapor in a sealed chamber for 30–60 sec; the pale yellow areas were quickly outlined using a dental probe, and the plates exposed to air until the iodine had evaporated from the spots. When a permanent record of developed plates was desired, plates were sprayed lightly with 10 N H_2SO_4, then heated at 110° for 15 min.

The silica gel in each spot was scraped with the aid of a sharp-edged polyethylene blade onto glassine weighing paper, then transferred to a 12 ml conical centrifuge tube. Phospholipids were extracted by suspending each portion of silica gel (Vortex mixer, 30–60 sec) in four successive 2 ml portions of chloroform–methanol–water–formic acid 97:97:4:2 (v/v), separating the extract at each stage by centrifugation at 1200 rpm for 2 min. The same extraction procedure was carried out on silica gel from blank portions of each chromatoplate. Pooled extracts were concentrated by evaporation under purified nitrogen, and quantitative phosphorus analyses performed on suitable aliquots by the method of Bartlett (6), as modified by Marinetti (4).

Figure 1 shows a representative two-dimensional chromatogram demonstrating the separation of a synthetic mixture of eight reference phospholipids.

TABLE 1 Recovery of Reference Phospholipids after TLC

Compound	Reference Solution*	Recovery Procedure†,‡ A	B	C
	μmole P		%	
Phosphatidyl choline	0.200	100.1	98.6	98.6
Phosphatidyl ethanolamine	0.200	97.5	98.0	100.0
Phosphatidyl inositol	0.166	100.2	97.0	96.4
Phosphatidyl serine	0.200	95.5	87.0	84.0
Phosphatidic acid	0.100	99.0	99.0	99.0
Lysolecithin	0.200	100.4	99.0	98.0
Sphingomyelin	0.200	99.0	95.5	98.0

* μmoles of lipid P in a 20 μl aliquot of reference solution.

† Procedure A: 20 μl of reference solution applied to chromatoplate, then immediately extracted from the adsorbent. Procedure B: 20 μl of each reference solution applied and developed in one dimension. Procedure C: 5 μl of each of the seven phospholipid reference solutions pooled and applied as a single spot to each of four chromatoplates; after two-dimensional chromatography corresponding spots from the four plates were pooled, extracted, and analyzed.

‡ All values are corrected for Silica Gel G blanks; typically, these were equivalent to less than 0.001 μmole of P per plate.

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TABLE 2 Quantitative Analysis of Rat Thymus Phospholipids by TLC

Compound Isolated	mμmoles P
Phosphatidyl choline	146.3 ± 0.7*
Phosphatidyl ethanolamine	57.0 ± 0.1
Phosphatidyl inositol	34.2 ± 1.3
Phosphatidyl serine	19.3 ± 1.3
Phosphatidic acid	3.3 ± 0.6
Lysolecithin	4.2 ± 0.6
Cardiolipin	5.8 ± 0.4
Sphingomyelin	32.3 ± 0.2

* Mean ± standard error. Number of replicate determinations = 5. The original phospholipid mixture contained 326.0 ± 5.0 mμmoles of lipid P. Recovery, 302.4 mg.

Figure 2 shows a two-dimensional chromatogram of a phospholipid mixture isolated from rat thymus. Separations of individual phospholipids were essentially complete in both cases. It should be noted that R_F values in the second dimension were somewhat variable from run to run because of variations in room temperature; however, the observed variation was merely a small shift of the entire chromatogram, the degree of separation of the spots remaining unimpaired.

That the separation and extraction procedures were quantitative was checked by experiments with purified reference phospholipids, alone and in combination (Table 1). With the exception of phosphatidyl serine, which was recoverable after one- and two-dimensional TLC in yields of only 87 and 84% respectively, all other phospholipids tested were recoverable in over 95% yield.

A phospholipid mixture isolated from rat thymus gave results listed in Table 2. Recovery of lipid phosphorus was about 93%. Some portion of this loss is presumably attributable to poor recovery of phosphatidyl serine; lipid phosphorus remaining at the origin amounted to less than 1% of the total phosphorus applied to the plate.

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