An improved two dimensional thin-layer chromatography system for the separation of phosphatidylglycerol and its derivatives

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Summary A two dimensional thin-layer chromatography system has been devised for the improved separation of phosphatidylglycerol and its derivatives, cardiolipin and bis(monoacylglyceryl)phosphate, from the other phospholipid components of tissue total lipid extracts. The system employs silica gel G plates prepared with 0.4 M boric acid. Linear recovery of added phosphatidylglycerol was found, and phosphatidylglycerol did not cochromatograph with *N*,*N*-dimethylphosphatidylethanolamine in this system. The phospholipid class composition of various rat tissues and a Morris 7777 hepatoma has been determined and compared with values from the literature.

Phosphatidylglycerol[1] is an acidic phospholipid that has been found in trace amounts in many mammalian tissues. It serves as the precursor of cardiolipin in mitochondria (1–3) but is also synthesized at many subcellular sites in the liver cell where its function is less clear, including the plasma membrane (4), the golgi (5), and the microsomes (2, 6). Evidence has recently been presented showing that phosphatidylglycerol can serve as the precursor of bis(monoacylglyceryl)phosphate (7), a compound reported to accumulate in Niemann-Pick disease (8, 9) and in certain drug-induced lipidoses (10). Phosphatidylglycerol has also been shown to be a component of lung surfactant. This subject has recently been reviewed by Goerke (11).

Several methods have been reported for the determination of phosphatidylglycerol by thin-layer chromatography including one dimensional systems of Skipski et al. (12), and two dimensional systems of Broekhuyse and Veerkamp (13), and Getz et al. (14). In the former, phosphatidylglycerol is separated as a single component, as are cardiolipin and phosphatidic acid, but other phospholipids are not clearly separated. In the latter two systems, cardiolipin migrates too near to the solvent front to allow reliable separation from bis(monoacylglyceryl)phosphate (formerly called lysobisphosphatidic acid), another derivative of phosphatidylglycerol (7). A modification of the system of Getz et al. (14) recently reported by Eichberg and co-workers (15) has a similar disadvantage. Rouser and co-workers (16, 17) have reported several two dimensional thin-layer chromatography systems that separate phosphatidylglycerol from other components in complex mixtures of lipids. However, we have found that phosphatidylglycerol and phosphatidylethanolamine frequently overlap or cochromatograph in these systems, making quantitation difficult or impossible.

An improved two dimensional thin-layer chromatography system has recently been developed that allows a highly reproducible separation of phosphatidylglycerol, cardiolipin, bis(monoacylglyceryl)phosphate, as well as other phospholipids present in the total lipid extracts of most tissues. This paper reports the phospholipid class composition of a rat hepatoma and various rat tissues as determined by this new technique.

Materials and methods

A male Sprague-Dawley rat and a male Buffalo rat carrying a Morris 7777 hepatoma were killed after an overnight fast. The respective tissues were quickly removed, cut into small pieces and washed with ice-cold 0.9% sodium chloride. The tissues were homogenized for several minutes in a Waring blender containing 10 volumes of cold methanol. Chloroform was then added and the proportions adjusted to chloroform–methanol 2:1 (v/v). The total lipid extract was prepared and washed with 0.73% sodium chloride as described by Folch, Lees and Sloane Stanley (18). Lipid phosphorus was determined on aliquots of the washed total lipid extract by the method of Rouser, Fleischer, and Yamamoto (19).

Phosphatidylglycerol was prepared by the transferase action of phospholipase D (Calbiochem, La Jolla, CA) on egg lecithin in the presence of 2M glycerol and purified by silicic acid column chromatography as described by Dawson (20). Bis(monoacylglyceryl)phosphate was purified from rat liver by the method of Poorthuis and Hostetler (7). *N*,*N*-dimethylphosphatidylethanolamine and cardiolipin were obtained from Sigma Corporation, St. Louis, MO. Phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol were purchased from Avanti Biochemicals, Birmingham, Alabama. Silica gel G (Type 60) and silicic acid (45 to 325 mesh

[1] The nomenclature employed for this and other phospholipids is not meant to indicate the exclusive presence of diacyl forms. In the absence of analytical data we recognize the possible presence of alkyl and alk-1-enyl forms as well.

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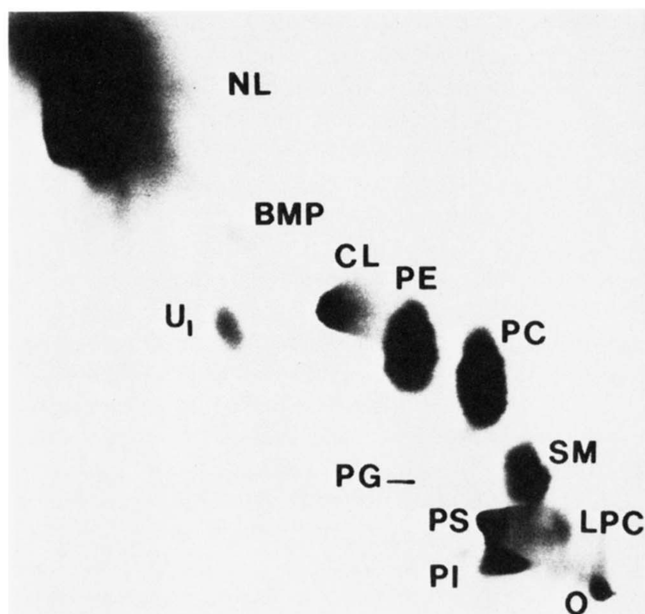

Fig. 1. Two dimensional thin-layer chromatogram of rat kidney lipids as described in Methods. Abbreviations used: NL, neutral lipids; UI, unidentified; BMP, bis(monoacylglyceryl)phosphate; CL, cardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PG, phosphatidylglycerol; SM, sphingomyelin; PS, phosphatidylserine; PI, phosphatidylinositol; LPC, lysophosphatidylcholine; O, origin. The solvent for development in the vertical direction was chloroform–methanol–water–concentrated ammonia 70:30:3:2 (by vol); the solvent for development in the horizontal direction was chloroform–methanol–water, 65:35:5 (by vol).

ASTM) were obtained from EM reagents (Brinkmann Instruments, Burlingame, CA). Solvents were obtained from the usual commercial sources and were redistilled before use. Other chemicals were of analytic reagent grade quality from commercial sources.

A slurry of silica gel G was prepared by mixing 25 g of silica gel with 50 ml of 0.4 M boric acid. A 0.25 mm layer was spread on 20 × 20 cm glass plates and allowed to air dry. The plates were then activated for 1 hr at 120°C and the total lipid extract was applied to the origin immediately after the plates had cooled to room temperature. Normally 250–500 nmoles of phospholipid was applied. Developing tanks were lined with Whatman 3MM filter paper, which had been dried in the oven for 5 min at 120°C. The developing solvents (300 ml) were allowed to equilibrate in the tanks for 1 hr before chromatography was carried out. The solvent for the first dimension was chloroform–methanol–water–concentrated ammonia 70:30:3:2 (by vol). After development in the first dimension, the plates were placed in a modified TLC tank and dried with a stream of nitrogen at room temperature for 30 min. Immediately after drying, the plates were developed in the second dimension with chloroform–methanol–water 65:35:5 (by vol). The lipids were visualized with iodine vapors and photographs were made with a Polaroid MP3 Camera and Type 51 high-contrast film. A discrete spot containing 2 nmoles or more of phospholipid could be visualized with this technique.

Phospholipids were identified by comparison with pure reference compounds and by mild alkaline hydrolysis and chromatography of the water-soluble products (21). The areas of silica representing the respective phospholipids were scraped into tubes and phosphorus was determined by the method of Rouser et al. (19).

Results and discussion

Figs. 1 and **2** are pictures of thin-layer chromatograms of the total lipid extracts from rat kidney and lung, respectively. Basically, the relative positions of the phospholipids in these chromatograms are not too dissimilar from the widely-used two dimensional system of Rouser et al. (19) with two important exceptions. The positions of phosphatidylinositol and phosphatidylserine are reversed and, in addition, phosphatidylglycerol is more distinctly separated from phosphatidylethanolamine with which it often cochromatographs in the system of Rouser et al. (19). Both of these differences are due to the presence in

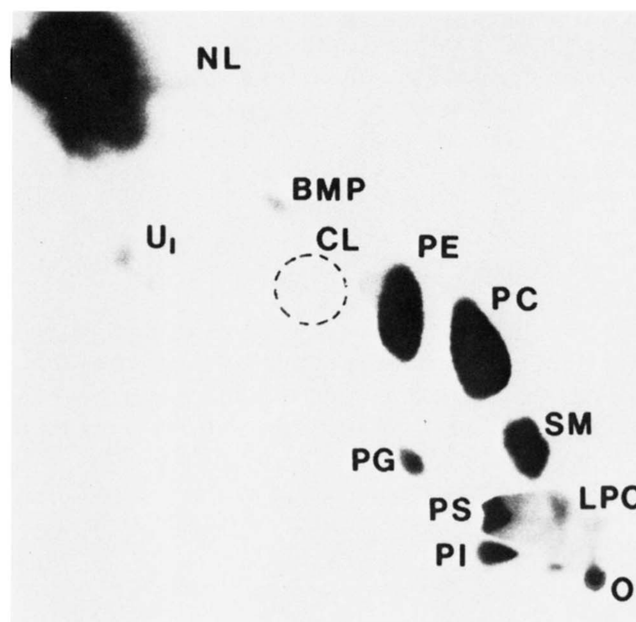

Fig. 2. Two dimensional thin-layer chromatogram of rat lung lipids as described in Methods. Abbreviations used are the same as for Fig. 1.

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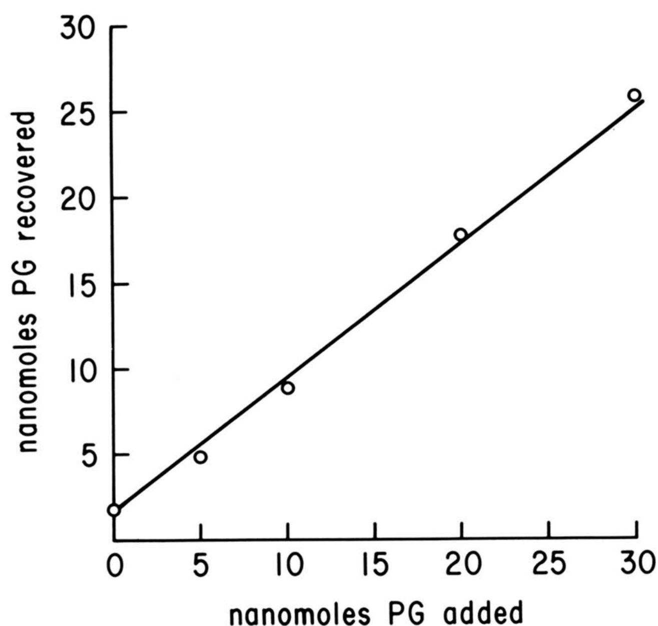

Fig. 3. Recovery of reference phosphatidylglycerol (PG) added to rat liver total lipids. Rat liver total lipid extract containing 400 nmoles of phospholipid was applied to the origin, and increasing amounts of PG were subsequently applied as noted on the abscissa. The plates were developed and phosphorus determined as described in Methods. PG recovery was linear; no significant changes were noted in the recovery of other phospholipid classes.

the silica gel of boric acid, which forms complexes with compounds containing vicinal hydroxyl groups and retards their mobility (22, 23).

Phosphatidic acid, an important trace phospholipid, was not regularly detected in significant quantities in these total lipid extracts. However, when larger loads are applied, it can be identified. It is found at the left of and slightly superior to phosphatidylinositol. Its position was confirmed by the chromatography of pure reference phosphatidic acid and phosphatidylinositol.

Recovery of phosphatidylglycerol added to total lipid extract. **Fig. 3** shows the results of an experiment in which 400 nmoles of rat liver phospholipid was applied to the origins of silica gel G–boric acid plates. Increasing amounts of reference phosphatidylglycerol were spotted on the origin, which already contained 400 nmoles of rat liver phospholipid. The plates were developed and a phospholipid analysis was carried out. The recovery of the added phosphatidylglycerol was linear and averaged about 80%. This is somewhat lower than the usual >95% recovery of total phospholipid, and is probably due to the presence of some impurities in the phosphatidylglycerol reference and to the affinity of acidic phospholipids for glass surfaces (e.g., the Hamilton syringe barrel).

Separation of phosphatidylglycerol from N,N-dimethylphosphatidylethanolamine. Several published reports indicate that *N*,*N*-dimethylphosphatidylethanolamine can be difficult to separate from phosphatidylglycerol (24–26). **Fig. 4** shows the position of these two compounds in this chromatography system. Reference *N*,*N*-dimethylphosphatidylethanolamine appears slightly above the place where phosphatidylethanolamine is normally found, while phosphatidylglycerol occupies a lower position due to the retardation of its movement caused by the effect of boric acid on vicinal hydroxyl-containing compounds. Thus, this system clearly presents no ambiguities in the separation of these two compounds.

Phospholipid analysis of tissue total lipid extracts. **Table 1** shows the phospholipid composition of various rat tissues and a Morris 7777 hepatoma as determined by this method of two dimensional thin-layer chromatography. Small, but readily detectable amounts of phosphatidylglycerol were observed in all tissues studied. Rat lung tissue had the highest phosphatidylglycerol content, 4.1% of the total phospholipid phosphorus, while rat liver and the Morris 7777 hepatoma had the lowest content, 0.63 and 0.70%, respectively. Bis(monoacylglyceryl)-phosphate, a derivative of phosphatidylglycerol (7), was detected in most rat tissues with the exception of the heart. Rat spleen contained the most bis(monoacylglyceryl)phosphate, 3.0% of the total lipid phosphorus. Cardiolipin, also a derivative of phosphatidylglycerol (1–3), was also present in all tissue lipid

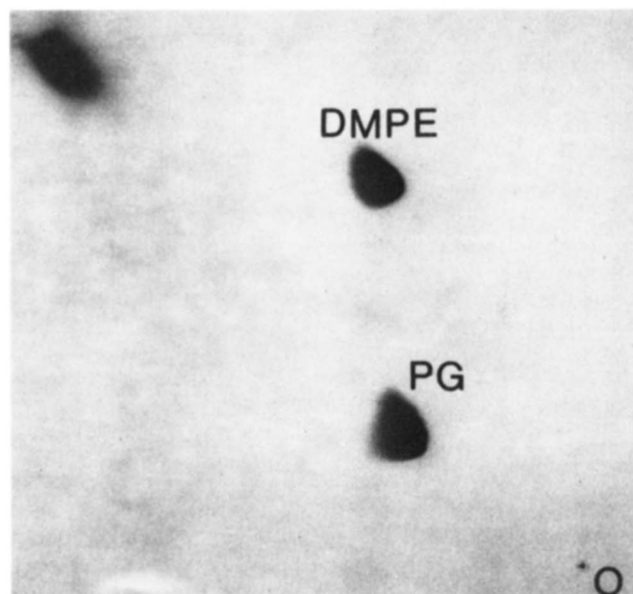

Fig. 4. Two dimensional thin-layer chromatograph of reference phosphatidylglycerol (PG) and *N*,*N*-dimethylphosphatidylethanolamine (DMPE).

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TABLE 1. Phosphatidylglycerol class composition of rat tissues and a rat hepatoma[a]

	Heart (3)[b]	Liver (4)	Lung (3)	Kidney (5)	Spleen (4)	Morris 7777 Hepatoma (3)
Bis(monoacylglyceryl)phosphate	n.d.[c]	0.41 ± 0.12	0.77 ± 0.22	1.2 ± 0.50	3.0 ± 0.37	0.62 ± 0.13
Cardiolipin	14.7 ± 0.17	5.7 ± 0.27	0.76 ± 0.28	7.0 ± 0.65	2.9 ± 0.35	3.5 ± 0.07
Phosphatidylglycerol	1.5 ± 0.15	0.63 ± 0.17	4.1 ± 0.15	1.5 ± 0.26	1.1 ± 0.25	0.70 ± 0.15
Phosphatidylethanolamine	32.0 ± 0.44	21.6 ± 0.17	18.6 ± 1.2	24.7 ± 0.92	23.9 ± 1.0	25.8 ± 0.54
Phosphatidylcholine	41.2 ± 0.52	52.0 ± 0.64	54.3 ± 0.15	31.4 ± 1.0	42.6 ± 2.2	45.6 ± 0.19
Phosphatidylinositol	2.9 ± 0.17	10.3 ± 0.30	3.8 ± 0.35	6.9 ± 0.38	6.8 ± 0.30	8.8 ± 0.12
Phosphatidylserine	2.5 ± 0.64	2.9 ± 0.49	5.8 ± 0.52	6.3 ± 0.24	8.4 ± 0.24	4.4 ± 0.17
Sphingomyelin	1.9 ± 0.15	3.6 ± 0.23	8.1 ± 0.50	12.1 ± 0.40	6.2 ± 0.38	8.0 ± 0.17
Lysophosphatidylcholine	1.0 ± 0.17	1.1 ± 0.15	2.4 ± 0.03	2.5 ± 0.59	2.4 ± 0.44	0.38 ± 0.09
Unidentified	0.22 ± 0.03	0.78 ± 0.16		3.4 ± 0.20	1.3 ± 0.09	0.60 ± 0.31
Origin	1.6 ± 0.25	1.2 ± 0.49	1.1 ± 0.27	2.7 ± 0.42	2.0 ± 0.40	1.6 ± 0.56

[a] Percent of total phospholipids ± SEM.
[b] Number of determinations.
[c] n.d., not detected.

extracts examined; heart contained the most cardiolipin, 14.7%, and lung the least, 0.76%, in accordance with the values reported in the literature (17, 27). The remainder of the phospholipid analysis was found to agree reasonably well with previously published reports for rat heart (17), liver (16), lung (27), and kidney and spleen (16). Recoveries of phospholipid phosphorus were greater than 95% and reproducibility was satisfactory as indicated in Table 1. This method is simple, reliable, and has proven to be quite useful in our ongoing studies of the metabolism of phosphatidylglycerol and its derivatives. It should also be useful in analysis of lung and surfactant phospholipids.

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