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Note

Improved thin-layer chromatographic method for the separation of major phospholipids and glycolipids from plant lipid extracts and phosphatidyl glycerol and bis(monoacylglyceryl) phosphate from animal lipid extracts

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A large number of solvents and stationary phases exist for the separation of both plant and animal glycerolipids using both one- and two-dimensional thin-layer chromatography (TLC)[1–4]. Two-dimensional TLC, while providing the best separation, is time consuming and often results in oxidation of some of the poly-unsaturated fatty acids of the lipids. A further disadvantage is that the amount of lipid that can be applied to a plate is very limited. On the whole, the one-dimensional TLC systems that exist do not completely separate all plant lipids. Similarly no one-dimensional TLC system has been reported that satisfactorily and consistently separates phosphatidyl glycerol and bis(monoacylglyceryl) phosphate from animal tissues.

In our work with phospho- and glycolipids from plant tissue we needed a rapid and complete separation of individual lipids for quantitative analysis by gas–liquid chromatography. Many of the TLC systems used to separate plant lipids did not separate phospholipids from the galactolipids, particularly digalactosyl diglyceride (DGDG), and often resulted in considerable overlap of other phospholipids and glycolipids. The solvent system of Pohl *et al.*[1] provided good separation of monogalactosyl diglyceride (MGDG) and DGDG but did not separate phosphatidyl glycerol and phosphatidylethanolamine consistently. We report here a TLC system which not only allows a highly reproducible separation of plant lipids but also separates phosphatidyl glycerol (PG) and bis(monoacylglyceryl) phosphate (bis-MP) from the crude lipid extracts of various mammalian tissues.

MATERIALS AND METHODS

Plant lipids

Lipids were extracted from leaves of *Vicia faba* by homogenization in a high-speed blender (VirTis Model 45, Fisher Scientific) with chloroform–methanol (2:1, v/v). Lipid extracts were filtered through PTFE-coated Millipore filters and washed thoroughly with chloroform–methanol (2:1) and chloroform. Water soluble non-lipid contaminants were removed by the method of Williams and Merrilees[5]. The chloroform solution of lipids was dried in a flash evaporator, redissolved in a suitable volume of chloroform and stored under nitrogen in a freezer until use. Labelled

lipids (^{14}C) were extracted from leaf discs infiltrated with [^{14}C]acetate solution or fed $^{14}CO_2$ in light (1100 ft-c.)[6,7].

Animal lipids

Male rats of the Wistar strain, weighing 200–250 g, received chlorphentermine (50 mg/kg) intraperitoneally for two weeks[8]. Animals were killed after fasting for 16 h, livers and spleens were removed and the lipids were extracted with 19 volumes of chloroform–methanol (2:1). The crude lipids were purified according to the method of Folch *et al.*[9].

Purification of bis(monoacylglyceryl) phosphate

Male Wistar rats weighing 200–250 g were fasted for 16 h before sacrifice. Livers from 2 rats were removed and the lipids were extracted as described previously. Lipids were dissolved in 100 ml of methanol–chloroform–water (60:30:8) and put on to a DEAE-Sephadex column, 30 × 2.5 cm (ref. 10). The column was eluted sequentially with 1 l of methanol–chloroform–water (60:30:8) and 1 l of methanol–chloroform–0.2 *M* sodium acetate (60:30:8). The acidic phospholipids, containing bis-MP, were eluted with the second eluting medium and were concentrated. The lipids were redissolved in 25 ml of chloroform–methanol (2:1) and sodium acetate was removed by washing with water. Lipids were concentrated and chromatographed to separate bis-MP[11]. After elution from the gel the lipid was rechromatographed to check the purity[12]. A single spot of bis-MP was detected after spraying sulphuric acid and charring at 150°. The purified bis-MP was used as a reference compound. Phosphatidyl glycerol standard was purchased from Supelco.

Preparation of TLC plates

Normal silica gel G plates were prepared by applying a slurry of 40 g of gel in 100 ml of water to glass plates (20 × 20 cm) at a thickness of about 0.37 mm using a Desaga spreader. Ammonium sulphate impregnated plates were prepared by substituting 0.15 *M* ammonium sulphate solution for water. Both types of plates were left at room temperature for 5 min and then dried in an oven at 110° for 4 h. The plates were cooled to room temperature just prior to use. If the plates are stored at room temperature they must be activated for 1 h at 110° before use.

Application of samples and developing solvents

The samples and reference compounds were dissolved in chloroform or chloroform–methanol (2:1) and applied to the plate in a band with a glass applicator. The TLC chambers were lined on both sides with filter paper saturated with the chromatographic solvents. The ammonium sulphate impregnated plates were developed in acetone–benzene–water (91:30:8). For comparison normal plates were developed in the same solvent and also in chloroform–methanol–water (65:25:4)[2]. The plates were developed by the ascending technique until the solvent front had reached a distance of 2 cm from the top whereupon they were removed and dried under nitrogen.

Identification of lipids

Lipid bands were identified using reference compounds and appropriate spray

reagents. The following spray reagents were used: phospholipid spray reagent of Vaskovsky and Kostetsky[13], ninhydrin (2% in ethanol) for phosphatidylethanolamine and phosphatidyl serine, cresyl-violet acetate for sulphoquinovosyl diglyceride[14]. Lipids were also visualized by charring the plates after spraying with sulphuric acid. When the lipids were used for subsequent GLC analysis, chromatoplates were sprayed with 2′,7′-dichlorofluorescein and visualized under UV light.

Gas–liquid chromatography

Fatty acid methyl esters of individual lipids were prepared as described previously[6] and analysed on a dual-column gas chromatograph (Packard Model 7401) equipped with flame ionization detector and a digital integrator (Infotronics Model CRS 208). Samples were injected onto a glass column (4 mm × 180 cm) packed with 10% EGSS-X on Chromosorb P and run isothermally at 180°.

RESULTS

Plant lipids

The autoradiographs of ^{14}C-labelled lipids separated by three different chromatographic systems are shown in Fig. 1. Although the solvent system chloroform–methanol–water (65:25:4) allowed a distinct separation of MGDG from the rest of the lipids, the nearly equal R_F values of PG and PC, PE and DGDG and the complete overlapping of PE with an unidentified lipid made the whole system difficult to use for qualitative as well as quantitative studies (see legend of Fig. 1 for abbreviations). Besides, the lipid bands were often contaminated with pigments. The TLC system recommended by Pohl *et al.*[1] was used to separate galactosyl diglycerides but the separation of phospholipids was found to be very inconsistent and often resulted in the overlapping of PG with PE, and SL with the unidentified lipid. The method described here offered advantages over the above TLC techniques because of the complete separation of PC, PE, PG and SL as well as the galactosyl diglycerides. With the exception of PG and the unidentified lipid, the mobilities of the glycolipids and the phospholipids were not significantly different from the system used by Pohl *et al.* In order to check the purity of each of these lipids, individual lipids were separated by preparative TLC, eluted from the gel and rechromatographed separately by the above three systems; each lipid gave a single band.

Fatty acid methyl esters of individual lipids separated on ammonium sulphate plates were prepared[6] and analysed by GLC; each lipid showed its characteristic fatty acid profile (Table I). *Trans*-Δ^3-hexadecenoic acid is only found in PG and thus used as a marker for PG in green photosynthetic tissues. Galactosyl diglycerides were obtained free of contaminants. GLC analyses of deacylated products of MGDG and DGDG, as reported previously[7], was used to confirm their identity.

Animal lipids

PG and bis-MP are present in mammalian tissues in such small quantities that it becomes impossible to separate and detect them by two-dimensional TLC unless the lipids are previously labelled with radioactive precursors. Attempts were made to increase the level of bis-MP in liver and spleen by drug treatment and the total lipids were chromatographed by using our chromatographic system. The details of

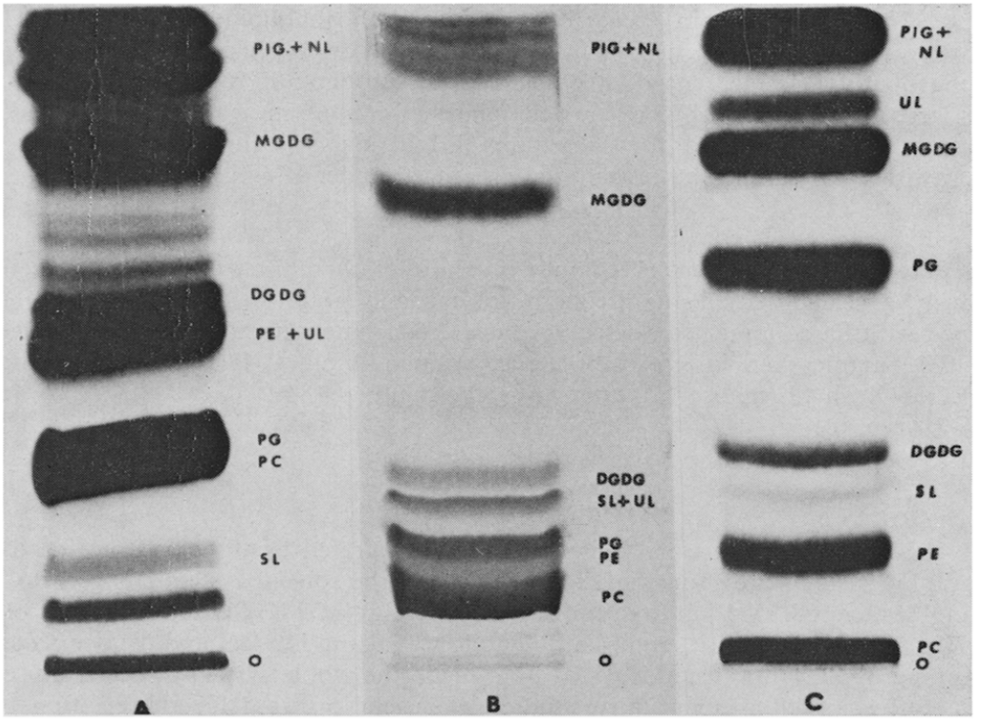

Fig. 1. Autoradiographs of TLC plates comparing the three different systems employed during the separation of ^{14}C-labelled lipids from *Vicia faba* leaves. A, silica gel G plates in chloroform–methanol–water (65:25:4); B, silica gel G plates in acetone–benzene–water (91:30:8): C, ammonium sulphate impregnated silica gel G plates in acetone–benzene–water (91:30:8). O = origin; PC = phosphatidyl choline; PE = phosphatidylethanolamine; PG = phosphatidyl glycerol; MGDG = monogalactosyl diglyceride; DGDG = digalactosyl diglyceride; SL = sulphoquinovosyl diglyceride; UL = unidentified lipid; PIG + NL = pigments and neutral lipids.

TABLE I

FATTY ACID COMPOSITIONS (mole %) OF PHOSPHOLIPIDS AND GLYCOLIPIDS FROM GREEN LEAVES OF *VICIA FABA*

For abbreviations of lipids, see legend to Fig. 1.

Lipids	*No. of C at. in fatty acid: no. of unsaturated bonds*						*Quantity (μmole/g fresh wt.)*
	16:0	*16:1*	*18:0*	*18:1*	*18:2*	*18:3*	
PC	19	—	3	14	49	15	1.90
PE	28	—	3	6	45	18	0.64
PG	40	11	2	6	16	25	0.83
MGDG	3	—	trace	2	9	85	2.96
DGDG	10	—	3	1	4	82	1.72
SL	28	—	4	5	17	46	0.29
UL	23	—	4	6	17	50	0.16

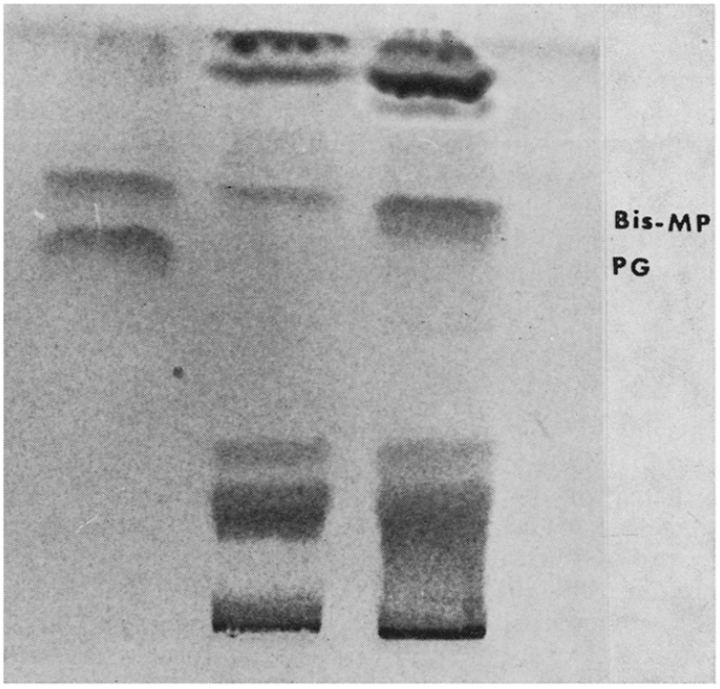

Fig. 2. TLC of total lipids from rat liver and spleen. Samples in lanes 1–3 are reference compounds of PG + bis-MP, spleen and liver lipids, respectively. Samples were applied to silica gel G plates impregnated with ammonium sulphate and developed with acetone–benzene–water (91:30:8).

TABLE II

FATTY ACID COMPOSITION OF BIS(MONOACYLGLYCERYL) PHOSPHATE DERIVED FROM RAT LIVER AND SPLEEN TISSUES AFTER TREATMENT WITH CHLORPHENTERMINE (50 mg/kg)

Fatty acid (no. of C at.: no. of unsaturated bonds)	*% by weight*	
	Liver	*Spleen*
14:0	tr*	2
16:0	3	9
16:1	1	3
18:0	3	2
18:1	8	37
18:2	7	19
20:0	1	tr*
20:1	1	2
Unidentified	tr*	2
22:0	3	4
20:5	2	2
24:0	1	4
24:1	2	2
Unidentified	3	2
22:6	64	6
Unidentified	1	3

* Trace amount, $< 0.5\%$.

the drug effect on the metabolism of phospho- and glycolipids will be published elsewhere[15].

Fig. 2 shows the separation of bis-MP from total lipids extracted from liver and spleen tissues of rats previously treated with chlorphentermine. Most non-acidic phospholipids remained near the origin whereas bis-MP moved further up the plate and also separated distinctly from its closely related lipid, PG. Thus the two lipids can be scraped out of the plate with the silica gel and recovered quantitatively. Fatty acid methyl esters were prepared from bis-MP and analysed by GLC. The percent composition of the fatty acids of rat liver bis-MP (Table II) were very similar to the results obtained from liver "tritosomes"[16].

DISCUSSION

The use of ammonium sulphate to minimize absorption effects has previously been recommended by Chalvardjian *et al.*[17]. Mangold and Kammereck[18] have used silica gel G TLC plates impregnated with 10% ammonium sulphate for the separation of phospholipids and strong acidic fatty acid derivatives. Horrocks[19] has employed ammonium sulphate to suppress the streaking problem in some phospholipids. Recently, Walker[20] has reported a technique to detect the lipids separated on silica gel G TLC plates impregnated with ammonium sulphate, simply by charring.

The present method utilizes the solvent system of Pohl *et al.* that was used to separate plant phospho- and glycolipids on silica gel G plates, and the purpose of using ammonium sulphate impregnated silica gel G plates is to modify the mobility of some acidic phospholipids. In contrast to many methods for the separation of lipids this system has proved to be a rapid technique which gives reproducible results and allows a complete separation of the major phospholipids and glycolipids. The chromatoplates can be loaded with the plant lipid extract equivalent to 1.5–2 mg of chlorophyll (8–10 μmoles of lipids) and separation is complete in 40–45 min.

Since PG and bis-MP are present in most mammalian tissues in extremely small quantities[21] a large amount of total lipid extract should be loaded onto a TLC plate in order to detect these lipids. The advantage of the TLC system reported here is that it can be loaded with approximately 10–12 mg of total lipids and still give excellent separation of PG and bis-MP. We believe this method should be useful for scanning and quantitative recovery of phosphatidyl glycerol and its derivatives synthesized during incubations of rat liver mitochondrial preparations with cytidine diphosphate diglyceride and radioactive *sn*-glycerol-3-phosphate.

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