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Two-dimensional thin-layer chromatography of rat liver phosphatides

W. D. Skidmore and C. Entenman

U. S. Naval Radiological Defense Laboratory, San Francisco, California

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SUMMARY

A system of two-dimensional thin-layer chromatography was developed that separated rat liver phosphatides into several phosphate-positive spots in about 2 hr developing time. Characteristic hydrolysis products derived from phosphatidyl serine, phosphatidyl ethanolamine, phosphatidyl inositol, phosphatidyl choline, sphingomyelin, and lysophosphatidyl choline were identified. The hydrolytic products of "phosphatidic acid" were not definitely characterized. The application of thin-layer chromatography as described for rat liver phosphatides can be extended to phosphatide extracts of other tissues.

The trend toward widespread use of thin-layer chromatography (TLC) was accelerated by the work of Stahl (1–3) who demonstrated its potential usefulness in lipid research. In an excellent review, Mangold (4) presented a detailed description of commercially available apparatus and several techniques used in TLC as well as applications of the method to lipids in general. One-dimensional TLC has been applied to the separation of phosphatides from brain (5, 6), serum (7–9), and other sources (10) with varying degrees of success. It was observed in this laboratory that one-dimensional TLC did not separate all of the phosphatides found in a rat liver extract with the solvent systems used. Therefore, a system of two-dimensional TLC was applied. Tentative identification of the phosphatides was made possible by the application of different color-developing reagents. Further identification was accomplished by eluting the phosphatides, hydrolyzing them, and identifying the hydrolytic cleavage products with standards by TLC. In this way, the seven phosphatide spots were identified as "phosphatidic acid" (PhA), phosphatidyl serine (PhS), phosphatidyl ethanolamine (PhE), phosphatidyl inositol (PhI), phosphatidyl choline (PhC), sphingomyelin (Sph), and lysophosphatidyl choline (L-phC).

MATERIALS

1. *Detection reagents for phosphatides and their hydrolytic products.*

a. *Iodine vapor* (I_2) to detect lipids nonspecifically. Dry plates were exposed for about 1 min in a plastic box containing iodine vapor produced from iodine crystals (4).

b. *Ninhydrin* (Nin) to detect amino phosphatides. Dry plates were sprayed with a solution of 0.3 g ninhydrin in 5 ml lutidine and 95 ml *n*-butanol saturated with water. As the plates were dried at room temperature, red-violet spots appeared on a white background.

c. *Molybdic acid* (Mo) to detect phosphatides. Dry plates were sprayed with a solution of 5 ml 60% w/v perchloric acid, 10 ml N HCl, and 25 ml 4% w/v ammonium molybdate (11). Blue spots appeared on a white background as the plates were dried at room temperature.

d. *Ferric chloride-sulfosalicylic acid* (Fe) to detect phosphate groups. Dry plates were sprayed with a solution of 7.0 g sulfosalicylic acid, 0.1 g $FeCl_3 \cdot 6H_2O$, and 25 ml water diluted to 100 ml with 95% ethanol (12). White fluorescent spots appeared on a purple background as the plates were dried at room temperature.

e. *Ammoniacal silver nitrate* (Ag) to detect glycerol and inositol. Dry plates were sprayed with a mixture of equal volumes of 0.1 N $AgNO_3$ and 7 N ammonium hydroxide (13). The plates were then heated at 110° until dark brown spots appeared on a white background.

f. *Dragendorf reagent* (Bi) to detect choline. Dry plates were sprayed with a mixture of 4 ml solution I, 1 ml solution II, and 20 ml distilled water. Solution I contained 1.7 g $Bi(NO_3)_3 \cdot 5H_2O$ diluted to 100 ml with 20% v/v acetic acid. Solution II contained 40 g KI in 100 ml water. As the plates were dried at room temperature, free choline produced a purple spot and choline-containing compounds produced orange spots (6).

g. *Fuchsin-sulfurous acid* (Schiff's reagent) to detect aldehyde groups. Dry plates were sprayed with a solution of 1 ml Fuchsin-sulfite reagent (14), 1 ml 0.05 M $HgCl_2$, and 10 ml 0.05 M H_2SO_3 diluted to 100 ml with distilled water. Violet spots appeared rapidly on a pale violet background.

h. *Hydroxylamine-ferric chloride* to detect esterified fatty acids (EFA). Dry plates were sprayed with alkaline hydroxylamine reagent, dried briefly, and then sprayed with ethereal acid ferric chloride reagent. Purple spots appeared quickly on a yellow background (15).

The alkaline hydroxylamine reagent was prepared by dissolving 10 g $HONH_2 \cdot HCl$ in 25 ml water and diluting it to 100 ml with ethanol and mixing this reagent with 26 ml of saturated aqueous NaOH diluted to 200 ml with ethanol. The

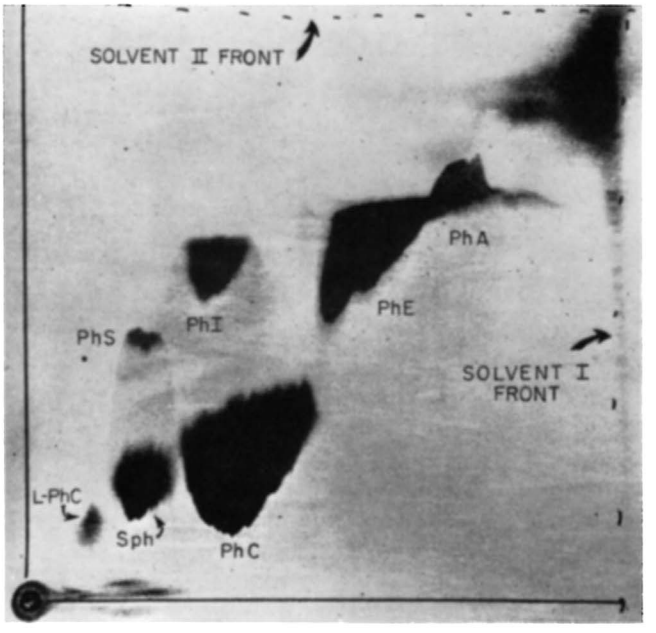

FIG. 1. Two-dimensional TLC chromatogram of mixed phosphatides. A portion (10 μl, 45 μg P) of rat liver phosphatide extract was applied to the origin of a TLC plate. The first dimension was developed for 1 hr with Solvent I, and the second dimension was developed for 1 hr with Solvent II. The phosphatides were detected with iodine vapor. The following abbreviations are used: L-PhC (lysophosphatidyl choline), Sph (sphingomyelin), PhC (phosphatidyl choline), PhS (phosphatidyl serine), PhI (phosphatidyl inositol), PhE (phosphatidyl ethanolamine), and PhA ("phosphatidic acid").

sodium chloride precipitate was removed by filtration. The ethereal acid ferric chloride reagent was prepared by first grinding together a mixture of 10 g $FeCl_3 \cdot 6H_2O$ and 20 ml HCl (37% w/v) with a mortar and pestle and then shaking the resulting solution with 300 ml ether.

2. *Developing solvents for TLC.*

a. *Solvent I.* Chloroform–methanol–7 N ammonium hydroxide were mixed in the ratio of 60:35:5 in a glass-stoppered, graduated cylinder.

b. *Solvent II.* Chloroform–methanol–7 N ammonium hydroxide were mixed in the ratio of 35:60:5.

c. *Solvent III.* Methanol–water–7 N ammonium hydroxide were mixed in the ratio of 6:3:1.

d. *Solvent IV.* Isopropanol–acetic acid–water were mixed in the ratio of 3:1:1.

3. *Other reagents.*

a. *Chloroform.* Reagent grade chloroform was distilled and a volume of absolute ethanol, equivalent to 0.4% of the chloroform volume, was added to retard decomposition.

b. *Methanol.* Redistilled reagent grade methanol was used.

c. *Methanolic HCl* (1.7 N anhydrous).

d. *Ammonium hydroxide* (7 N). Equal volumes of concentrated ammonium hydroxide (28% w/v) and distilled water were mixed.

METHODS

Chromatographic Technique. Glass plates (20 cm x 20 cm and 5 cm x 20 cm) were thoroughly cleaned with a detergent and then with a 5% w/v solution of potassium hydroxide in ethanol. Silica Gel G[1] was applied to the plates according to Stahl (3). Samples were dissolved in an appropriate solvent (chloroform–methanol 2:1) and small volumes (2–50 μl) of solution were applied with a calibrated pipette. The plate was developed for a specific time interval or until the solvent front had moved a predetermined distance from the origin. The plate was then removed from the container and the solvent front marked before air-drying the plate. For two-dimensional TLC, the dry plate was rotated 90° clockwise and placed in another container with a different solvent. The solvent front moved 13 cm in 1 hr with solvents I and II. The lipid spots were located by placing the developed plate face down in a horizontal position in an iodine atmosphere

[1] E. Merck, A. G., Darmstadt, Germany.

TABLE 1. COLOR REACTIONS AND R_f VALUES OF PHOSPHATIDES

Phosphatide	R_f Values*		Color Reactions†							
	Solvent I	Solvent II	I_2	Nin	Mo	Fe	Ag	Bi	Schiff's	EFA
"Phosphatidic acid"	0.73	0.71	+	−	+	+	−	−	+	+
Phosphatidyl serine	0.19	0.44	+	+	+	+	−	−	+	+
Phosphatidyl ethanolamine	0.58	0.61	+	+	+	+	−	−	+	+
Phosphatidyl inositol	0.31	0.58	+	−	+	+	+	−	+	+
Phosphatidyl choline	0.35	0.24	+	−	+	+	−	+	+	+
Sphingomyelin	0.19	0.20	+	−	+	+	−	+	+	−
Lysophosphatidyl choline	0.11	0.13	+	−	+	+	−	+	+	+

* The R_f values were determined from Fig. 1.

† For the abbreviations used for the color reagents see section on Materials.

in an enclosed plastic box. After dark yellow spots on a white background were observed, the plate was removed and the iodine on the plate was then allowed to evaporate and the spots allowed to disappear. The plate then could be sprayed with a variety of different detection reagents. A permanent record of the chromatograms was obtained by photographing them with a Polaroid camera.

Only chromatograms on which the lipid had been revealed with iodine were used for elution of lipid from the spot. After the iodine had evaporated, the spots were scraped off the chromatogram, collected separately on weighing papers, and transferred to centrifuge tubes. Solvent I was added to each tube. The mixture was then agitated, centrifuged, and the supernatant fluid decanted into another tube. The thin-layer residue was extracted two more times with methanol. All supernatants were combined. Portions were taken for phosphorus and esterified fatty acid determinations, for hydrolysis studies, and for rechromatography on TLC.

Preparation of Rat Liver Phosphatide Solution. Phosphatides were extracted from fresh rat liver by the method of Folch *et al.* (16) and finally precipitated with acetone (17).

Hydrolysis of Phosphatides. Portions of each eluted phosphatide were heated in sealed ampules with 1.7 N methanolic HCl (anhydrous) for 4 hr at 100° (18). The hydrolysates were taken to dryness under reduced pressure at 75°. Distilled water was added to each ampule, and a portion of each sample was applied to TLC. Samples of each of the expected characteristic products of hydrolysis were carried through this same procedure to check for possible further degradation.

Chemical Methods. Phosphorus (P) was determined by the method of Bartlett (19) and esterified fatty acids (EFA) by the method of Skidmore and Entenman (20).

RESULTS

The phosphatide mixture prepared from rat liver was dissolved in chloroform, and a portion was applied to a TLC plate and developed with Solvent I in the first dimension and Solvent II in the second. Seven iodine-positive spots were observed between the origin and the solvent fronts. The plate was immediately photographed (Fig. 1). The phosphatides were tentatively identified by observing the color reactions of the spots (Table 1).

In order to identify the seven phosphate-positive spots, it was necessary to obtain a sufficient quantity of each phosphatide to characterize it by hydrolysis. Partial separation of the phosphatides was accomplished with a silicic acid column (17). The seven phosphatides were finally isolated by selecting partially separated column fractions, which contained one or two different phosphatides as major components, and applying large portions to TLC plates. The plates were developed with Solvent I in one dimension. The individual phosphatides were eluted as described in the Methods section, and a portion of each was applied to TLC and developed in one dimension with Solvent I (Fig. 2). It was evident that some of the phosphatides had deteriorated. The P/EFA ratio and the R_f values of the rechromatographed phosphatides are listed in Table 2. Another portion of each isolated phosphatide was hydrolyzed, and a portion of each hydrolyzed sample was

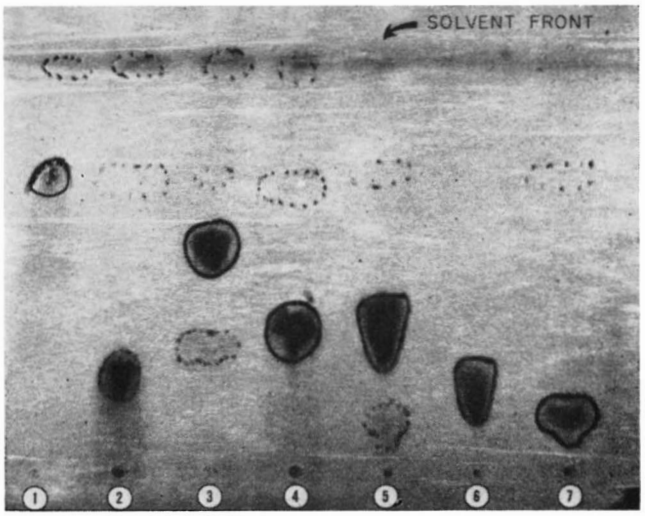

FIG. 2. Rechromatography of phosphatides eluted from a TLC chromatogram. The chromatogram was developed in one dimension with Solvent I for 10 cm. The spots were detected with iodine vapor and circled. The dotted circles indicate weakly stained spots. The iodine was allowed to evaporate and the plate then sprayed for phosphatides with the molybdic acid reagent. The photograph shows the phosphate-positive spots. The phosphatide and the number of micrograms of P applied to the origin were as follows: (1) PhA, 2.6; (2) PhS, 1.4; (3) PhE, 2.7; (4) PhI, 2 3; (5) PhC, 2.5; (6) Sph, 2.1; (7) L-PhC, 2.7.

TABLE 2. R_f VALUES AND PHOSPHORUS/FATTY ACID RATIOS OF PURIFIED PHOSPHATIDES

Phosphatide Eluted	R_f Values with Solvent I*	P/EFA Molar Ratio
"Phosphatidic acid"	0.70	1:1.4
Phosphatidyl serine	0.23	1:1.85
Phosphatidyl ethanolamine	0.52	1:2.1
Phosphatidyl inositol	0.33	1:1.5
Phosphatidyl choline	0.32	1:1.8
Sphingomyelin	0.17	1:0.05
Lysophosphatidyl choline	0.12	1:0.9

* The R_f values were determined from Fig. 2.

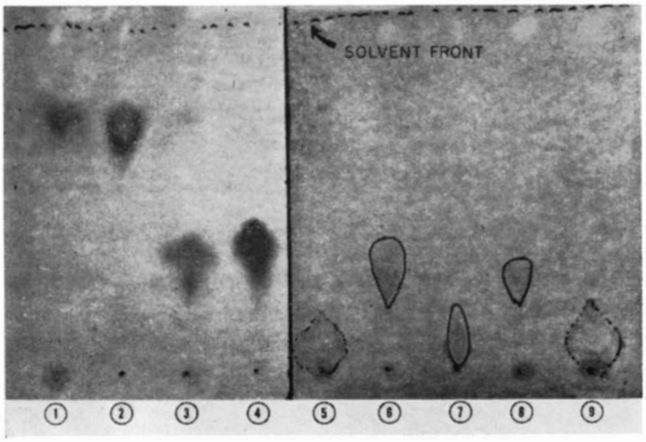

FIG. 3. TLC chromatogram of standards and of hydrolysis products of phosphatides. The chromatogram was developed with Solvent III for 10 cm. The left half of the plate was sprayed with ninhydrin and the right half with Dragendorf reagent. The substances hydrolyzed and the amounts applied to TLC were as follows: (1) PhS, 8 μg P; (2) serine, 50 μg; (3) PhE, 3 μg P; (4) ethanolamine, 50 μg; (5) PhC, 8 μg P; (6) phosphoryl choline, 150 μg; (7) choline, 50 μg; (8) Sph, 3 μg P; (9) L-PhC, 8 μg P.

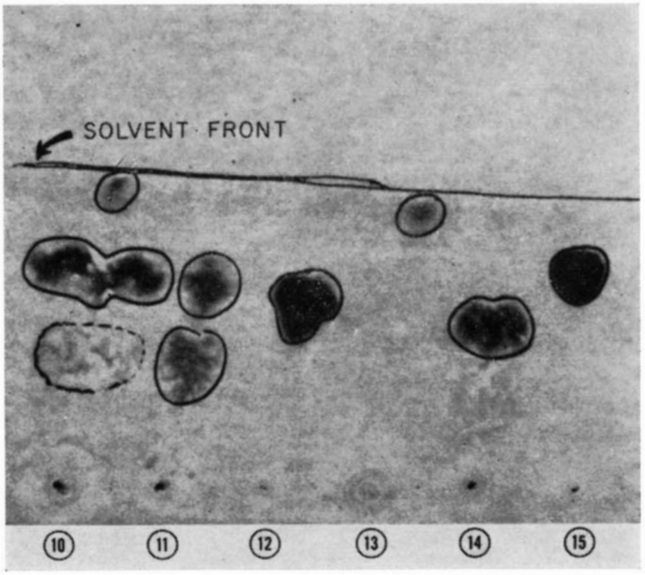

FIG. 4. TLC chromatogram of standards and of hydrolysis products of phosphatides. The chromatogram was developed in one dimension with Solvent IV for 5 cm. The spots were detected with ammoniacal silver nitrate. The substances hydrolyzed and the amounts applied to TLC plate were as follows: (10) PhE, 4 μg P; (11) inositol-1-PO_4, 30 μg; (12) inositol, 20 μg; (13) PhA, 8 μg P; (14) α-glycerolphosphate, 100 μg; (15) glycerol, 50 μg.

chromatographed by TLC (Figs. 3 and 4). PhS was shown to contain serine and no ethanolamine. PhE was shown to contain ethanolamine and no serine. PhC and L-PhC produced choline and no phosphoryl choline. Sph gave phosphoryl choline and no choline. PhI produced inositol and 1-phosphoryl inositol. PhA was not definitely characterized. R_f values and color reactions of the hydrolysis products of phosphatides and standards are presented in Table 3.

Preliminary studies of the recovery of P indicated that essentially all of the P that moved in the two-dimensional TLC chromatogram could be recovered from the phosphatide spots. About 25% of the P applied to the plates, however, could not be recovered from the Silica Gel G by this method of elution. Apparently there is a certain amount of P-containing material that does not move with either Solvent I or Solvent II and remains at the origin. It is understandable, then, that this same material resists elution with a similar eluting solvent.

Since breakdown of phosphatides occurs on storage, especially after silicic acid chromatography, information on TLC behavior of the breakdown products, including the lysophosphatides, is of interest. The movement of lysophosphatide spots has been observed on two-dimensional TLC chromatograms. Lysophosphatidyl ethanolamine moves with the PhI spot on TLC and has been noted to be present as a component on old extracts of PhE by using the ninhydrin spray. Lysophosphatidyl serine moves closely behind PhS and appears as a spot between PhS and the origin. The eluted phosphatides were apparently unstable when stored in the eluting solvent at 4°. Traces of iodine-positive substances were observed on TLC with R_f values similar to the corresponding lysocompounds and free fatty acids (Fig. 2).

TABLE 3. COLOR REACTIONS AND R_f VALUES OF STANDARDS AND OF HYDROLYSIS PRODUCTS OF PHOSPHATIDES USING THIN-LAYER CHROMATOGRAPHY

No.*	Substance	R_f Values of Characteristic Products		Color Reaction†		
		Solvent III	Solvent IV	Nin	Bi	Ag
1	Phosphatidyl serine	0.74		+	−	
2	Serine	0.72		+	−	
3	Phosphatidyl ethanolamine	0.34		+	−	
4	Ethanolamine	0.36		+	−	
5	Phosphatidyl choline	0.08		−	+	
6	Phosphoryl choline	0.30		−	+	
7	Choline	0.09		−	+	
8	Sphingomyelin	0.27		−	+	
9	Lysophosphatidyl choline	0.08		−	+	
10	Phosphatidyl inositol		0.40, 0.64, and 0.95			+
11	Inositol-1-PO_4		0.40, 0.64			+
12	Inositol		0.64			+
13	"Phosphatidic acid"		0.95			+
14	α-Glycerol-PO_4		0.55			+
15	Glycerol		0.76			+

* The numbers are given to identify the substance applied to the plates for chromatography as shown in Figures 3 and 4.

† See section on Materials for abbreviations used for color reagents.

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