A METHOD FOR THE UNIDIMENSIONAL SEPARATION OF PHOSPHOLIPIDS BY THIN-LAYER CHROMATOGRAPHY*

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INTRODUCTION

A number of methods for thin-layer chromatography of phospholipids have been reviewed by MANGOLD[1]. SKIDMORE AND ENTENMAN[2] have employed a two-dimensional thin-layer system which separates seven phospholipids from a rat liver extract. The present communication describes a single solvent system which separates the radioactive phospholipids from pancreas slices into seven discrete spots. The phospholipids may be distinguished from their lyso derivatives by this technique. The lipids are detected by autoradiography and the radioactive spots can be quantitatively cut out from the thin-layer plate.

MATERIALS AND METHODS

Preparation of chromatographic plates

Silica gel G (Merck, Germany) was used directly from the bottle. It was applied to glass plates as a slurry (1/2 w/v), using a modified version of STAHL's[3] original applicator purchased from Research Specialties Co. The thin-layer plates were kept in an oven at a 110° and removed shortly before use.

Developing solvent

A phenol–water solution was prepared by dissolving 5 lbs. of phenol (reagent grade) in 520 ml of distilled water. This solution can be stored at room temperature for several months. The developing solvent was prepared just prior to chromatography by mixing 1.0 ml of concentrated ammonium hydroxide with 99 ml of the phenol stock solution.

Treatment of the chromatographic plates

The plates were developed at room temperature. After approximately 4 h, when the solvent had ascended 12 to 15 cm, the plates were removed and partially dried in an oven at 110°. When it was desired to determine radioactivity in the separated

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phospholipids the plates were sprayed with a water dispersion of polyvinylpropionate (Neatan®, purchased from Brinkmann Instrument Inc.). The plates were then returned to the oven and completely dried. The plates which were used to isolate the radioactive material for alkaline hydrolysis were not sprayed with Neatan®.

Detection of the phospholipids

The reagents and techniques for detecting amino groups, choline, phosphorus, aldehyde, and unsaturation which were used in this study have been described by MANGOLD[1] and SKIDMORE AND ENTENMAN[2]. Most of these reagents can be used after the application of the plastic coating with little loss in sensitivity. The radioactive phospholipids were detected by autoradiography and coincided with the chemically stained regions. The chromatographic plate was placed in a specially constructed holder made by gluing together two 1/8 × 10 × 12 in. sheets of pressed wood, the

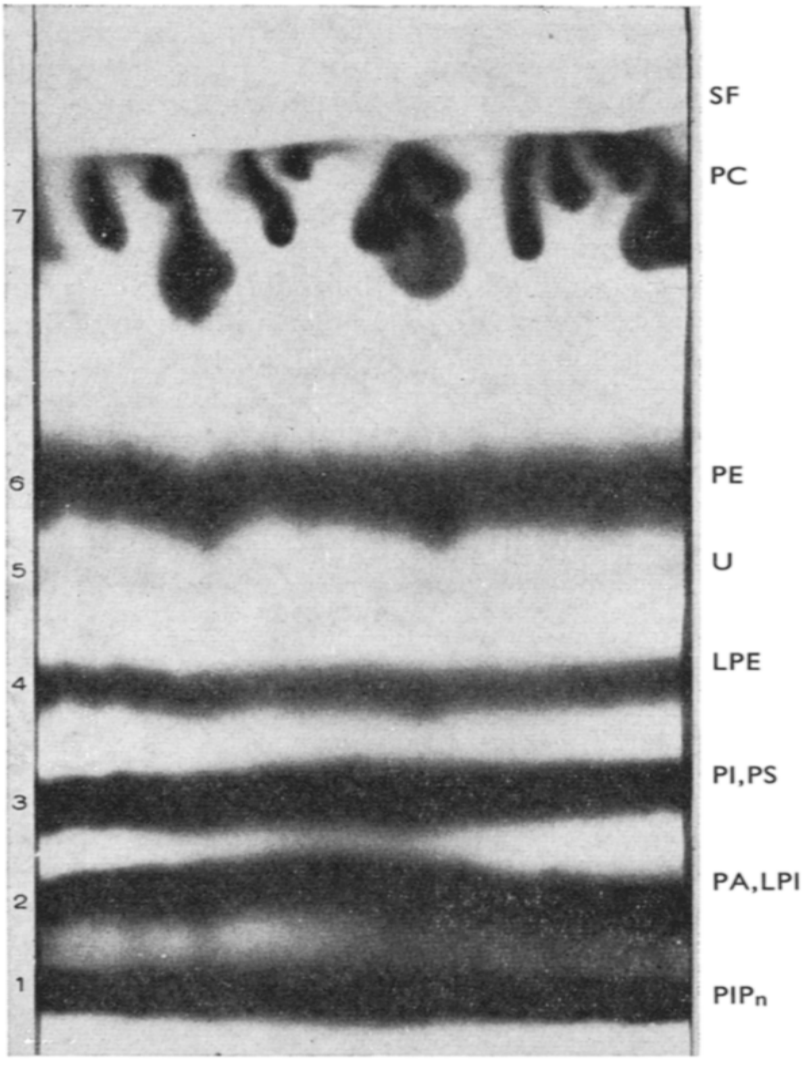

Fig. 1. An autoradiograph showing the separation of the phospholipids of pigeon pancreas tissue. The following abbreviations were used: SF (solvent front); PC (phosphatidyl choline); PE (phosphatidyl ethanolamine); PS (phosphatidyl serine); U (unknown); LPE (lysophosphatidyl ethanolamine); PI (phosphatidyl inositol); PA (phosphatidic acid); LPI (lysophosphatidyl inositol); PIP_n (phosphatidyl inositol polyphosphate).

upper sheet having an 8 × 8 in. square removed to accommodate the plate. An 8 × 10 in. Kodak No-Screen X-ray film was secured over the chromatographic plate by means of spring clamps and the holders were stored in a lightproof cabinet until the film was sufficiently exposed.

Quantitative determination

The radioactive phospholipids spots, detected by autoradiography, were marked with pencil and circled with a razor blade. After moistening with water, the plastic impregnated silica gel patches were lifted off the plate with a spatula and transferred to planchets for counting.

RESULTS AND DISCUSSION

Identification of separated phospholipids

Phospholipids, labelled with radioactive orthophosphate from pigeon pancreas slices[4], were applied to several thin-layer plates. The lipids separated into seven distinct bands (Fig. 1). Several thin-layer chromatograms were sprayed with various chemical reagents, as described in MATERIALS AND METHODS, to aid in the identification of these lipids. The separated phospholipids were further characterized by identifying their radioactive alkaline hydrolysis products (Table I). This was done by scraping the radioactive areas off the plates with a spatula and subjecting the phospholipids adsorbed to the silica gel to mild alkaline hydrolysis by the method of DAWSON[7] and identifying the hydrolysis products by their mobility in two chromatographic systems[7]. The R_F values of the separated phospholipids were also compared to the R_F values of standard phospholipids (Table I). The alkaline hydrolytic products of band 1 (Fig. 1) were not identified. Polyphosphoinositides are labelled with ^{32}P in brain[8] and red blood cells[9]. Chromatography of lipid extracts from these tissues showed that the

TABLE I

IDENTIFICATION OF THE INDIVIDUAL PHOSPHOLIPIDS

Standard compounds were prepared by chromatographing ^{32}P-labelled phospholipids from pancreas slices[4] according to the method of MARINETTI[5] and eluting the separated phospholipids from the paper with 3:1 chloroform–methanol. Lysophosphatidyl inositol was isolated as previously described[6], and sphingomyelin was obtained from the Nutritional Biochemical Corp. The following abbreviations were used: α-GP (α-glycerophosphate); GPI (glycerophosphorylinositol); IMP (inositol monophosphate); GPE (glycerylphosphorylethanolamine); GPC (glycerylphosphorylcholine).

Band No. (Fig. 1)	*R_F value*	*Ninhydrin*	*Dragendorf*	*Fuchsin-sulfurous acid*	*Principal radioactive alkaline hydrolysis products*	*Compound**
1	0.02	—	—	—	unknown	PIP_n**
2	0.12	—	—	—	α-GP, GPI, IMP	PA** and LPI**
3	0.28	—	—	—	GPI and IMP	PI** and PS**
4	0.44	+	—	—	GPE	LPE**
5	0.56	—	—	—	unknown	unknown
6	0.64	+	—	+	GPE	PE**
–	0.74	—	+	—	—	Sphingomyelin**
7	0.92	—	+	+	GPC	PC**

* For abbreviations see the legend to Fig. 1.
** Standard compounds had the same R_F value.

polyphosphoinositides did not move very far from the baseline. Polyphosphoinositides have not, however, been definitely identified in pancreas.

An unknown lipid was detected (Fig. 1, band 5). This lipid may be a diacylglycerophosphatide since it is attacked by bee venom, presumably to yield the lyso derivative (Fig. 2). On alkaline hydrolysis it gave α-glycerophosphate and an unidentified product. There was no positive reaction with the various stains used.

Fuchsin aldehyde tests were positive in bands 6 and 7 (Table I), indicating the presence of ethanolamine and choline plasmalogens.

This chromatographic system also separates the phospholipids from their lyso derivatives (Fig. 2).

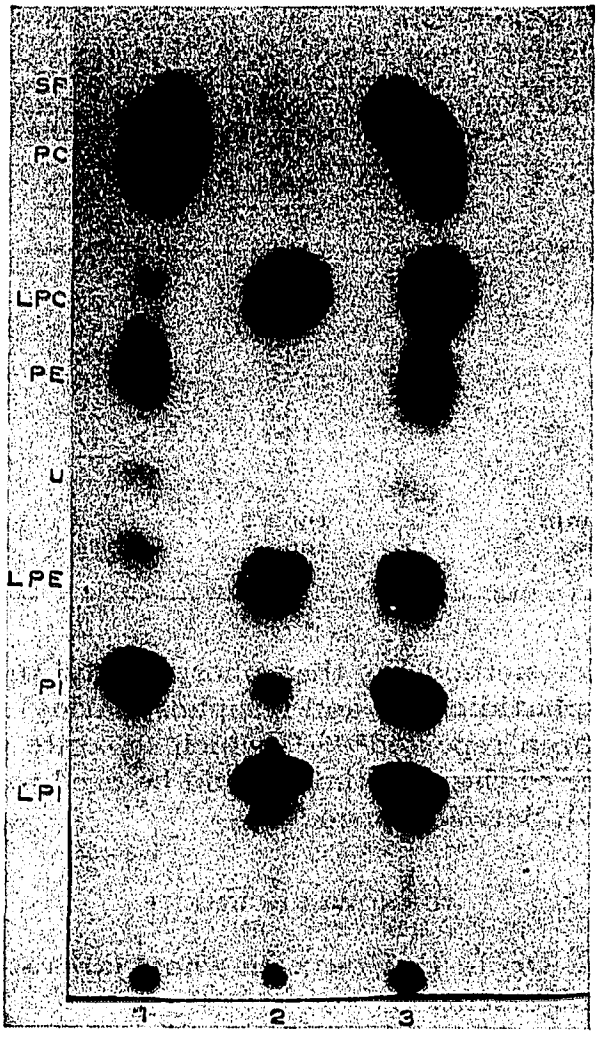

Fig. 2. An autoradiograph showing the separation of the phospholipids and the lysophospholipids. Aliquots of ^{32}P-labelled pancreas phospholipids were incubated with 0.1% bee venom (purchased from Sigma Chemical Company) in an ethereal system similar to that described by HANAHAN[10], except that several mg of alumina were added to promote hydrolysis[11]. The reaction mixture was dried under nitrogen and the lipids were dissolved in ethanol–chloroform–water (5:2:2). Aliquots of this solution were spotted. Phosphatidic acid, polyphosphoinositide and lyso derivatives of these compounds were strongly bound by the alumina and were not present in the lipid extract. Numbers 1 and 2 are duplicate samples of pancreas phospholipids incubated in the absence and presence of bee venom, and 3 is a mixture of the two samples. See Fig. 1 for abbreviations used.

Quantitative estimation of phospholipid radioactivity

The capacity of the plate was determined by spotting varying amounts of pancreas lipid extracts. A range of 1.8 to 18 μg of phospholipid phosphorus were plated and no change in the R_F values of the separated phospholipids was noticed.

Since the spots are relatively small, the autoradiographic method is extremely sensitive, and as little as 50 disintegrations/min of ^{32}P are sufficient to produce a discrete spot in one day. The absence of radioactive streaking allows the separated phospholipids to be counted accurately. The data in Table II show that very precise results can be obtained.

TABLE II

THE QUANTITATIVE ESTIMATION OF RADIOACTIVITY INCORPORATED INTO THE INDIVIDUAL PHOSPHOLIPIDS

Duplicate aliquots of a total lipid fraction (sample 1) and a partially purified phosphatidyl inositol fraction (sample 2) from pigeon pancreas stimulated with carbamylcholine[4] were chromatographed and the radioactivities of the individual phospholipids were determined as described under MATERIALS AND METHODS. Abbreviations are given in the legend to Fig. 1.

Compound	*Sample 1 (counts/min)*		*Sample 2 (counts/min)*	
	A	*B*	*A*	*B*
Origin	65	77	—	—
PIP_n	69	51	31	22
LPI + PA	543	519	193	203
PI	1128	1116	1672	1642
LPE	35	35	109	124
PE	130	127	200	213
PC	31	37	—	—
Total recovered counts/min	1992	1960	2205	2204
Total spotted counts/min	2065	2065	2310	2310

Disadvantages of this method are that phosphatidyl inositol and phosphatidyl serine (Band 3) and phosphatidic acid and lysophosphatidyl inositol (Band 2) do not separate. Methods have been reported[2,12,13] which separate phosphatidyl serine and phosphatidyl inositol. One of these methods could be used together with the phenol–NH_3 system to distinguish between them.

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SUMMARY

A phenol–water–ammonia solvent system has been utilized to separate most of the known phospholipids and lysophospholipids on thin-layer plates of silica gel G. These compounds were identified by chromatography of their mild alkaline hydrolysis products, various staining reactions, and comparison with known compounds. An unknown radioactive phospholipid has been detected in pigeon pancreas lipids by

means of this chromatographic method. A procedure is described which makes it possible to determine the radioactivity incorporated into the individual phospholipids with a high degree of precision.

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