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SOME CONTRIBUTIONS TO THE THIN-LAYER CHROMATOGRAPHIC ANALYSIS OF COMPLEX NATURAL PHOSPHOLIPID AND NEUTRAL LIPID MIXTURES

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SUMMARY

We have isolated a minor phosphatidyl ethanolamine component from pancreas and a minor phosphatidyl choline component from retina, which were revealed by their separate thin-layer chromatographic properties on silica gel and aluminum oxide sheets, respectively. We have described in some detail a number of modifications in thin-layer chromatography methodology which enhances the opportunity for assessing the glycerophospholipid and neutral lipid composition of tissues, as attested by a diverse set of examples, and have pointed out some of the associated technical problems.

INTRODUCTION

Our first contribution to chromatography of lipids [1] proposed that the chromatographic conditions be empirically modified, as required, to effect optimal separation for the purposes at hand. We have adhered to this precept in subsequent studies and continue to find manipulations of this kind to be essential to the adequate resolution of the lipid mixtures from diverse natural sources.

Reduction of the thin-layer chromatography (TLC) plate size from 20 to 10 cm produces chromatograms with smaller and more uniform spots (i.e.,

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markedly less ascending spot-size spread) and a concomitant decreased run time and reduced temperature dependence. The exploitation of these advantages through (a) changes in the solvent system(s) components and/or their ratios, (b) two-dimensional application, (c) examination of the usefulness of aluminum oxide TLC media, (d) optimizing on the principles of the extraction procedures of Folch et al. [2] and Bligh and Dyer [3] and (e) selected sequential spot-testing has resulted in a number of observations to be briefly documented here. The 20-cm runs, however, continue to have important uses.

EXPERIMENTAL

Thin-layer chromatography media

The following commercially prepared TLC precoated plastic sheets were used: Macherey-Nagel Polygram Sil G (MN-Sil G) (Düren, F.R.G.) Alox N, Baker-flex silica gel IB2 (J.T. Baker, Deventer, The Netherlands) and aluminum oxide IB. All of the above should be successively washed prior to use with chloroform–ethanol (2:1) and acetone. Both silica gel preparations were operationally equivalent, as were both aluminum oxide preparations.

Tissue extraction

Various animal tissues were quick-frozen, lyophilized, and lipid extracts prepared for TLC examination with chloroform–methanol (2:1), 1–5 ml per 100 mg freeze-dried tissue. If the non-lipid extractives (e.g. peptides, etc.) are bothersome to the analyses, they can be removed either by drying 1 ml of the chloroform–methanol extract under nitrogen at 40°C and redissolving the lipid in 0.2 ml methanol and 1 ml *n*-butanol–benzene (1:1) or by extracting the freeze-dried tissue directly with the methanol–butanol–benzene mixture. The extractives are then separated from the lipid (in both cases) by adding 8 ml of butanol-saturated 0.85% sodium chloride; the total mixture is then transferred to a 10-ml glass-stoppered cylinder or volumetric flask. A brief period of vigorous mixing is sufficient to transfer the extractives to the lower, aqueous phase with the lipid quantitatively occupying the upper phase, where it can be conveniently sampled. A tranfer to PTFE-lined, screw-capped tubes and centrifugation may be required to speed up the separation process. A 10–20 μl aliquot of extract is usually adequate for TLC analysis; 500–1000 μl can be required for the preparative TLC. For some applications butanol alone, for the upper phase, was found to be more quantitative.

Silica gel chromatography

We had earlier [4] employed a developing system (A) for the silica gel sheets consisting of ethyl acetate–isopropanol–chloroform–methanol–water (50:50:50:20:18), increasing the methanol if the mixture was cloudy. We have since observed that by reducing the proportion of chloroform to 30, and substituting the 1-propanol by 2-propanol (because of the boiling point difference), we produced a more stable and less temperature-dependent mixture, referred to here as A-2-30. Addition of 0.2 ml of ammonium hydroxide to this mixture permitted a more clear-cut resolution of certain phospholipids, particularly with a 30-min overrun (which is still substantially

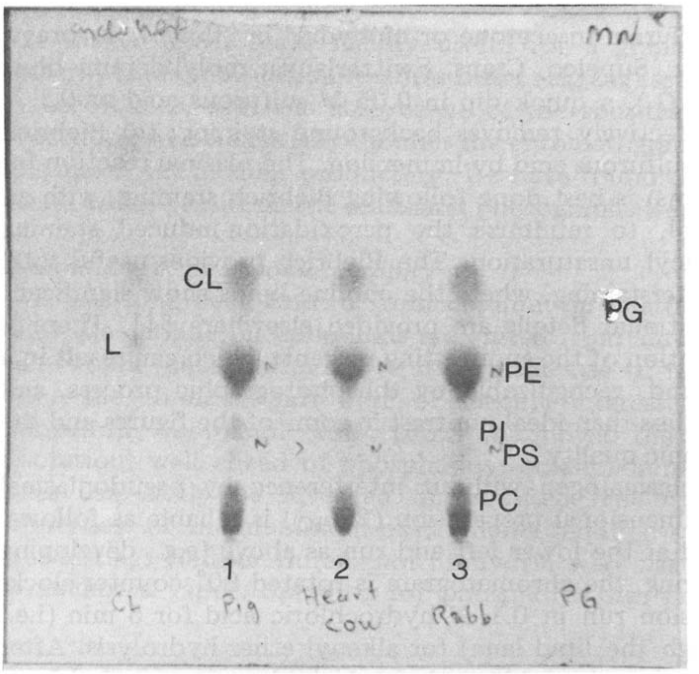


Fig. 1. Cyclohexane—isopropanol—water (30:40:6) chromatogram using an MN-Sil G 10 × 10 cm sheet, spot-tested for phosphorus. The numbers 1, 2 and 3 represent cardiac muscle extracts of pig, cow and rabbit, respectively. The CL standard (left lane) shows a lyso (L) contaminant; the right lane is a PG standard. Key: CL = diphosphatidyl glycerol; PE = phosphatidyl ethanolamine; PI = phosphatidyl inositol; PS = phosphatidyl serine; PC = phosphatidyl choline; PG = phosphatidyl glycerol.

shorter than the time required for 20-cm sheets). Addition of ammonia, for example, transposes the R_F relationships of cardiolipin (diphosphatidyl glycerol, CL) and N-acylphosphatidyl ethanolamine (NAPE), and generally retards the rate of migration of all the phospholipids. We have recently adapted a phospholipid high-performance liquid chromatographic (HPLC) elution system [5] to TLC application, using hexane—isopropanol—water (6:8:1.3). Substituting cyclohexane for hexane, with its higher boiling point, appeared to be more effective and addition of ammonium hydroxide behaved as described above (see Fig. 1). Sheets of 10 × 10 cm could comfortably accommodate six sample lanes. Rectangular developing chambers, appropriate for this size, were obtained from Alltech Europe (Eke, Belgium) and Applied Sciences Labs. (State college, PA, U.S.A.). They were used unlined and required 100 ml of developing solvent. Only experience determines whether an equilibration period is desirable for any given set of environmental conditions. Temperatures above 24°C can be contraindicated and the chambers should only be used in a draft-free location. The origin is 1 cm from the bottom edge and the solvent front 0.5 cm from the top edge, e.g., a run-span of 8.5 cm.

Detection

The spot-testing can be done separately or in sequence according to the information required. When more than one spot-test is done, the following

sequences are useful: (a) fluram in acetone or ninhydrin in ethanol, as sprays; (b) phosphorus (Phospray, Supelco, Crans, Switzerland); molybdenum blue, (Sigma, Taufkirchen, F.R.G.), a quick dip in 0.05 *M* sulfurous acid or 0.1 *M* ammonium hydroxide effectively removes background staining; (c) Biebrich Scarlet, 0.2%, in 0.05 *M* sulfurous acid by immersion. The plasmal reaction for alkenyl lipids (plasmalogens) is best done following Biebrich staining, with an mercuric chloride control, to minimize the peroxidation-induced staining (pseudoplasmal) due to acyl unsaturation. The Biebrich provides useful total lipid orientation, as counterstaining, where the choline lipids show significant preferential staining; additional details are provided elsewhere [4]. There is a varying amount of retention of the spot-testing reagents which can result in a less than white background, recognizable by the photographic process, and providing an unavoidable less-than-ideal contrast in some of the figures and the illusion of poor photographic quality.

To better assess the plasmalogen without interference by pseudoplasmal events, a one-lane, two-dimensional preparation ($2D_{HCl}$) is reliable as follows. The lipid sample is placed at the lower left and run as above (e.g., developing system A-2-30); after drying, the chromatogram is rotated 90° counter-clockwise for a second dimension run in 0.1 *M* hydrochloric acid for 5 min (i.e., until it has passed through the lipid lane) for alkenyl ether hydrolysis. After thorough air-drying (1 h), the released long-chain aldehydes are separated from the phospholipids by a run, in the same second dimension, in isooctane—isopropyl acetate (100:7) and subsequently spot-tested by the Schiff aldehyde reagent (i.e., the leucofuscin of the plasmal spot-test). Other spot-tests can be done prior to the Schiff as feasible, e.g. phosphorus. For the above purposes, half-saturated aqueous mercuric chloride, as an aldehyde releasing agent, was generally not a suitable substitute for hydrochloric acid because of the mercury-adduct formation often resulting in multiple aldehyde spots. A control sample can be safely placed in the lower right lane prior to the first-dimension run and also subjected to the hydrochloric acid, isooctane—isopropyl acetate (100:7) sequence (in the opposite direction) if desired.

Neutral lipids (NL) can be chromatographed in isooctane—ethyl acetate–isopropanol (100:20:2) and visualized by osmium tetraoxide vapor-phase staining; addition of ammonium hydroxide to this system leaves the fatty acids at the origin, enabling the cholesterol/diglyceride group to be more easily recognized. The NL plasmalogens (see ref. 6) can be effectively examined by the $2D_{HCL}$, modified to accommodate NL properties.

Assessment of the alkyl and alkenyl glyceryl ether phospholipids (and neutral lipid analogues) was accomplished by Vitride reduction [7] and the resulting 1-glyceryl ethers fractionated by a TLC run in chloroform–acetone (4:1) or hexane–isopropanol (10:3). Vitride is sodium bis(2-methoxyethoxy)-aluminum hydride and is a product of Eastman-Kodak obtained from Fisher Scientific (Springfield, NJ, U.S.A.), it is commercially available as 70% in toluene or benzene; for use it was diluted to 5% with benzene.

Densitometry

The compactness of the spots in these 10-cm chromatograms provided a more quantitative condition for densitometric scanning. The variation in spot

size makes linear scans mainly useful for a graphic demonstration of lipid pattern; true quantitation requires raster (zig zag) scan and a wavelength choice in accordance with the absorbance of the spot-test product. As a recording procedure, we sequentially number the chromatograms with a rubber ink stamp at some point in their production. We have found this system to be especially useful when densitometric scans and photographs were made.

Aluminum oxide chromatography

It has long been known from column chromatography that, unlike silicic acid, the choline phospholipids are eluted from aluminum oxide ahead of the non-choline phospholipids. We have made use of this characteristic for TLC when the choline lipids were of specific interest. Chromatographic runs in chloroform—methanol—water (65:30:4) moved the choline lipids, with good resolution, well ahead of phosphatidyl ethanolamine (PE); other solvent mixtures can easily be invented or modified. The hydrophobicity, and other properties, of the aluminum oxide media required separate considerations for spot-testing. Neither fluram nor ninhydrin were usable without first exposing to ammonia vapor. Spraying for phosphorus was precise and uncomplicated.

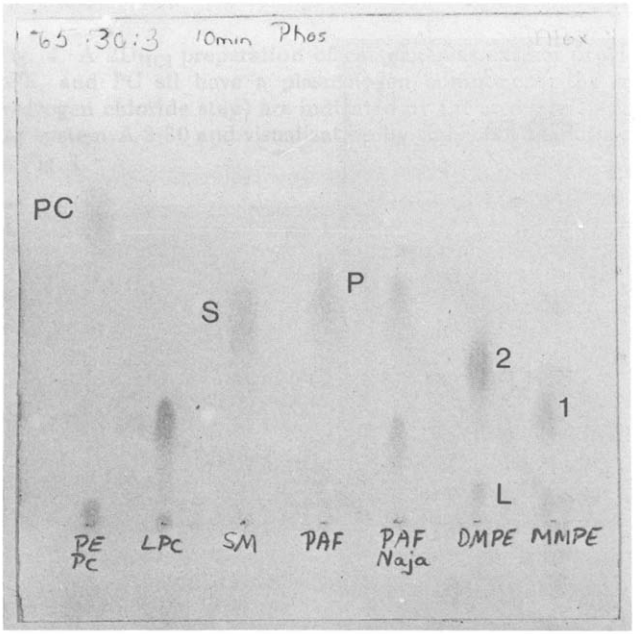


Fig. 2. Alox preparation, chloroform—methanol—water (65:30:3), of various phospholipid standards. The platelet activating factor (PAF)-Naja lane reflects the hydrolysis of PAF through incubation with Naja naja venom phospholipase A_2 (PLA_2). On the presumption that the PLA_2 hydrolysis was complete, this sample was nearly 50% sphingomyelin (SM). Visualization was by the phosphorus spot-test. The range of solvent usefulness ranges from proportions of 17 to 30 for the methanol and 2 to 5 for the water, with varying R_F and degree of resolution. Substitution of 4 *M* ammonium hydroxide for water can be useful. Relevant letters and numbers are placed next to some of the spots; the identity of each lane is clearly marked at the origin. Abbreviations as given in Fig. 1; DMPE = dimethyl phosphatidyl ethanolamine and MMPE = monomethyl phosphatidyl ethanolamine.

The phosphorus spray is transient. We, therefore, mark each phosphorus spot with a "P" prior to Biebrich staining. The latter is permanent and a useful choline differentiator. The Biebrich Scarlet (5 mg per 100 ml) required 0.5 *M* ammonium hydroxide as a staining vehicle and post-staining (differentiating) wash. The plasmalogen reaction required lengthy (overnight) staining and wash and was probably no longer sufficiently selective to justify (see Fig. 2).

RESULTS

A previously undetected plasmal positive pancreas PE (PE-X), of greater R_F, was easily resolvable from the main PE component(s) and was readily isolated under preparative run conditions using the same solvent system (Fig. 3). In the cat, its fatty acid composition was 40% 16:0 and 60% 18:1. The $2D_{HCl}$ (Fig. 4) confirmed its plasmalogen component and Vitride analysis established an alkyl/alkenyl ratio of 1:1; a diacyl contribution was not determined nor has an in vitro, artifactual origin been established. Similar PE-Xs were observed in chloroform—methanol extracts of freeze-dried pancreas from a number of other vertebrates and were hydrolyzable, in vitro, by their own pancreas phospholipase A_2. The details will be reported elsewhere.

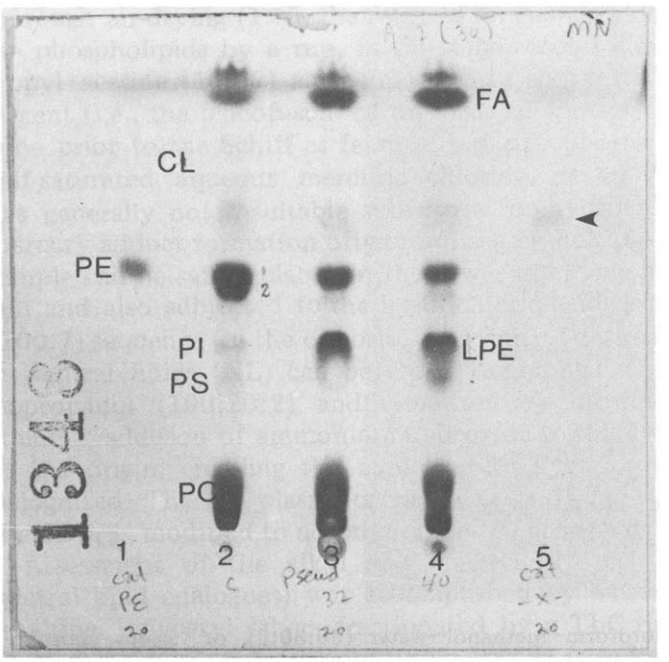


Fig. 3. A 10 × 10 cm MN-Sil G sheet run in developing system A-2-30 and stained with the Schiff mercuric chloride reagent and Biebrich Scarlet (some pseudoplasmal staining has occurred, obscured by the photography). Numbers 1 and 5 represent preparative TLC isolates of cat pancreas PE and PE-X, respectively; numbers 2, 3 and 4 represent pancreas of the turtle (*Pseudemys elegans*) control, 32 and 40°C incubations, respectively. The incubated samples show the LPE and LPC derived from PE and PC, respectively, by the endogenous PLA_2 of the samples. In addition, there are reductions in CL, PE-X, PE, and PC due to the same enzyme. Abbreviations as given in Fig. 1.

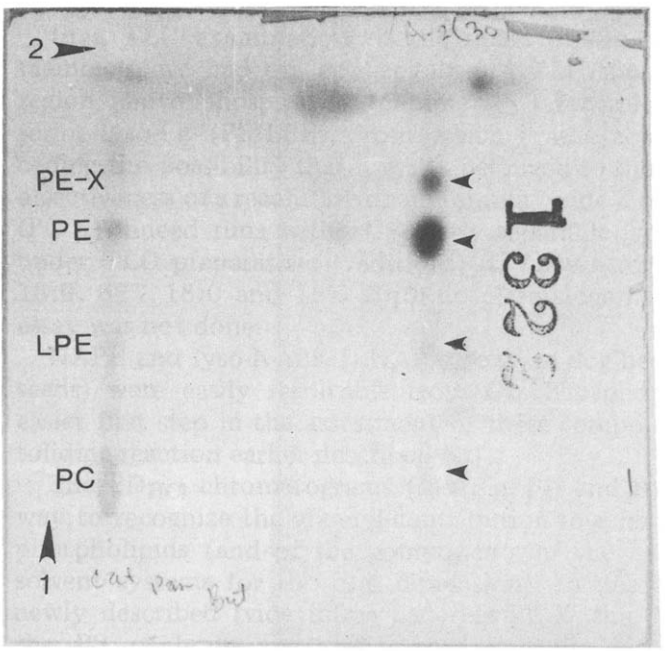


Fig. 4. A $2D_{HCl}$ preparation of cat pancreas extract providing the evidence that PE-X, PE, LPE, and PC all have a plasmalogen component; the released free aldehydes (from the hydrogen chloride step) are indicated by the arrows. The first-dimension run was in developing system A-2-30 and visualization by the Schiff leucofuscin reagent. Abbreviations as given in Fig. 1.

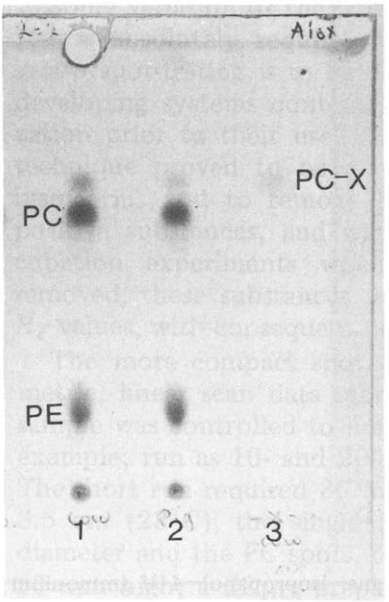


Fig. 5. Phosphorus-stained Alox preparation run in chloroform—methanol—isopropanol—water (100:25:2:2) showing the resolution of PC-X in cow (1) and pig (2) retina; 3 represents a preparative TLC isolate from cow retina. Abbreviations as given in Fig. 1.

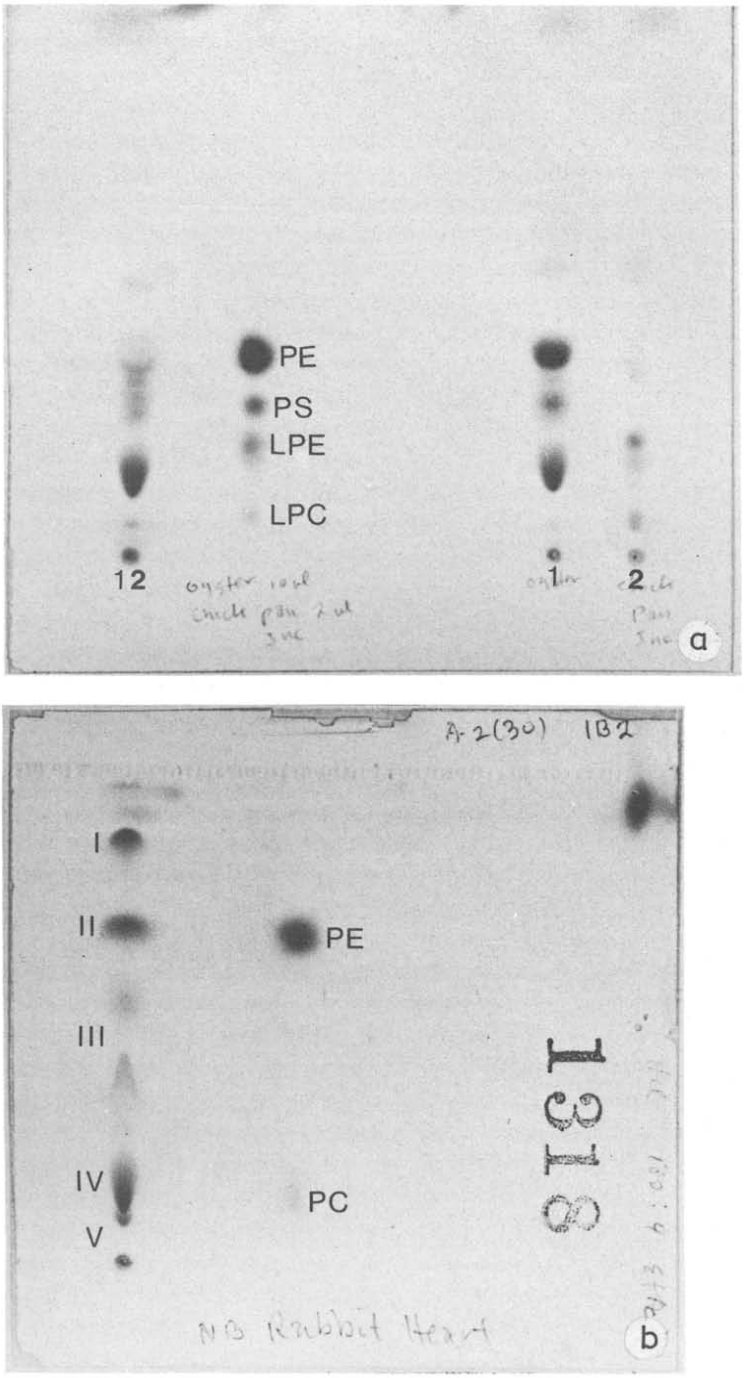


Fig. 6. (A) $2D_{HCl}$ preparation, first dimension cyclohexane—isopropanol—4 *M* ammonium hydroxide (30:40:6) with Schiff plasmal staining showing the resolution of PS from LPE (as plasmalogens), from a mixture of extracts from oyster (1) and incubated chicken pancreas (2) (left) and their respective unidimensional patterns (right). (B) $2D_{HCl}$ of heart of new born rabbit with free aldehydes from PE and PC. The left lane of this chromatogram illustrates well the zone conception discussed in the text where CL is in zone 1, etc. Abbreviations as given in Fig. 1.

In a TLC examination of the lipids of the tissues of the eye of various mammals, we had earlier recognized a TLC spot, solely in the retina, in the region above phosphatidyl choline (PC) generally occupied by phosphatidyl serine/lyso-PE (PS/LPE), from which it was not clearly differentiated. Conceding the possibility that it really belonged to the PC region, we explored the effectiveness of a resolution on aluminum oxide TLC sheets. Fig. 5 shows that it (PC-X) indeed runs with PC, clearly separable from PS and PE and isolatable under TLC preparative conditions. Its fatty acid composition (cow) was 21% 16:0, 62% 18:0 and 15% 20:0; no plasmalogen was detected and the Vitride assay was not done.

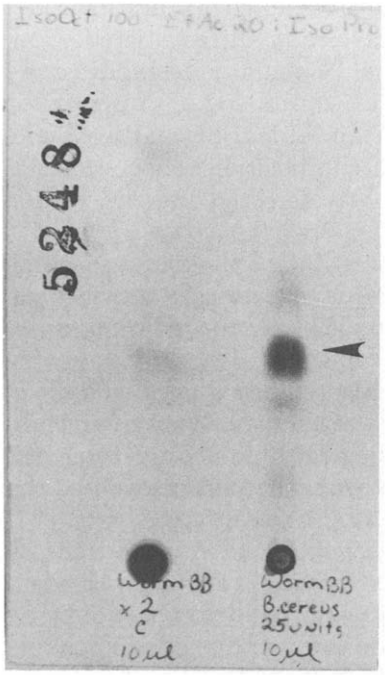
NAPE and lyso-NAPE (LNAPE) (e.g., in dog heart infact and various legume seeds) were easily resolvable from CL. Phosphorus spot-testing provided an easier first step in the assessment of these compounds than the chlorination/*o*-tolidine reaction earlier described [8].

The $2D_{HCl}$ chromatograms (see Fig. 6A and B) provided a rapid and clean way to recognize the alkenyl contribution to each of the base types of glycerophospholipids (and of the components of the neutral lipid group, using NL solvent systems for the first dimension). In this way, the plasmalogen of the newly described (vide infra) pancreas PE-X, the dog heart infarct NAPE [9], the PS of brain and spinal cord and the lysocomponents, produced by pancreatic [4] and snake venom phospholipase A_2, were all demonstrated.

The NL chromatography provided an ancillary way to detect phospholipase A_1 and A_2 hydrolysis via fatty acid release of lipase through loss of triglyceride, of phospholipase C through diglyceride formation (see Fig. 7), of sphingomyelinase and of ceramidase.

Some variation of the chloroform–methanol wash procedure of Folch et al. [2] is absolutely required when unambiguous ninhydrin (or fluram) amino group spot-testing is to be done. Neither works under acid conditions so that developing systems containing acetic acid require post-development neutralization prior to their use. The butanol–benzene extraction (or re-extraction) technique proved to be quantitative, for both the phospholipids and their lyso forms, and to remove completely the bothersome non-lipid ninhydrin-positive substances, and was therefore especially useful in phospholipase incubation experiments when trypsin was involved as an activator. If not removed, these substances often interfered with the establishment of reliable R_F values, with consequent possibility of identity misinterpretation.

The more compact spots of the 10-cm chromatograms made the densitometric, linear scan data more quantitative, particularly when the amount of sample was controlled to limit the spot size (see Fig. 8). Pig heart extracts, for example, run as 10- and 20-cm sheets in A-2-30, produced the following data. The short run required 30 min to reach the origin to solvent front distance of 8.5 cm (23°C); the single PE spot was contained within a circle 8 mm in diameter and the PC spots, together, contained within an oval 4 mm wide and 10 mm high; a $2D_{HCl}$ preparation showed the PE to consist of two plasmal spots. The 20-cm run required 2.5 h to attain an origin to solvent front distance of 17 cm with two PE spots in an area 8 × 12 mm and an oval PC 4 × 16 mm. The 20-cm chromatograms showed a generally sharper resolution of sphingomyelin (SPM) and lyso-PC (LPC); further manipulation of the developing



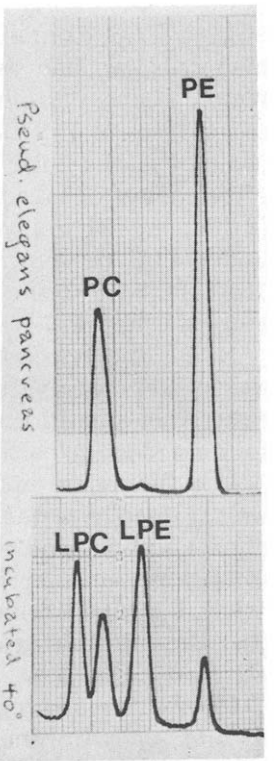


Fig. 7. The conversion of PE plasmalogen (origin, left lane) to the corresponding 1-alkenyl-2-acyl diglyceride (arrow) by incubation, in vitro, with *B. cereus* phospholipase C. The developing solvent was isooctane—ethyl acetate—isopropanol (100:20:2) and the Schiff mercuric chloride reagent provided the plasmalogen spot-test. The alkenyl nature of the DG zone lipids was more clearly established by $2D_{HCl}$ with the same first-dimension solvent system.

Fig. 8. Linear densitometric scan of control (upper) and 40°C incubated (lower) pancreas of turtle showing the evidence of phosphclipase A_1 hydrolysis in the reduction of PE and PC and appearance of LPE and LPC. From a plasmal stained A-2-30 (ammonium hydroxide) chromatogram. Abbreviations as given in Fig. 1.

solvent ratios can achieve this latter result with the 10-cm chromatograms when required. The long runs unmistakably remain thoroughly useful with their additional maneuvering space and increased opportunity for a favorable gradient to be established, suitable for resolving some of the major PE and PC molecular species [10, 11] with greater clarity.

The phospholipids can independently take three different shapes as they spot-out on the chromatogram. Some, characteristically, change shape when chromatographed in ammonia systems and, thus, provide a useful recognition test. In general, the acidic phospholipids [CL, phosphatidylglycerol (PG), bisphosphatidic acid (Bis-PA), phosphatidyl inositol (PI) and PS] have an upward pointing arrow shape, while the choline phospholiplids (PC, SPM) have a downward pointing arrow; the rest are circular.

From our experience, the silica gel phospholipid chromatograms can, conceptually, be divided into five zones between the origin and solvent front.

Because PE and PC are the dominant phospholipids of plant and animal tissues and appear to retain a constant separation under all TLC conditions, they each can be assigned separate zones which represent markers against which the content of other zones is examined. In this scheme, zone I occupies the region between PE and the solvent front; PE occupies zone II. Zone III is between PE and PC; PC is zone IV. Zone V lies between PC and the origin. Separate solvent manipulations can preferentially expand zone I, zone III or zone V with some disadvantages resulting for the remaining zones. Similarly, the NL chromatograms can be divided into separate zones according to where TG, DG and MG run.

Zone I can contain CL, NAPE, Bis-PA, PG and phosphatidyl cholesterol (as well as PE-X and the PE acetone imine [12]. In ammonia systems, the relative R_F values of CL and NAPE are reversed and all of the phospholipids have substantially lower R_F values than is customary with neutral development systems (a 30-min overrun corrects this situation). Zone II, in addition to PE, can contain PG and a lyso-CL. Zone III generally contains PI and PS, well resolved, with the amino group of PS permitting its spot-test detection. Monomethyl phosphatidyl ethanolamine (MMePE), dimethyl phosphatidyl ethanolamine (DMePE), PC-X and LPE can also appear here; resolution of PS from LPE can be achieved in ammonia systems (Fig. 6A). Zone IV appears to contain only PC. Zone V contains SPM generally co-chromatographing with platelet activating factor (PAF). PAF can be selectively removed by conversion to LPC through incubation with phospholipase A_2. Our commercial PAF sample had a small alkenyl component, and from the phospholipase A_2 test, about 50% SPM (Fig. 2).

The lyso derivatives of CL, PG, PI, PS, MMePE, DMePE and NAPE occupy zones lower than the respective parent and present their own co-chromatography problems, not yet completely solved. Quite obviously isotopic labeling and chromatographic autoradiography can effectively resolve some of these problems due to the selectivity of the autoradiographic spot-test. PS has proven to be the least reliable, commercially available, phospholipid standard, often with multiple contaminants and not all readily interpretable.

Co-chromatography often presents problems for the resolution of CL and PG from PE, MMePE and DMePE from PI and PS, respectively, and PS from LPE. Two-dimensional TLC has been utilized by others to effect some of these resolutions. These, however, must be single-lane chromatograms. Our approach for multi-lane chromatograms has been to make substitutions of ethanol for methanol, 2-propanol for 1-propanol, 4 *M* ammonium hydroxide for water, etc, to manipulate solvent ratios, and to rely on multiple spot-tests as described here.

In order to account for all of the phospholipids likely to be present, and the enzymatic products representing the activity of various phospholipases, several different TLC preparations are required, all using the 10 × 10 cm format and various plastic sheet silica gel or aluminum oxide preparations. The full details of our experience with these problems will be reported elsewhere.

DISCUSSION

The TLC literature on lipids is extensive with a moderate amount of redundancy and narrowness of application. There are, however, many lipid problems remaining to be solved. Many will require imaginative application of existing procedures, others will require entirely new approaches. A biological understanding of the meaning of the three forms (diacyl, alkyl-acyl, and alkenyl-acyl) of glycerophospholipids and simple glycerides is by no means at hand. Active cognizance of their existence is required. So, too, is the puzzle as to why all types of glycerophospholipid do not always exist together as the three forms, nor why their quantitative relationships do not appear to be orderly. What the breakdown is, situation by situation, remains to be worked out and the data conceptualized to form a consistent, generalized explanation metabolically relating these diverse forms.

The physiological relevance of molecular species of such a diverse group of compounds as the glycerophospholipids also remains to be established. For these compounds, the conception of molecular species must be considered to exist at, at least, two levels, i.e. (1) at the glyceryl ether level and (2) at the non-glyceryl ether level, which includes the diacyl family, for each of the relevant base classes. On occasion, a random distribution of fatty acids (on the basis of opportunity) has been proposed for certain, mainly dietary, situations. We believe alkyl and alkenyl distributions have a very high degree of deliberateness and that there is considerable probability that acyl distributions are largely deliberate as well, so that correct molecular species occur at the right place at the right time. At the present time, TLC appears to still have a place in this future.

ACKNOWLEDGEMENTS

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