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SILICA GEL THIN-LAYER CHROMATOGRAPHY OF ACIDIC PHOSPHOLIPIDS

II. CHROMATOGRAPHIC BEHAVIOUR OF PHOSPHATIDYLSERINE AND PHOSPHATIDIC ACID APPLIED WITH DIFFERENT CATION COMPOSITION ON ADSORBENTS EITHER FREE OF METAL IONS OR CONTAINING A SURPLUS OF DIVALENT METAL IONS

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SUMMARY

Different salt forms of phosphatidylserine and phosphatidic acid (two acidic phospholipids) have been subjected to thin-layer chromatography on two commonly used silica adsorbents, one of which (silica gel HR) is practically free of metal ions and the other (silica gel G) contains 13% of calcium sulphate as binder. The chromatographic behaviour was studied in an acidic, a neutral and a basic solvent. Both adsorbents provided usable systems for phosphatidylserine with each of the three solvents, except for silica gel G with the neutral solvent, in which system tailing was prominent. The inclusion of calcium sulphate in the silica gel tended to impair chromatography of phosphatidylserine in acidic and neutral solvents, but improved its chromatography in the basic solvent. In all the systems, the migration was independent of the cation composition of the applied phosphatidylserine samples. For the chromatography of phosphatidic acid, only three of the systems tested were usable, and in those three, the chromatographic behaviour was independent of the cation composition of the samples. The calcium sulphate in an adsorbent increased tailing of phosphatidic acid in acidic and neutral solvents, as it did for phosphatidylserine, whereas with the basic solvent, calcium sulphate in the adsorbent caused phosphatidic acid to remain at the origin. Two one-dimensional thin-layer chromatographic systems previously recommended for the chromatography of acidic phospholipids were unsuitable for the chromatography of phosphatidic acid under the conditions used here. For both phosphatidylserine and phosphatidic acid chromatographed in acidic systems, the solvent must contain water in addition to acetic acid if excessive tailing is to be avoided.

INTRODUCTION

In two previous papers, column chromatography (CC) and thin-layer chro-

matography (TLC) on silica gel of two acidic phospholipids (cardiolipin and phosphatidylinositol) were investigated[1,2]. It was found that, both in CC and TLC, the mobility of these phospholipids was influenced by the type of metal cation bound to them and by metal ions present in the adsorbent. When a monovalent metal ion is bound to a phospholipid, the chromatographic mobility is lower than when the phospholipid is in the divalent salt form. The influence of the metal ions of an adsorbent is exerted in part through ion exchange with the phospholipid, thereby converting the latter into another salt form with a different chromatographic mobility. In the present investigation, the TLC of two other common acidic phospholipids, phosphatidylserine and phosphatidic acid, has likewise been studied to see whether their chromatographic mobilities also depend on the metal ions of the phospholipids and the sorbents. As before[2], the chromatography of the phospholipids was studied on silica gel G Type 60 (E. Merck, Darmstadt, G.F.R.), which is abundant in Ca^{2+}, and silica gel HR (Merck), which is almost free of metal ions. For each phospholipid, the chromatographic behaviour was studied when applied with different cation compositions to the two adsorbents and chromatographed in an acidic, a neutral and a basic solvent. The less satisfactory behaviour of phosphatidic acid in most of these systems prompted us to examine its chromatography in other TLC systems commonly used for the analysis of phospholipids. With two of these systems, results were inconsistent with those of the original report[3]. Since phosphatidic acid is a key compound in the synthesis of glycerophospholipids, it appears important for the future use of these systems to report these observations. We have done this under the heading *Additional studies on the chromatography of phosphatidic acid* at the end of the section Results and discussion.

MATERIALS AND METHODS

Chemicals

Chloroform and methanol were of analytical-reagent grade. The acetone and light petroleum (b.p. below 50°) used in the preparation of phosphatidylserine were of purum grade. Chloroform–methanol mixtures for extraction and for CC of the lipids were deoxygenated before use under reduced pressure from a water pump, followed by return to atmospheric pressure under pure nitrogen, and the antioxidant butylated hydroxytoluene (2,6-di-*tert.*-butyl-*p*-cresol; Sigma, St. Louis, Mo., U.S.A.) was added to give a concentration of 50 mg/l. The lecithin and phosphatidylethanolamine used as reference compounds were obtained by CC of acetone-precipitated egg-yolk phospholipids on silica gel. Reference phosphatidylserine and phosphatidic acid were obtained from Supelco (Bellefonte, Pa., U.S.A.) (phosphatidylserine), Sigma (phosphatidic acid from egg lecithin, sodium salt, grade I, No. P-9511) and Sedary Research Laboratories Inc. (London, Ont., Canada) (phosphatidic acid, dioleoyl, No. B-82). The two silica adsorbents used for the main TLC were silica gel G Type 60, catalogue No. 7731, control No. 2548637, and silica gel HR, catalogue No. 7744, control No. 6228064, both products of Merck. In addition, silica gel type D-O without calcium sulphate binder (Camag, Muttenz, Switzerland), silica gel Woelm TLC without calcium sulphate binder (M. Woelm, Eschwege, G.F.R.) and silica gel H (Merck) were used in the separate examination of the behaviour of phos-

phatidic acid as described under *Additional studies on the chromatography of phosphatidic acid.*

Preparation of phosphatidylserine and phosphatidic acid

Phosphatidylserine was prepared from ox brain by silica gel CC[4]. Phosphatidic acid was prepared enzymically from egg lecithin with subsequent isolation of the liberated phosphatidic acid by CC on Mallinckrodt (St. Louis, Mo., U.S.A.) silicic acid[5]. TLC in the two-dimensional system of Nichols[6], with authentic phosphatidylserine and phosphatidic acid as reference compounds, confirmed the identities of the isolated phospholipids. Polar contaminants amounted to less than 10% as judged by visual estimation of the charred chromatograms.

Conversion of the phospholipids into different salt forms

The procedure described by Shimojo *et al.*[7] for conversion of acidic phospholipids into their monovalent or divalent salt forms was used, except that contaminating inorganic salt was removed from the converted phospholipids by gel filtration on a Sephadex column[8] instead of by passage through a cellulose column.

Analytical methods

The metal ion composition of the phospholipids was determined as described previously[1], phosphorus being determined by Bartlett's method[9] and the metals by atomic-absorption spectrophotometry.

Conditions for thin-layer chromatography and rendering the chromatograms visible

Plates (20 × 20 cm) were coated with a 0.25-mm-thick layer of adsorbent by use of a Desaga spreader (Desaga, Heidelberg G.F.R.). Before use, the plates were activated for 1 h at 100–105° and delineated into 2-cm lanes. In addition, a front line was delineated, allowing a run of 15 cm with the samples being applied 2 cm from the bottom edge. Samples of a phospholipid having different cation compositions were applied on neighbouring lanes to facilitate comparison of their chromatographic behaviour. Chromatography was studied for loads of phospholipid corresponding to 1, 3, 5 and 8 μg of phosphorus. In addition, lecithin (and sometimes also phosphatidylethanolamine) was chromatographed on the plates to show the migration of the acidic phospholipids relative to these neutral phospholipids. The chromatograms were developed at room temperature in jars lined with filter-paper and equilibrated for 3–4 h before use. Chloroform–methanol mixture containing acetic acid and water (acidic solvents), water (neutral solvents) or ammonia (basic solvents) were used for development. After development, the chromatograms were rendered visible by spraying with 66% sulphuric acid and charring for 1–2 h at 150–160°.

RESULTS AND DISCUSSION

Metal ion content (sodium, potassium, calcium and magnesium) of the phosphatidylserine and phosphatidic acid used

Phosphatidylserine contains two acidic and one basic group in its molecule. A metal ion:P equivalence ratio of 1 indicates that one acidic proton has been exchanged for an equivalent of metal ion. If there were quantitative intramolecular

neutralisation of the other acidic group by the basic group of the molecule, no further uptake of metal ions would be possible, and metal ion:P equivalence ratios no higher than 1 would be expected. However, if intramolecular neutralisation were partial, equivalence ratios higher than 1 would be possible; values less than 1 would signify a mixture of the free acid form and a salt form. Phosphatidic acid contains two acidic and no basic groups in its molecule, and a metal ion:P equivalence ratio of 2 indicates that both acidic protons have been replaced by equivalents of metal ions. An equivalence ratio of 1 means that, on average, each molecule has one acidic proton replaced by an equivalent of metal ion. This indicates that either the phospholipid is in the acidic salt form, or that equal amounts of the neutral salt and the free acid form are present. However, since pK_1 and pK_2 for phosphatidic acid are presumably different, an equivalence ratio of 1 probably means that the phospholipid is present solely as the acidic salt. An equivalence ratio less than 1 signifies a mixture of the free acid form and the acidic salt, and values between 1 and 2 signify mixtures of the acidic salt and the neutral salt.

TABLE I

METAL ION COMPOSITION OF THE PHOSPHOLIPIDS USED IN THE CHROMATOGRAPHIC INVESTIGATIONS EXPRESSED AS METAL ION: P EQUIVALENCE RATIO

In calculating the equivalence ratio, 1 atom of P was considered as 1 equivalent, while 1 equivalent of metal ion equals 1 singly charged ion.

Phospholipid	*Equiv. metal cation:equiv. P*				
	Na	*K*	*Ca*	*Mg*	*Total metal cations:P*
Sodium phosphatidylserine	1.19	0.015	0.037	0.018	1.26
Magnesium phosphatidylserine	0.014	0.000	0.006	0.40	0.42
Sodium phosphatidic acid	1.46	0.007	0.063	0.056	1.59
Magnesium phosphatidic acid	0.030	0.004	0.045	0.81	0.90

Table I shows the metal ion:P equivalence ratios for the phospholipids used. It is apparent that each phospholipid contains almost solely the kind of metal ion intended by the conversion procedure used. Since the actual values of metal ion:P equivalence ratio are not whole numbers, a phospholipid is present as a mixture of different forms in relative proportions corresponding to the experimentally determined equivalence ratios. For the sake of convenience, the phospholipid samples have been named according to their main metal cation as sodium phosphatidylserine, magnesium phosphatidylserine, and so on, although each actually represents a mixture. Sodium phosphatidic acid, having a Na:P equivalence ratio of 1.46, may be considered as being a mixture of equal amounts of disodium phosphatidate and sodium hydrogen phosphatidate. Magnesium phosphatidylserine, having a Mg:P equivalence ratio of 0.42, may be considered as being a mixture of the fully protonated form of phosphatidylserine and its magnesium hydrogen salt, the latter form amounting to some 40% of the total. Magnesium phosphatidic acid and sodium phosphatidylserine, having equivalence ratios about 1, are mainly the acidic salts of the metals and the phospholipids.

TABLE II

CHROMATOGRAPHIC SYSTEMS INVESTIGATED FOR TLC OF PHOSPHATIDYLSERINE AND PHOSPHATIDIC ACID

A plus sign indicates that the phospholipid in question displays true chromatographic migration (*i.e.*, tailing is either absent or negligible) in the system. A minus sign indicates that the system is unsuitable for chromatography of the phospholipid in question.

Figure	*Adsorbent*	*Solvent*	*Phospholipid*	*Applicability*
1a	Silica gel G Type 60	$CHCl_3$–CH_3OH–CH_3COOH–H_2O (62.5:37.5:10:5)	Phosphatidylserine	+
1b		$CHCl_3$–CH_3OH–H_2O (65:25:4)	Phosphatidylserine	–
1c		$CHCl_3$–CH_3OH–7 *N* NH_4OH (50:50:5)	Phosphatidylserine	+
1d	Silica gel HR	$CHCl_3$–CH_3OH–CH_3COOH–H_2O (62.5:37.5:10:5)	Phosphatidylserine	+
1e		$CHCl_3$–CH_3OH–H_2O (65:25:4)	Phosphatidylserine	+
1f		$CHCl_3$–CH_3OH–7 *N* NH_4OH (50:50:5)	Phosphatidylserine	+
2a	Silica gel G Type 60	$CHCl_3$–CH_3OH–CH_3COOH–H_2O (62.5:37.5:10:5)	Phosphatidic acid	+
2b		$CHCl_3$–CH_3OH–H_2O (65:25:4)	Phosphatidic acid	–
2c		$CHCl_3$–CH_3OH–7 *N* NH_4OH (30:70:5)	Phosphatidic acid	–
2d	Silica gel HR	$CHCl_3$–CH_3OH–CH_3COOH–H_2O (62.5:37.5:10:5)	Phosphatidic acid	+
2e		$CHCl_3$–CH_3OH–H_2O (65:25:4)	Phosphatidic acid	–
2f		$CHCl_3$–CH_3OH–7 *N* NH_4OH (30:70:5)	Phosphatidic acid	+

Chromatographic systems used for main investigation of the TLC of phosphatidylserine and phosphatidic acid applied with different cation composition

Table II summarizes the systems in which TLC of phosphatidylserine and phosphatidic acid was investigated. The column marked *Applicability* indicates whether a system is suitable for chromatography of the phospholipids (+) or not (–). The chromatographic results are presented below in the form of selected representative segments of the chromatograms (Figs. 1a–f and 2a–f). Only the lanes with phospholipid corresponding to 1 and 5 μg of phosphorus have been shown; together with the comments given on each chromatogram, this permits a sufficiently detailed description of the chromatographic results.

Chromatography of phosphatidylserine on silica gel G and silica gel HR

Figs. 1a, b and c show representative segments of the chromatograms obtained for phosphatidylserine on silica gel G, Type 60, with acidic, neutral and basic solvents, respectively. It appears that silica gel G provides a usable chromatographic system for phosphatidylserine with either a basic or an acidic solvent, (although with the latter solvent, there is some tailing with increasing loads, and, in addition, the chro-

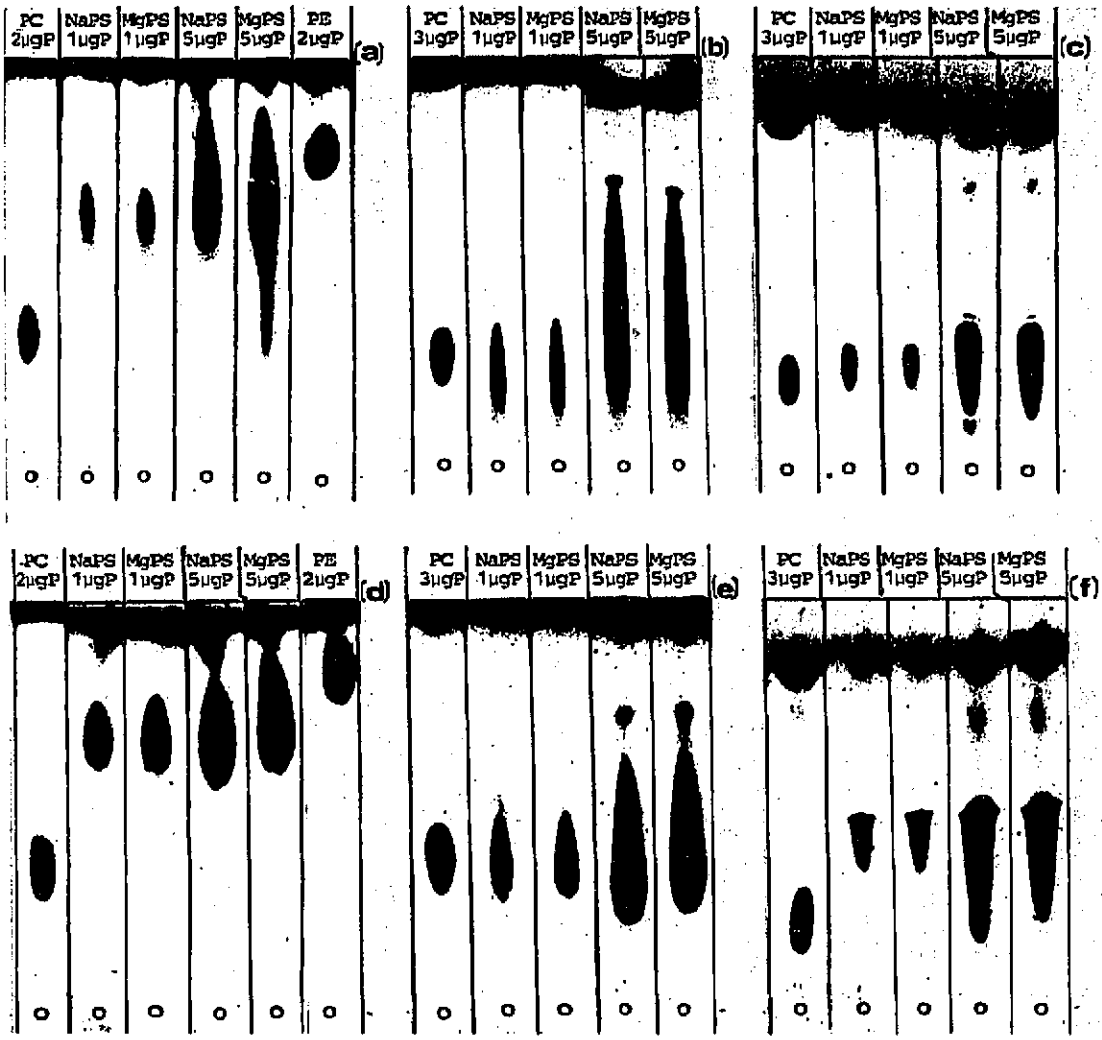

Fig. 1. TLC of the acidic phospholipid phosphatidylserine (PS) on silica gel G Type 60 (a, b and c) and on silica gel HR (d, e and f). In (a) and (d), the acidic solvent chloroform–methanol–acetic acid–water (62.5:37.5:10:5) was used; in (b) and (e), the solvent was the neutral chloroform–methanol–water (65:25:4), and the basic solvent chloroform–methanol–7 *N* ammonia (50:50:5) was used in (c) and (f). The phospholipid was applied in chloroform solution (0.5 µg/µl of P) corresponding to the amounts of P listed on the chromatograms. Samples of different cation composition (detailed compositions are shown in Table I) were applied on neighbouring lanes, as indicated on the chromatograms by Na-PS and Mg-PS, respectively, according to their principal metal cation. Lecithin (PC) was included on each chromatogram to show the migration of phosphatidylserine relative to a well-known neutral phospholipid. On (a) and (d), phosphatidylethanolamine (PE), another neutral phospholipid, was also included. The chromatograms were rendered visible by charring with sulphuric acid. The material migrating with, or close to, the front on all chromatograms consists of non-phosphorous contaminants of adsorbent and phospholipid. O = Origin.

matographic mobility depends on the load). With the neutral solvent, tailing occurs to an extent that makes the system unfit for chromatography of phosphatidylserine. The fact that migration relative to the neutral phospholipid lecithin is considerably faster in the acidic system (a) than in the neutral system (b) may indicate that some protonated form of phosphatidylserine is migrating in the acidic system. In the neutral system (b), phosphatidylserine probably migrates as its calcium salt, owing to the high concentration of Ca^{2+} in the adsorbent. Of all the systems tested, silica gel G with the basis solvent (c) provided the best chromatographic system. In this system, phosphatidylserine migrates as a dense, well-defined spot with an R_F value independent of the load. The satisfactory chromatography in this system is probably due, at least in part, to quantitative conversion of phosphatidylserine into its ammonium salt form, which is then the sole migrating form of the phospholipid.

Chromatography of phosphatidylserine on silica gel HR is shown in Figs. 1d–f. The chromatographic behaviour of phosphatidylserine on this adsorbent differs slightly from that on silica gel G. With the acidic and neutral solvents (d and e, respectively), tailing is diminished and chromatography is to some extent improved. With the basic solvent (f), spots are distorted and chromatography is inferior to that on silica gel G (c). Since the 13% of calcium sulphate in silica gel G is the only difference between the two adsorbents, this salt appears to cause tailing of phosphatidylserine in acidic and neutral solvents, whereas it improves the chromatography of this phospholipid in a basic solvent.

Rouser *et al.*[10] consider TLC on silica gel to be largely partition chromatography, in contrast to the more general view that it is adsorption chromatography. They suggest that the tailing of acidic phospholipids observed on adsorbents containing calcium sulphate is due to binding of the phospholipid anionic groups to the Ca^{2+} of the adsorbent. Such binding would cause the chromatography to become to some extent adsorption chromatography, and that would account for the tailing. The results presented here for phosphatidylserine in the acidic and neutral systems are consistent with this view [compare (a) with (d), and (b) with (e) in Fig. 1]. On the other hand, when the solvent is basic, the presence of calcium sulphate in an adsorbent obviously does not cause adsorption to be important, since no tailing occurs under these conditions (Fig. 1c). This may be because effective shielding of the Ca^{2+} by OH^- of the solvent prevents binding of the anionic groups of the phospholipid to this metal ion.

For both adsorbents it was most important that the acidic solvent should contain water in addition to acetic acid. When no water was included in the solvent, tailing occurred to an extent that made the systems almost useless for the TLC of phosphatidylserine. This favourable effect of water in the chromatographic solvent may be due to a facilitated protonation of the phospholipid anionic groups in the presence of water, thereby decreasing their binding to the cations of the adsorbent; tailing due to adsorption would thereby be diminished. It is also possible that water adsorbed on the surface of the silica gel and saturated with the other components of the solvent constitutes a stationary phase favouring partition chromatography over adsorption chromatography.

Further, on both adsorbents and with all the solvents tested, the chromatographic behaviour of phosphatidylserine was not influenced by the difference in cation composition of the phosphatidylserine samples used. This is in contrast to the earlier

findings for cardiolipin and phosphatidylinositol, which demonstrated that the cation composition of these phospholipids considerably influences their chromatographic behaviour in some systems[2]. However, a similar effect of the cation composition on the chromatographic behaviour of phosphatidylserine may occur if the differences in cation composition are more pronounced than in the present investigation. In fact, such an influence was probably observed when the elution of phosphatidylserine from a silica gel column was localized by TLC in the isolation of ox-brain phosphatidylserine by the method[4] mentioned above. In this method, a phosphatidylserine-enriched fraction obtained by solvent fractionation of ox-brain phospholipid is washed with sodium chloride solution, whereby the acidic phospholipids of the fraction are more or less converted into their sodium salt form, and phosphatidylserine is subsequently isolated by CC. Aliquots withdrawn at intervals from the eluate were subjected to TLC to localize the elution of phosphatidylserine. The first portion of phosphatidylserine eluted had the same R_F value as authentic phosphatidylserine in a neutral system, but the material eluted later gave rise to an additional, slower-moving, ninhydrin-positive spot. However, when examined in a basic system containing ammonia, only one spot appeared, the R_F value of which was that of phosphatidylserine. Thus, the two ninhydrin-positive spots observed are considered to reflect the migration of two different salt forms of phosphatidylserine.

Chromatography of phosphatidic acid on silica gel G Type 60 and silica gel HR

Figs. 2a–c show the chromatographic behaviour of phosphatidic acid on silica gel G, Type 60, in an acidic solvent (a), a neutral solvent (b) and a basic solvent (c). Only the acidic system (a) may be of some use for the chromatography of amounts of phosphatidic acid equivalent to 1 μg or less of P; with higher loads considerable tailing occurs. The neutral system (b) is useless for the chromatography of phosphatidic acid because of severe tailing for all loads of the phospholipid. The fact that practically no migration occurred with the basic solvent (c) indicates that phosphatidic acid is strongly adsorbed to the adsorbent in that system. In part, this is due to phosphatidic acid being in the very polar form of its diammonium salt, but the calcium sulphate of the adsorbent may also contribute, since migration does occur when no calcium sulphate is present (see Fig. 2f). The effect of calcium sulphate is probably due to strong binding between the anionic groups of the phospholipid and Ca^{2+}.

When silica gel HR is used as adsorbent, the chromatograms shown in (d), (e) and (f) of Fig. 2 are obtained. It appears that tailing in the acidic and neutral systems, (d) and (e) respectively, has been somewhat diminished by using silica gel HR instead of silica gel G. Hence, the calcium sulphate of the silica gel G obviously causes tailing of phosphatidic acid on that adsorbent in acidic and neutral solvents. As mentioned above, the same applies to phosphatidylserine. With the basic solvent, phosphatidic acid migrated on silica gel HR, and this system may be useful for the chromatography of phosphatidic acid equivalent to less than 1 μg of P; at higher loads, the compound will be spread over a considerable distance along the chromatogram. It appears to be important that only the particular silica gel HR is used with the basic solvent. Thus, when Merck's silica gel H, which does not contain calcium sulphate binder, is used, phosphatidic acid remains at or near the origin, much in the same way as is observed on silica gel G (Fig. 2c).

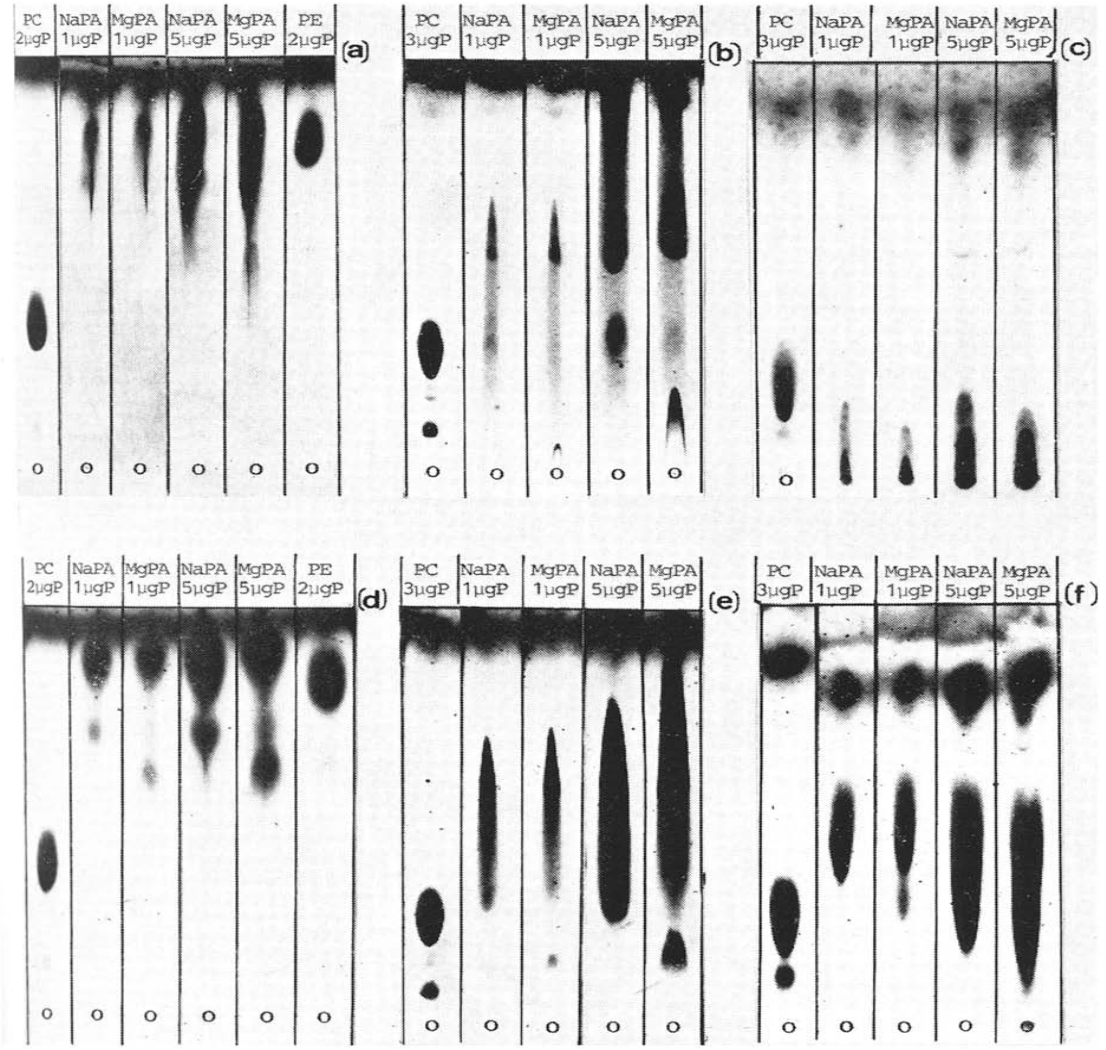

Fig. 2. TLC of the acidic phospholipid phosphatidic acid (PA) on silica gel G Type 60 (a, b and c) and on silica gel HR (d, e and f). In (a) and (d), the acidic solvent chloroform–methanol–acetic acid–water (62.5:37.5:10:5) was used; in (b) and (e), the solvent was the neutral chloroform–methanol–water (65:25:4), and the basic solvent chloroform–methanol–7 *N* ammonia (30:70:5) was used in (c) and (f). The phospholipid was applied in chloroform solution (0.5 µg/µl of P) corresponding to the amounts of P listed on the chromatograms. Samples of different cation composition (detailed compositions are shown in Table I) were applied on neighbouring lanes as indicated on the chromatograms by Na-PA and Mg-PA, respectively, according to their principal metal cation. Lecithin (PC) was included on each chromatogram to show the migration of phosphatidic acid relative to a well-known neutral phospholipid. On (a) and (d), phosphatidylethanolamine (PE), another neutral phospholipid, was also included. The chromatograms were rendered visible by charring with sulphuric acid. The material migrating with the front consists of non-phosphorus contaminants of adsorbent and phospholipid, except in (a), (b) and (d) where the area occupied by PA extends into the front. O = Origin.

Omission of water from the acidic solvent had the same effect on the chromatography of phosphatidic acid in causing severe tailing as was found for phosphatidylserine. The fact that water in the acidic solvent diminished tailing, and the effect of calcium sulphate in the adsorbent in causing tailing of phosphatidic acid in the acidic and neutral systems (Figs. 2a and b) may, as suggested above for phosphatidylserine, be due to water favouring partition chromatography, and to Ca^{2+} causing (to some extent) adsorption chromatography. However, whereas in the basic solvent, the chromatographic behaviour of phosphatidylserine on the adsorbent containing calcium sulphate (Fig. 1c) indicates partition chromatography, the immobility of phosphatidic acid on that adsorbent in a basic solvent suggests that strong adsorption is the decisive factor responsible for its behaviour. It seems natural to suggest that a strong affinity of the phosphate group of phosphatidic acid (carrying two net negative charges) for the Ca^{2+} of the adsorbent accounts for this adsorption.

With both adsorbents there was no essential dependence of chromatographic behaviour on the cation composition of phosphatidic acid.

The less satisfactory chromatographic behaviour of phosphatidic acid in most of the above-mentioned systems made it desirable to study its behaviour in other systems reported for the TLC of phospholipids in general and acidic phospholipids in particular. The findings of these supplementary experiments are summarized below.

Additional studies on the chromatography of phosphatidic acid

Three one-dimensional systems (I, II, III) for the TLC of acidic phospholipids have been reported by Skipski *et al.*[3]. We have examined the chromatography in these systems of phosphatidic acid samples differing in cation composition and samples of phosphatidylethanolamine and cardiolipin; the chromatograms obtained were compared with those presented by the above workers.

The chromatogram obtained with system I and silica gel HR as adsorbent essentially agreed with that reported[3], and the behaviour of the phosphatidic acid samples was not affected by the difference in cation composition. Some tailing was observed with increasing loads, and this may be due to the higher loads used in the present work (0.2–3 μg of P). On the other hand, the results obtained for phosphatidic acid with systems II and III on any of the gels tested [silica gel HR (Merck); silica gel H (Merck); silica gel D-O (Camag); and silica gel Woelm TLC], containing the appropriate amounts of sodium carbonate (system II) or sodium hydrogen carbonate (system III), differed most significantly from those reported[3]. Phosphatidic acid was reported to migrate as well-defined spots in systems II and III (with R_F values of about 0.3 and 0.6, respectively), but we found it at the origin in system II, and both at the origin and in streaks extending therefrom in system III. The chromatographic behaviour of phosphatidylethanolamine and cardiolipin, however, agreed with their reported behaviour in these systems. The cation composition of the phosphatidic acid samples (sodium and magnesium salts of phosphatidic acid of Table I) had no effect. The chromatograms were repeated with commercial phosphatidic acid (phosphatidic acid, sodium salt, from Sigma, and synthetic phosphatidic acid from Sedary Research Labs.), and with the calcium salt form of phosphatidic acid (Ca:P equivalence ratio = 1.5) prepared from the sodium salt form mentioned in Table I (sodium salt of phosphatidic acid) by shaking it for 5 min in the Folch chloroform–methanol–water (8:4:3) system containing calcium acetate in the upper phase to a concentration

of 5 m*M*. The chromatograms obtained for these samples of phosphatidic acid did not differ from those already obtained with the sodium and magnesium salts of phosphatidic acid of Table I. The reason for the discrepancy between the chromatographic behaviour of phosphatidic acid observed in our laboratory and that reported by Skipski *et al.*[3] is at present unknown. It seems natural to suggest that the silica gels used by us may differ from that used by Skipski *et al.* in some way crucial to the chromatography of phosphatidic acid (but obviously not critical to other phospholipids). As we included in our investigation the silica gel used in the original work (Camag silica gel D-O), some change in the manufacturing conditions for this gel seems to have taken place after the original work was published.

Two-dimensional TLC using a basic solvent in the first direction and an acidic solvent in the second direction was used by Nichols[6] and by Rouser *et al.*[11,12] for the separation of complex mixtures of phospholipids. The chromatography of phosphatidic acid was examined in the system of Nichols[6] (Fig. 3). The chromatographic behaviour was found to be independent of the cation composition of the applied sample, and its reported[6] location on the chromatogram in an area free of any other phospholipid was confirmed. There was a difference in the chromatographic behaviour

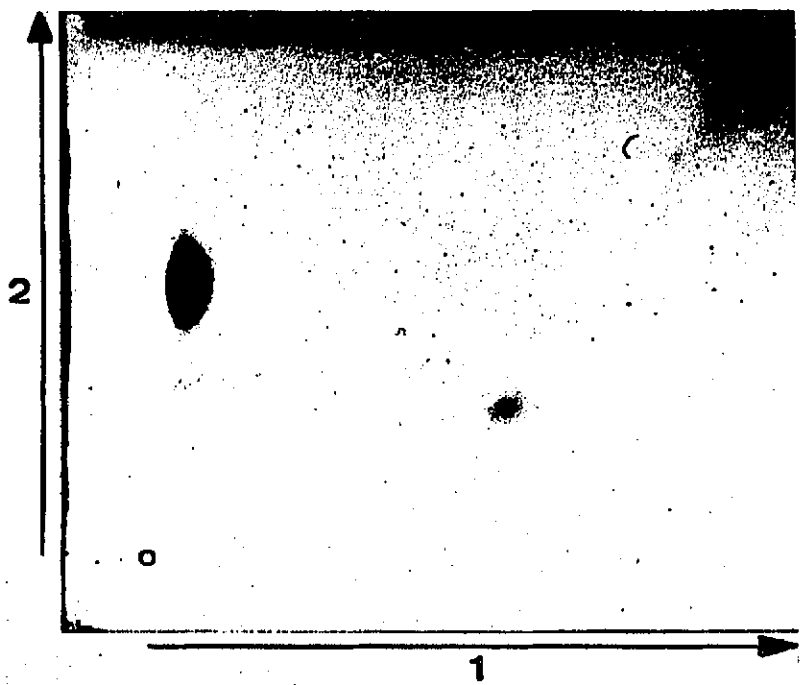

Fig. 3. TLC of phosphatidic acid in the two-dimensional system of Nichols[6], with silica gel HR as adsorbent. Phosphatidic acid containing Na as its principal metal cation (Na-PA of Table I) was applied in the lower left corner in an amount corresponding to 3 μg of P. The plate was developed first (direction 1 on the figure) with chloroform–methanol–7 *N* ammonia (65:30:4), then the solvent was completely removed (*i.e.*, until ammonia could no longer be smelt) in a stream of air at room temperature before development with the second solvent, chloroform–methanol–acetic acid–water (85:12.5:12.5:3). The chromatogram was rendered visible by charring with sulphuric acid. O = Origin.

on silica gel G and silica gel HR: on the former adsorbent (the one used by Nichols[6]), phosphatidic acid may migrate as two overlapping fractions in the second direction, whereas it migrated as one spot (Fig. 3) on silica gel HR.

CONCLUSION

The present work allows the following statements to be made:

(1) In general, it is an advantage to use silica gel without calcium sulphate binder as adsorbent for the TLC of phosphatidylserine and phosphatidic acid, except for TLC of phosphatidylserine in a basic solvent.

(2) It is obligatory that acidic solvents contain water to prevent excessive streaking of the two phospholipids.

(3) For one-dimensional TLC of phosphatidylserine, the following systems may preferably be used: silica gel without calcium sulphate binder using an acidic or a neutral solvent; and silica gel containing calcium sulphate binder using a basic (containing ammonia) solvent.

(4) For one-dimensional TLC of phosphatidic acid, silica gel without calcium sulphate binder and an acidic solvent may be used. The acidic solvent should be made less polar than the one used here by increasing the proportion of chloroform to the polar components of the solvent. A basic (containing ammonia) solvent may also be used, but only if the adsorbent is Merck's silica gel HR.

(5) The chromatographic behaviour of phosphatidylserine and phosphatidic acid is not affected by the cation composition of the phospholipids, at least with the different forms used by us.

(6) Chromatographic systems using silica gel impregnated with sodium carbonate or sodium hydrogen carbonate and basic solvents reported to be suitable for the chromatography of phosphatidic acid were unfit for its chromatography under our conditions. Some difference in the properties of the silica gels used may be the reason for this discrepancy.

Although the present investigation did not reveal any dependence of the chromatographic behaviour of phosphatidylserine and phosphatidic acid on their cation composition —in contrast to the findings for cardiolipin and phosphatidylinositol[2]— the possibility of such an influence still exists for samples whose cation compositions differ more than those used in the present work; this possibility was mentioned for phosphatidylserine in the section *Chromatography of phosphatidylserine on silica gel G and silica gel HR*. A difference in the chromatographic behaviour of samples of an acidic phospholipid due to different cation composition will be eliminated in a basic chromatographic system, because of quantitative conversion of the applied samples to the same salt form. Thus, when two spots appearing in the TLC of a sample are suspected to arise from different salt forms of the same phospholipid, the sample should be examined in a basic system.

From the various one-dimensional TLC systems investigated in this and the previous paper[2] with respect to the chromatography of four important acidic phospholipids of animal tissue, it is possible to select for each of these phospholipids two or three systems (one of which should be basic) that will allow their identification in mixtures consisting of a few phospholipids. One such important application of one-dimensional TLC is the localization of phospholipids during the fractionation of

complex mixtures by CC. In CC, acidic phospholipids are generally eluted together with one or two of the neutral phospholipids, and one-dimensional TLC of the eluate (allowing examination of several fractions in a limited time) is the method of choice for establishing the positions of their elution. When specific sprays are used for detection of the lipids containing a phosphate group[13] and those containing an amino group (ninhydrin spray), the information obtained is considerably increased.

Analysis of complex mixtures of phospholipids by one-dimensional TLC is not possible at present, because of overlapping of fractions, but it can be done by two-dimensional TLC. Two systems are available (those of Nichols[6] and Rouser *et al.*[11]), in both of which a basic solvent (containing ammonia) is used in the first direction, and an acidic solvent in the second direction. The defects of one-dimensional TLC are largely absent from these systems. In the first place, initial application of a basic solvent converts each of the acidic phospholipids quantitatively into one salt form and thus eliminates the possibility that an acidic phospholipid may give rise to more than one spot (due to different salt forms being applied). Secondly, the combination of a basic and an acidic solvent as a whole favours separation of the neutral and the acidic phospholipids. This is because the chromatographic mobility of acidic phospholipids is relatively low in basic solvents and relatively high in acidic solvents. Thirdly, the systems allow sufficient material to be applied to the plate for quantitative analysis of the separated components by analysis of the spots for phosphorus[11,12].

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