Journal of Chromatography, 89 (1974) 275–285

CHROM. 7113

SILICA GEL THIN-LAYER CHROMATOGRAPHY OF ACIDIC PHOSPHOLIPIDS

I. EFFECT OF METAL IONS OF THE ADSORBENT AND PHOSPHOLIPID ON THE CHROMATOGRAPHIC MOBILITY OF CARDIOLIPIN AND PHOSPHATIDYLINOSITOL

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(Received September 27th, 1973)

SUMMARY

The monovalent and divalent salt forms of two acidic phospholipids (cardiolipin and phosphatidylinositol) have been subjected to thin-layer chromatography on two different adsorbents commonly used for the chromatography of lipids. It was found that in some thin-layer chromatographic systems, an acidic phospholipid displays different chromatographic behaviour when applied to the plate as its monovalent salt and when applied as its divalent salt.

The results show that misleading results will often occur in the thin-layer chromatography of acidic phospholipids unless the ionic states of both the phospholipid and the adsorbent are properly appreciated.

INTRODUCTION

In the preceding paper[1], it was shown that the chromatographic behaviour of acidic phospholipids on silica gel columns may be influenced by both the type of metal cation bound to them and the metal ions in the adsorbent. Thus, the monovalent salt form has a considerably lower chromatographic mobility than the divalent salt form. The influence of metal ion present in an adsorbent for column chromatography (CC) is exerted mainly through its ion exchange with the phospholipid, thereby converting it into another salt form with a different chromatographic mobility. The different metal ion contents of different adsorbents were shown to constitute a source of variability in chromatographic results by this mechanism.

In the thin-layer chromatography (TLC) of lipids, silica gel is also used as an adsorbent, and as this technique is extensively used both for routine work and in work on the isolation and identification of individual lipids, it appears important to establish whether metal ions of acidic phospholipids and adsorbents may have similar effects in this type of chromatography. For this investigation, two adsorbents were chosen, which represent extremes with respect to metal ion content. The one adsor-

bent, silica gel G Type 60 (E. Merck, Darmstadt, G.F.R.), which contains 13% of plaster of Paris as a binder, represents one extreme, being rich in metal ions (Ca^{2+}). The other adsorbent, Merck's silica gel HR, contains a relatively small amount of metal ions. As representatives of acidic phospholipids, cardiolipin and phosphatidylinositol were chosen. For each phospholipid, the chromatographic mobility was investigated when applied as its monovalent and divalent salts on the two adsorbents and chromatographed in an acidic, a neutral and a basic solvent.

MATERIALS AND METHODS

Chemicals

Cardiolipin and phosphatidylinositol were isolated from ox liver as previously described[1,2]. Lecithin was isolated by silica gel CC of acetone-precipitated egg yolk phospholipid[3]. The acidic phospholipids were converted into their monovalent (sodium) and divalent (magnesium) salts as described by Shimojo *et al.*[4]. Analytical-reagent grade chemicals and deoxygenated solvents containing butylated hydroxytoluene (2,6-di-*tert.*-butyl-*p*-cresol, St. Louis, Mo., U.S.A.) as antioxidant (50 mg/l) were used throughout the work. The two silica adsorbents used for 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.

Analytical methods

The determination of the metal ion content (sodium, potassium, calcium and magnesium) of silica gel HR and the metal ion:P equivalence ratio for the lipids to be chromatographed was carried out as described previously[5].

Conditions for thin-layer chromatography and rendering the chromatograms visible

Plates of dimensions 20 × 20 cm coated with a 0.25-mm thick layer of adsorbent were prepared 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. The chromatograms were developed at room temperature in jars lined with filter-paper and equilibrated for 3–4 h before use. Chloroform–methanol mixtures containing either acetic acid (acidic solvents), water (neutral solvents) or ammonia (basic solvents) were used for development. After development, the chromatograms were rendered visible by spraying them with 66% sulphuric acid and charring for 2 h at 150–160°.

RESULTS AND DISCUSSION

Metal ion content (sodium, potassium, calcium and magnesium) of silica gel HR and phospholipids

The amounts of metal ions (sodium, potassium, calcium and magnesium) in silica gel HR which are extracted by four successive extractions with 0.1 *N* hydrochloric acid are as follows (the metal ion content is expressed as micromoles of metal per gram of non-activated adsorbent): sodium, 23.7; potassium, 0.08; calcium, 0.39; and magnesium, 0.28.

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 cardiolipin	0.91	0.003	0.031	0.017	0.96
Magnesium cardiolipin	0.042	0.001	0.015	0.83	0.89
Sodium phosphatidylinositol	0.91	0.005	0.044	0.010	0.97
Magnesium phosphatidylinositol	0.045	0.001	0.029	0.95	1.03

The cation composition of the phospholipids is given in Table I. The figures show Na^+ to constitute some 90% of the cation equivalents of sodium cardiolipin and sodium phosphatidylinositol with the remainder being made up by Ca^{2+}, Mg^{2+} and presumably H^+. Mg^{2+} constitutes 83% of the cation equivalents of magnesium cardiolipin, while Na^+ and Ca^{2+} make up a few per cent and H^+ presumably about 10%. Magnesium phosphatidylinositol has 95% of its cations as Mg^{2+} and a few per cent as Na^+ and Ca^{2+}. The analyses of the cation:P equivalence ratios thus show that the phospholipid preparations obtained are mainly in the intended salt forms.

Chromatographic mobility of cardiolipin and phosphatidylinositol in different chromatographic systems when applied as their monovalent and divalent salts

Table II summarizes the results of the comparison of the chromatographic mobilities of an acidic phospholipid subjected to TLC as monovalent and divalent salts. An observed difference in the chromatographic mobility is indicated by a plus sign together with reference to the figure which shows that particular chromatogram.

The interpretation given below of the individual chromatograms is based partly on the knowledge obtained in the preceding work[1] concerning the mechanism by which metal ions of acidic phospholipids and silica adsorbents influence the elution profile in CC. It seems reasonable to draw this analogy to TLC, because here too the adsorbents are silica gels with varying metal ion contents and one would therefore expect the same basic phenomena to occur in the two chromatographic techniques.

Chromatogram No. 1 (Fig. 1a). Chromatogram No. 1, which is shown in Fig. 1a, illustrates the chromatography of cardiolipin on silica gel G Type 60 in an acidic solvent. With the lowest amount of phospholipid being applied (1 μg of P), there is no difference in the chromatographic mobilities of cardiolipin applied as its monovalent and divalent salts. However, as the load is increased, a difference appears, and cardiolipin applied as the monovalent salt displays a streaking which grows more pronounced the higher the load. Presumably, at the lower load, the ratio of Ca^{2+} contained in the adsorbent to cardiolipin is sufficiently high to cause complete conversion of the applied monovalent salt of cardiolipin into the calcium salt form. Therefore, no difference in chromatographic mobility is observed between cardiolipin

TABLE II

CHROMATOGRAPHIC MOBILITY OF TWO ACIDIC PHOSPHOLIPIDS IN DIFFERENT TLC SYSTEMS WHEN APPLIED AS THEIR MONOVALENT AND DIVALENT SALTS

An observed difference in mobility of a phospholipid applied as its monovalent and divalent salts is indicated by a plus sign. A minus sign indicates that such a difference was not observed.

Chromatogram No.	*Adsorbent*	*Solvent*	*Phospholipid*	*Difference beween chromatographic mobility of monovalent and divalent salt forms*
1	Silica gel G Type 60	$CHCl_3$–CH_3OH–CH_3COOH (65:25:4)	Cardiolipin	+ (Fig. 1a)
2		$CHCl_3$–CH_3OH (75:20) satd. with H_2O	Cardiolipin	–
3		$CHCl_3$–CH_3OH–7 *N* NH_4OH (65:40:4)	Cardiolipin	–
4		$CHCl_3$–CH_3OH–CH_3COOH (65:25:4)	Phosphatidylinositol	+ (Fig. 1b)
5		$CHCl_3$–CH_3OH–H_2O (65:25:4)	Phosphatidylinositol	–
6		$CHCl_3$–CH_3OH–7 *N* NH_4OH (50:50:5)	Phosphatidylinositol	–
7	Silica gel HR	$CHCl_3$–CH_3OH–CH_3COOH (75:20:4)	Cardiolipin	+ (Fig. 2a)
8		$CHCl_3$–CH_3OH (75:20) satd. with H_2O	Cardiolipin	+ (Fig. 2b)
9		$CHCl_3$–CH_3OH–7 *N* NH_4OH (65:40:4)	Cardiolipin	–
10		$CHCl_3$–CH_3OH–CH_3COOH (65:25:4)	Phosphatidylinositol	+ (Fig. 2c)
11		$CHCl_3$–CH_3OH–H_2O (65:25:4)	Phosphatidylinositol	+ (Fig. 2d)
12		$CHCl_3$–CH_3OH–7 *N* NH_4OH (50:50:5)	Phosphatidylinositol	–

applied as its monovalent salt and as its divalent salt, both samples displaying the chromatographic characteristics of the divalent salt form of cardiolipin. On the other hand, the tailing which appears at the higher loads for those samples applied as the monovalent salt form most probably arises from incomplete conversion of the applied sodium cardiolipin to calcium cardiolipin at the spot of application. The proportion of cardiolipin which is not immediately converted into the divalent salt form will be delayed because the monovalent salt form of cardiolipin has a lower chromatographic mobility than the divalent salt form. As the sodium cardiolipin migrates to new areas of adsorbent with Ca^{2+} ions available for ion exchange, it will gradually be converted into the fast-moving calcium salt and give rise to the observed streaking. With the largest amount of sodium cardiolipin applied on to the plate (8 μg of P), not all of it is converted into the calcium salt during the chromatography. A minor part of the sodium cardiolipin applied appears as a small dense spot with an R_F value of 0.14. This spot is considered to be sodium cardiolipin, and the R_F value of 0.14 is that

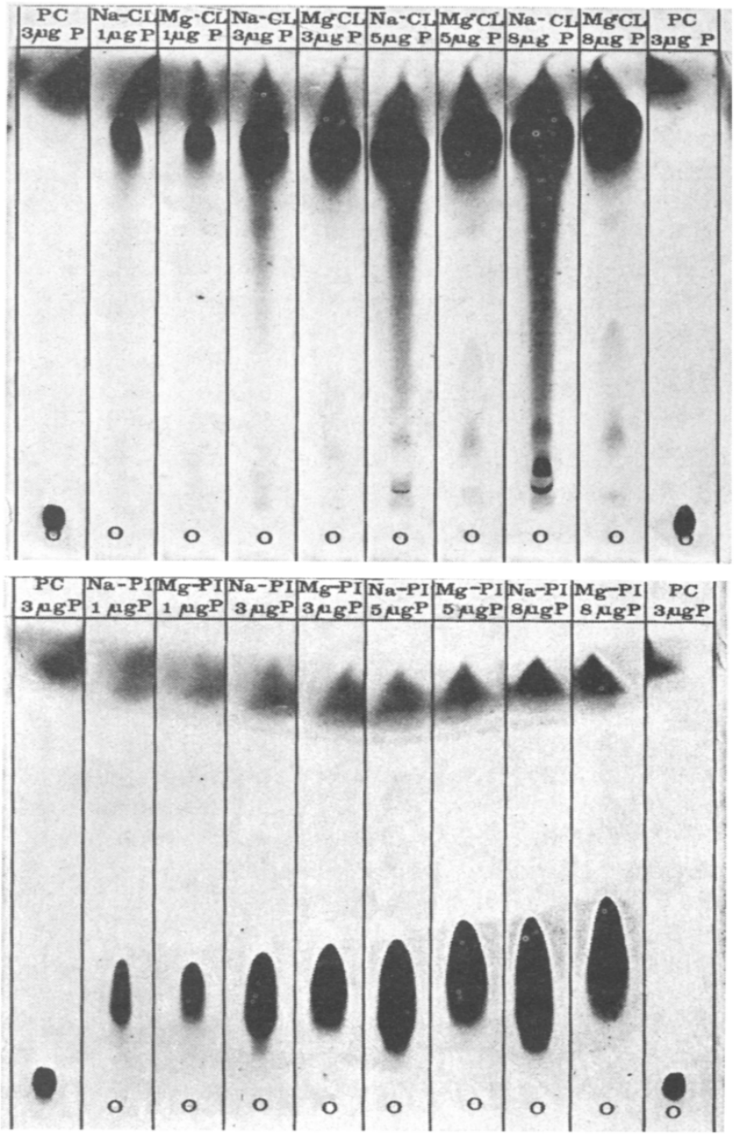


Fig. 1. TLC of the acidic phospholipids cardiolipin (CL) and phosphatidylinositol (PI), and the neutral phospholipid lecithin (PC), on silica gel G Type 60 using as solvents (a) chloroform–methanol–acetic acid (65:25:4) and (b) chloroform–methanol–acetic acid (65:25:4). The compounds were applied in chloroform solution (0.5 µg/µl of P) corresponding to the amounts of P listed on the chromatograms. The acidic phospholipids were applied as their monovalent and divalent salts to establish whether the observed chromatographic mobility depends on the salt form of the applied phospholipid. The chromatograms were rendered visible by charring with sulphuric acid. The compound running in the front on both chromatograms is the antioxidant butylated hydroxytoluene, which had been added to all solvents used for isolation of the phospholipids. O = Origin.

characteristic of sodium cardiolipin in the present system. It is instructive at this point to inspect the corresponding chromatogram obtained with silica gel HR as adsorbent

(chromatogram No. 7, Fig. 2a), because here the effects of shortage of divalent metal ions in the adsorbent are pronounced.

Chromatogram No. 2. No difference in chromatographic mobilities was observed between cardiolipin applied as its monovalent and divalent salts, the chromatographic mobility displayed by the samples being that of the divalent salt form. It is suggested that the water-saturated solvent, at the moment it arrives at the spot of applied sodium cardiolipin, contains sufficient Ca^{2+} ions to complete the conversion of sodium cardiolipin into calcium cardiolipin.

Chromatogram No. 3. No difference in the chromatographic mobilities of cardiolipin applied as its monovalent and divalent salts was observed in this chromatographic system. The applied samples migrated as well defined spots with an R_F value of about 0.5. Such an R_F value makes it less probable that the divalent salt should be the migrating form of cardiolipin. This is seen from a comparison with the mobility of the divalent salt in the less polar solvent of chromatogram No. 1 (Fig. 1a). In that chromatographic system, the divalent salt form has an R_F value of 0.8. It appears reasonable to assume that cardiolipin in the present system migrates in the form of its ammonium salt.

Chromatogram No. 4 (Fig. 1b). It appears that the migration of phosphatidylinositol applied as its sodium salt is slower than when it is applied as its divalent salt. The difference is more pronounced at higher loads. Probably the relative retardation of phosphatidylinositol applied as its sodium salt is due to the lower chromatographic mobility of that salt form. It should be realized, however, that sooner or later the sodium phosphatidylinositol applied will probably be converted into the calcium salt form on the adsorbent. Thus, the observed migration will depend on how rapidly conversion into the calcium salt form occurs and how complete it is. At lower loads, conversion may be complete at the spot of application, and the observed chromatographic mobility will be that of calcium phosphatidylinositol. At higher loads, the conversion of sodium phosphatidylinositol into the calcium salt will be less rapid, and retardation will consequently occur.

Chromatogram No. 5. No difference was observed between the chromatographic mobilities of phosphatidylinositol applied as its monovalent salt and as its divalent salt. This result is similar to that found for cardiolipin in the corresponding experiment (chromatogram No. 2), and the explanation given there probably also applies to the present chromatogram. Thus, the observed chromatographic mobility is that of the divalent salt form of phosphatidylinositol. It is of interest that the chromatographic mobility of phosphatidylinositol depended considerably on the load. At the lowest load, the R_F value was 0.28, with a load of 3 μg of P the R_F value was 0.36 and with loads of 5 and 8 μg of P the R_F values were nearly identical (0.42 and 0.43, respectively). This observation emphasizes the necessity for chromatographing markers and an unknown compound at the same load.

Chromatogram No. 6. Phosphatidylinositol applied as its monovalent and divalent salts had identical chromatographic mobilities in this basic system. This is similar to the findings for cardiolipin in the corresponding system (chromatogram No. 3). As suggested there, the phospholipid probably migrates as its ammonium salt.

The six chromatograms discussed below were obtained using silica gel HR as adsorbent. As this adsorbent contains a limited amount of divalent metal ions relative to the amount of phospholipid applied, one would expect that at least

part of a phospholipid applied as its monovalent salt would display the chromatographic behaviour of that salt form, provided that an acidic or neutral solvent is used for development. This expectation turned out to be satisfactorily fulfilled.

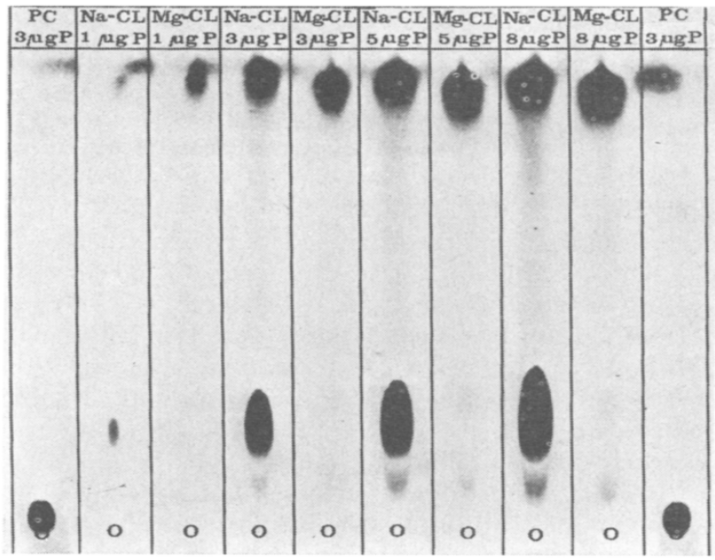
Chromatogram No. 7 (Fig. 2a). It can be seen that the chromatographic behaviours of cardiolipin applied as its monovalent and divalent salts are completely different. Cardiolipin applied as its divalent salt migrates close to the solvent front, while cardiolipin applied as its monovalent salt has an R_F value of about 0.24. Thus, in this system, cardiolipin migrates essentially in the form of the particular salt applied. However, on the lanes on which sodium cardiolipin was applied, a streaking running from the main sodium cardiolipin spot to the front was visible. A similar, although much more pronounced, streaking was observed for cardiolipin on the silica gel G Type 60 adsorbent (chromatogram No. 1, Fig. 1a). Ion-exchange reactions by which sodium cardiolipin is gradually converted into the fast-moving divalent salt form was suggested to account for the occurrence of that streaking. The streaking on the present chromatogram probably arises by a similar mechanism.

Chromatogram No. 8 (Fig. 2b). This chromatogram also shows a striking difference in the chromatographic behaviour of cardiolipin applied as its monovalent and divalent salts. When applied as its divalent salt, cardiolipin migrates as one well defined spot, whereas it migrates as two spots when applied as its monovalent salt. The faster moving spot corresponds to the divalent salt, and the slower moving spot to the monovalent salt. Apparently, part of the sodium cardiolipin has been converted into the divalent salt at the spot of application, and consequently will display the chromatographic behaviour of that salt form. The sodium cardiolipin that remains unconverted will migrate as that salt form, giving rise to the slower moving spot. It will, however, also give rise to the streaking which runs the whole length between the slower moving spot and the faster moving spot. The streaking probably arises by the mechanism already described (see the comments on chromatograms Nos. 1 and 7).

Chromatogram No. 9. Consistent with the results of the previous chromatographic runs using basic solvents (chromatograms Nos. 3 and 6), cardiolipin applied as the monovalent and divalent salts displays the same chromatographic behaviour in the present system because of conversion into the ammonium salt form.

Chromatogram No. 10 (Fig. 2c). It is obvious that the chromatographic mobility of phosphatidylinositol applied as the monovalent salt is lower than that of phosphatidylinositol applied as the divalent salt. A similar result was obtained in the corresponding experiment with cardiolipin (chromatogram No. 7, Fig. 2a). By analogy with the interpretation of that experiment, it is suggested that phosphatidylinositol in the present system migrates in the form of the ionic species subjected to chromatography.

Chromatogram No. 11 (Fig. 2d). By analogy with the corresponding results for cardiolipin (chromatogram No. 8, Fig. 2b), one might expect part of the phosphatidylinositol applied as the monovalent salt to be converted into the divalent salt and to display the chromatographic behaviour of that salt form, while the remainder of the sodium phosphatidylinositol applied will migrate as the sodium salt. The chromatogram obtained actually showed a similar chromatographic behaviour to that of phosphatidylinositol applied as its monovalent and divalent salts (Fig. 2d). This probably means that sodium phosphatidylinositol was converted into the divalent salt form at the spot of application. It is not possible to decide whether the conversion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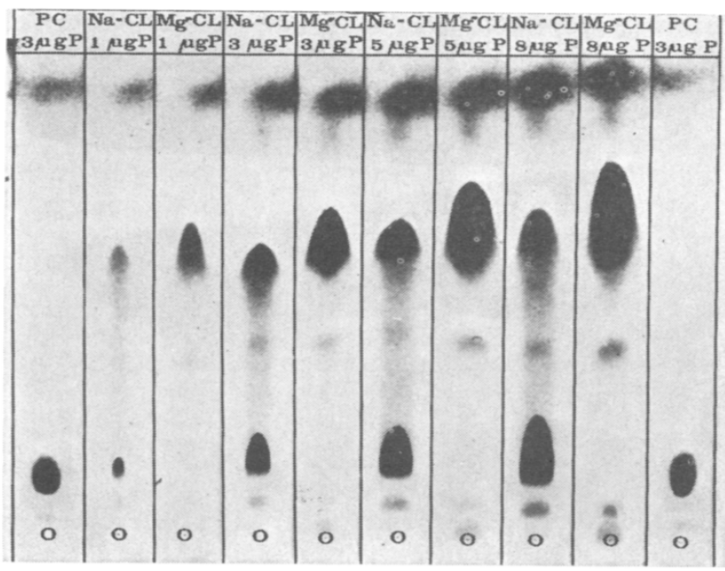
Na-CL 1 µg P
Mg-CL 1 µg P
Na-CL 3 µg P
Mg-CL 3µgP
Na-CL 5 µgP
Mg-CL 5µgP
Na-CL 8µgP
Mg-CL 8 µgP
PC 3µgP

(a)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Mg-CL 1 µgP
Na-CL 3 µgP
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Mg-CL 8µg P
PC 3µg P

(b)

fig. 2.

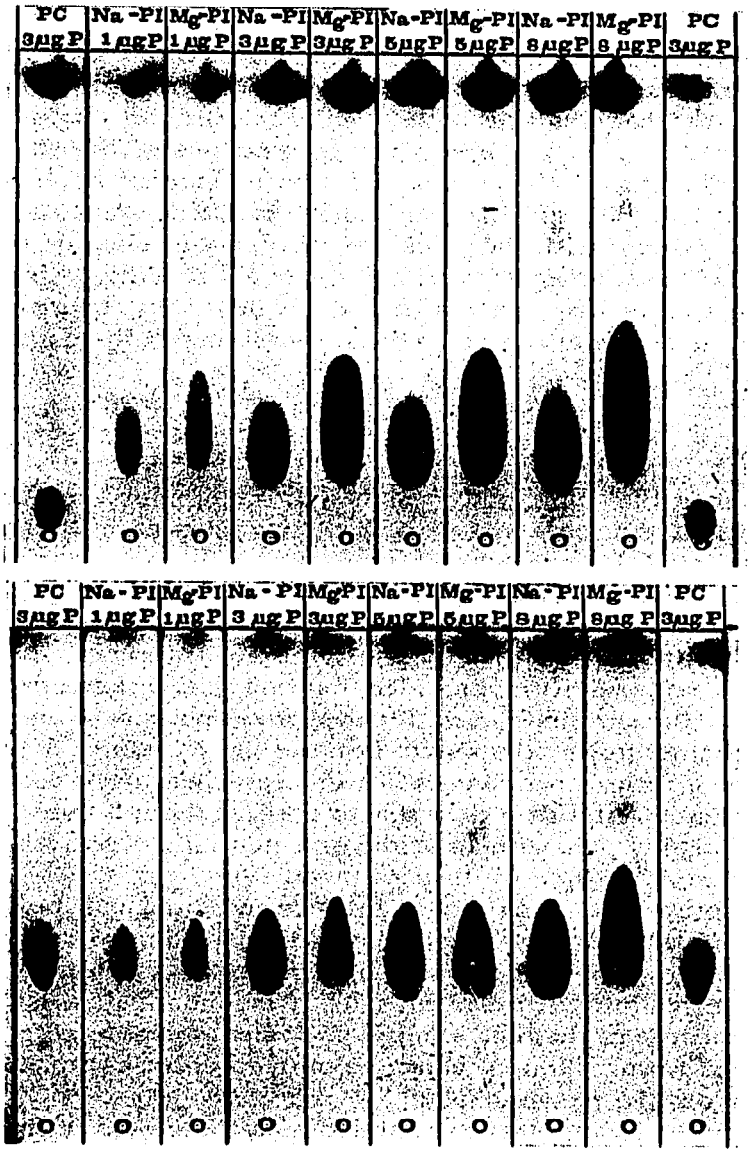

Fig. 2. TLC of the acidic phospholipids cardiolipin (CL) and phosphatidylinositol (PI), and the neutral phospholipid lecithin (PC), on silica gel HR using as solvents (a) chloroform–methanol–acetic acid (75:20:4); (b) chloroform–methanol (75:20) saturated with water; (c) chloroform–methanol–acetic acid (65:25:4); (d) chloroform–methanol–water (65:25:4). The compounds were applied in chloroform solution (0.5 μg/μl of P) corresponding to the amounts of P listed on the chromatograms. The acidic phospholipids were applied as their monovalent and divalent salts to establish whether the observed chromatographic mobility depends on the salt form of the applied phospholipid. The chromatograms were rendered visible by charring with sulphuric acid. The compound running in the front on each chromatogram is the antioxidant butylated hydroxytoluene, which had been added to all solvents used for isolation of the phospholipids. O = Origin.

was complete or only partial, because the monovalent and divalent salt of phosphatidylinositol may have nearly equal mobilities in the present chromatographic system. A close inspection of the chromatogram reveals that the spots corresponding to phosphatidylinositol applied as the sodium salt have a more dense and slightly slower moving lower half than the spots corresponding to phosphatidylinositol applied as the magnesium salt. This may indicate that phosphatidylinositol applied as the sodium salt migrates partly as that salt form and partly as the divalent salt.

Chromatogram No. 12. Consistent with the results of the previous chromatographic runs using basic solvents, no difference in chromatographic mobility exists between phosphatidylinositol applied as the monovalent and divalent salts, because in both instances phosphatidylinositol is converted into the ammonium salt form and migrates as that salt.

CONCLUSIONS

The present work has shown that misleading results may be obtained in the TLC of acidic phospholipids when the ionic states of the phospholipids and the adsorbent are not properly appreciated. The precautions to be taken will depend on the problem under investigation and must be considered in each individual instance. Thus, for example, for the purpose of identification of the components of a lipid mixture by TLC, it is essential to apply the authentic phospholipids (markers) and the unknown phospholipids as the same salt form, provided that an acidic or a neutral solvent is used for development of the chromatogram. Furthermore, markers should be applied at the same load as the unknown compound, because the R_F values may depend on the load. A divalent salt form is necessary when the adsorbent itself contains divalent metal ions (which many do). If it is desirable to chromatograph monovalent salt forms of the acidic phospholipids with an acidic or a neutral solvent, it is essential that the adsorbent is completely free of divalent metal ions.

The acidic phospholipids present in an extract of total animal lipid will be a mixture of monovalent and divalent salts[5]. By washing the lipid (dissolved in an organic solvent) with an aqueous solution of a divalent salt (*e.g.*, magnesium chloride), the acidic phospholipids are converted into their divalent salts. If the monovalent salt form is desired, it is necessary, because of the high affinity of divalent metal ions for acidic phospholipids, to use some form of ion-exchange column to effect complete removal of divalent metal ions from the phospholipids[4,6].

Solvents that contain ammonia in amounts comparable with those used in the present work cause the acidic phospholipids to be chromatographed as their ammonium salts, regardless of the ionic states of the applied lipid and the adsorbents. Thus, the use of a basic solvent system seems to be the easiest way to avoid those errors in the TLC of acidic phospholipids which are caused by a phospholipid migrating partly as its monovalent and partly as its divalent salt.

The fact that the results obtained for cardiolipin and phosphatidylinositol are analogous makes it likely that they apply to acidic phospholipids in general. In this connection, it may be mentioned that both phosphatidic acid and phosphatidylserine (acidic phospholipids) have previously been observed to migrate as two spots on silica gel thin-layers (unpublished results). At that time, the observation was not understood, but it now appears that it may be interpreted in terms of the effects of ion exchange

and different chromatographic mobilities of the monovalent and divalent salt forms of the phospholipids.

Finally, as TLC is used for the fractionation of a variety of other acidic compounds, the possibility should be kept in mind that similar ion-exchange effects may influence the chromatography of those compounds.

ACKNOWLEDGEMENT

The skilful assistance of Miss Bente Vilsen is gratefully acknowledged.

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