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SILICA GEL COLUMN CHROMATOGRAPHY OF ACIDIC PHOSPHOLIPIDS

EFFECTS OF METAL IONS OF ADSORBENT AND PHOSPHOLIPID ON THE ELUTION PROFILES*

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SUMMARY

The metal ion (Na, K, Ca, Mg, Fe and Cu) content of different silica adsorbents and filter aids has been measured and varying amounts were found in the different materials. The extent to which the presence of metal ions in three commonly used silica adsorbents influences the chromatography of acidic phospholipids was studied for cardiolipin and phosphatidylinositol. When a monovalent salt form of the phospholipid is subjected to chromatography, divalent metal ions of an adsorbent may replace the monovalent metal ion and convert the phospholipid into the divalent salt form. As that salt form is more readily eluted than the monovalent salt form, the elution profiles will be influenced by this ion exchange and consequently depend on the amount of divalent metal ions present in an adsorbent.

INTRODUCTION

Although silica gel column chromatography (CC) has been used for about 20 years for the fractionation of lipids into classes, the resolution of phospholipids is still incomplete. It appears that the acidic phospholipids undergo unsatisfactory chromatographic fractionation compared with the neutral phospholipids. The elution of acidic phospholipids often occurs over a broad interval of the elution scheme, overlapping the neutral phospholipids. Furthermore, it seems as if their elution is subject to uncontrolled variability, as conflicting data occasionally appear concerning their elution.

Recently, two papers have appeared reporting on the silica gel column chromatography of acidic phospholipids[1,2]. In one paper[1], the chromatographic behaviour on Mallinckrodt silica gel of different salt forms of acidic phospholipids was investigated. It was found that divalent salt forms (Ca, Mg) are eluted more readily from a

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silica gel column than monovalent salt forms (Na, K). The second paper[2] reported on the ion-exchange reactions that take place when cardiolipin and phosphatidylinositol are chromatographed on Merck silica gel. Cations of the phospholipids were shown to be exchanged for metal ions present in the silica adsorbent. It is logical to assume from these papers that differences in the metal ion contents of the different silica adsorbents may give rise to different elution profiles for an acidic phospholipid on those adsorbents.

The aim of the present study was to assess for two representative acidic phospholipids (cardiolipin and phosphatidylinositol) to what extent differences in the metal ion content of silica adsorbents actually do account for differences in elution profiles on these adsorbents.

For this purpose, the monovalent and divalent salts of the phospholipids were chromatographed on three commonly used adsorbents. The elution profiles were determined on the adsorbents without pre-treatment and extracted with hydrochloric acid before use. In addition, the ion exchange that occurred during the chromatography was determined. Comparison of the results obtained on the three adsorbents enables conclusions to be drawn concerning the influence of metal ions of the adsorbents on the elution of acidic phospholipids.

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical-reagent grade. The organic solvents were deoxygenated under reduced pressure from a water pump, followed by return to atmospheric pressure with pure nitrogen. The antioxidant butylated hydroxytoluene (2,6-di-*tert.*-butyl-*p*-cresol, Sigma, St. Louis, Mo., U.S.A.) was added to a concentration of 50 mg/l. Chromatographically pure reference lipids were obtained from Supelco, Bellefonte, Pa., U.S.A. (phosphatidylinositol) and Koch-Light, Colnbrook, Great Britain (cardiolipin *ex* bovine heart, sodium salt).

Preparation of cardiolipin

The preparation of cardiolipin by silica gel CC of either total liver lipid or total heart lipid, as well as examination of purity by thin-layer chromatography (TLC), was carried out as previously described[3]. Rat liver cardiolipin was used in the experiments in which the sodium salt form was subjected to chromatography, and ox heart cardiolipin was used in the experiments with the divalent salt form of cardiolipin.

Preparation of phosphatidylinositol

Phosphatidylinositol was prepared from ox liver, which was obtained fresh from the local slaughter-house and kept on ice until used within a few hours. After homogenization in a Waring blender, the tissue was partially dried by treating it with acetone for about 2 h at 0° with occasional stirring. About 2.5 l of acetone were used per kilogram of liver. The acetone was then squeezed from the tissue through a four-layered gauze, and the dried tissue was repeatedly extracted with chloroform–methanol (2:1). In a typical preparation, approximately 2 l of solvent per kilogram of liver were used in each of the first two extractions, and approximately 1 l of solvent per kilogram of liver in the third extraction. The crude lipid obtained in first, second and third extractions corresponded to 890, 240 and 40 mg of P, respectively, per kilogram of liver. The combined extracts were concentrated to about one tenth of volume under reduced pressure at 30° and dried overnight with sodium sulphate. The sodium sulphate was then removed by filtration and washed with about 150 ml of chloroform to ensure recovery of the lipid. Ethanol (99.9 %) in an amount of about 5 l per kilogram of liver was then added to the filtrate with stirring, followed by 70 ml of 0.1 *M* calcium chloride solution per kilogram of liver. Finally, the pH was adjusted to 8.5 with 4 *N* sodium hydroxide solution and the solution left overnight at 2° to complete precipitation. The precipitate was collected by centrifugation for 15 min at 600 *g*. Although precipitation occurs as soon as ethanol is added, it is important to add the calcium chloride, as its omission may cause only a minor fraction of the acidic phospholipids to be precipitated. The collected precipitate, which corresponded to about 175 mg of P per kilogram of liver, was dried overnight *in vacuo* in a nitrogen atmosphere over concentrated sulphuric acid. The composition of the precipitated phospholipids, as found by analysis of P after resolution by TLC, was: phosphatidylinositol, 37 %; phosphatidylethanolamine, 28 %; phosphatidylserine, 14 %; cardiolipin, 12 %; phosphatidylcholine, 8 %. The precipitated phospholipids were then converted mainly into the sodium salt form by washing with Na_2-EDTA in a two-phase system similar to that described by Brockerhoff[4] for conversion of acidic phospholipids into their triethylammonium salts, except that the initial conversion to the calcium salt form was omitted, and Na_2-EDTA was substituted for triethylammonium-EDTA. This washing with Na_2-EDTA is necessary to obtain phosphatidylinositol in a pure state by CC to be carried out later in the procedure. The dry lipid was subsequently treated with 1-fluoro-2,4-dinitrobenzene by the method of Wheeldon and Collins[5] as modified by Brown and Clark[6]. CC fractionation of the dinitrophenylated lipid was carried out on a column made from a mixture of 50 g of activated Mallinckrodt (St. Louis, Mo., U.S.A.) silica gel and 25 g of activated Celite (Koch-Light's 7470h Celite, A.R.). The silica gel and the filter aid were slurried into chloroform, degassed by suction from a water pump and poured into a chromatographic tube 2.7 cm in diameter. The lipid was applied as a 25-ml chloroform solution containing 2–3 mg/ml of P. Elution was carried out with 1000 ml of chloroform–methanol (8:2) followed by chloroform–methanol (6:4), and the eluate was monitored by TLC. Fractions containing phosphatidylinositol as the sole or major component were pooled and re-chromatographed if necessary.

Conversion of the acidic phospholipids into either the sodium or calcium salt form

The sodium salt form of the phospholipids was obtained by washing repeatedly with Na_2-EDTA in a two-phase system as previously described[3], except that phosphatidylinositol was only treated twice with Na_2-EDTA .The magnesium salt form of the phospholipids was prepared as described by Shimojo *et al.*[1].

Conditions for the chromatographic studies

The elution profiles of phospholipids may be influenced by several factors other than the metal ion content of the adsorbent. Thus, the load of phospholipids per gram of adsorbent, dimensions of the column, volumes of eluting solvent, degree of inactivation of adsorbent, flow-rate and temperature may influence the profile for given phospholipids. Therefore, it is important that these parameters are fixed for the chromatographic experiments to be compared, if definite conclusions are to be drawn concerning the effect of metal ions contained in the adsorbents. Conditions for the chromatographic experiments were as follows. In order to prepare a column, 5 g of adsorbent, activated overnight at 105°, were slurried into chloroform, degassed by suction from a water pump and poured into a chromatographic tube 1.1 cm in diameter provided with a plug of glass-wool as support for the adsorbent. The adsorbent was allowed to settle, and 10 ml of chloroform were passed through the column at 2°. The lipid sample, corresponding to 1.25 mg of P, was applied as a chloroform solution at a concentration of 1 mg/ml of P, giving a load of 0.25 mg of P per gram of silica gel. Discontinuous gradient elution was carried out at 2° with chloroform–methanol mixtures of increasing polarity using 50 ml of each of the following solvents: chloroform; chloroform–methanol (9:1); chloroform–methanol (8:2); chloroform–methanol (7:3); chloroform–methanol (6:4); chloroform–methanol (5:5); chloroform–methanol (4:6). The flow-rate was maintained at 0.5 ± 0.07 ml/min by hydrostatic pressure, and fractions of 10 ml were collected.

Gel filtration of various lipid preparations

As chloroform–methanol mixtures themselves elute inorganic material from a silica gel column, it is necessary to free the lipid obtained by CC from these contaminants before the metal ion composition is determined. Gel filtration in the version described by Wuthier[7] was used to remove inorganic contaminants from lipid preparations.

Extraction of metal ions from silica adsorbents and filter aids

The contents of metal ions of various silica adsorbents and filter aids listed in Table I refer to the amounts extracted by four successive extractions with 0.1 *N* hydrochloric acid. A 6-g amount of silica gel or 3 g of filter aid were shaken with 30 ml of 0.1 *N* hydrochloric acid for 15 min at room temperature. The suspension was then centrifuged for 15 min at 600 *g* and the supernatant filtered through a Whatman No. 1 filter which had been washed with ten 5-ml portions of 0.1 *N* hydrochloric acid.

The removal of metal ions from the three silica adsorbents to be used for chromatographic experiments was carried out by four successive extractions with 1 *N* hydrochloric acid (60 g of adsorbent being stirred mechanically with 600 ml of 1 *N* hydrochloric acid for 20 min at room temperature followed by centrifugation for 15 min at 600 *g* and discarding of the supernatant). The adsorbents were then washed nine times with deionized water. The pH remained constant in the last three washings, and chloride ions were not detectable.

Analytical methods

Phosphorus determination was performed according to Bartlett[8]. Sodium, potassium, calcium, magnesium, iron and copper were determined by atomic-absorption spectrophotometry with a Pye-Unicam SP 90A atomic-absorption spectrophotometer. Before analysis of the metal ions of a lipid preparation, the lipid was ashed with a mixture of concentrated nitric acid and 70% perchloric acid[9]. The ash was acidified with 5 μl of concentrated hydrochloric acid and dissolved in water. The concentrations of the metal ions and phosphorus were then determined.

RESULTS AND DISCUSSION

Determination of sodium, potassium, calcium, magnesium, iron and copper present in some silica adsorbents and filter aids

In Table I are given the amounts of metal ions that were extracted by four successive extractions with 0.1 *N* hydrochloric acid from commonly used silica adsorbents and from different filter aids. As the amount of metal ion extracted in the fourth extraction amounted to about only 1% of the total amount extracted in the first three extractions, the amounts listed state closely the contents of metal ions that can be extracted with 0.1 *N* hydrochloric acid. Different amounts of metal ions are

TABLE I

METAL ION CONTENTS OF DIFFERENT SILICA ADSORBENTS AND FILTER AIDS EXPRESSED AS MICROMOLES OF METAL PER GRAM OF NON-ACTIVATED MATERIAL

Material	*Na*	*K*	*Ca*	*Mg*	*Fe*	*Cu*	*Total metal ions*
Merck silica gel (0.05–0.2 mm, für die Säulen-Chromatographie, catalogue No. 7734, control No. 9733760)*	41.5	1.04	18.1	3.86	0.239	ND*** (<0.003)	64.7
Bio-Rad silicic acid (Bio-Sil HA, −325 mesh, catalogue No. 62380**, control No. 9413)*	6.62	0.195	0.74	0.574	0.054	<0.03	8.2
Mallinckrodt silicic acid (100 mesh, A.R., marked "Suitable for chromatographic analysis by the method of Ramsey and Patterson")*	1.02	0.069	0.061	0.041	0.055	ND*** (<0.003)	1.25
Merck silica gel (0.05–0.2 mm, reinst für die Säulen-Chromatographie, catalogue No. 7754, control No. 9748253)	7.90	0.089	1.14	0.439	ND*** (<0.03)	ND*** (<0.003)	9.6
Bio-Rad silicic acid (Bio-Sil A, 100–200 mesh, catalogue No. 62310, control No. 6537)	0.80	0.039	0.053	0.035	0.029	<0.005	0.96
Filter aid (Koch-Light, Celite, A.R., catalogue No. 7470h)	0.63	0.303	0.76	0.50	0.132	ND*** (<0.005)	2.32
Filter aid (Celite 545, Johns-Manville, Celite Division, Denver, Colo., U.S.A.)	12.7	0.95	9.4	1.53	1.08	<0.01	25.6
Filter aid (Kieselguhr Hyflo Supercel, Johns-Manville, Celite Division)	21.4	1.49	9.6	2.33	1.39	<0.02	36.2

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* Adsorbents used in the investigation of the importance in silica gel CC of metal ions of adsorbents and phospholipid.

** Bio-Sil HA of this catalogue number is no longer marketed. At present, Bio-Sil HA, catalogue No. 1311180 is marketed. This adsorbent (control No. 10508) appears to be practically free from metal ions (Na, Ca and Mg were measured).

*** ND = not detected.

present in the various silica adsorbents. As for the three silica products (marked with an asterisk in Table I) whose properties as adsorbents for the chromatography of acidic phospholipids have been studied in the present work, it appears that the E. Merck (Darmstadt, G.F.R.) product has by far the highest contents, some eight times the amount present in the Bio-Rad Labs. (Richmond, Calif., U.S.A.) product. On the other hand, the Mallinckrodt silica gel is almost free from metal ions. The quantitatively important metal ions are sodium, calcium and magnesium. It is noteworthy that very different amounts of metal ions may be present in different silica adsorbents obtained from the same manufacturer. For example, the metal ion content of Merck silica gel, catalogue No. 7734, contains seven times as much metal ions as Merck silica gel, catalogue No. 7754. An equally high relative difference in the metal ion content exists between the two different silica adsorbents from Bio-Rad (catalogue Nos. 62380 and 62310). The metal ion contents of the filter aids likewise turned out to be very different. The amounts of divalent metal ions present are of such magnitudes that even for the filter aid with the lowest content (Koch-Light Celite, catalogue No. 7470h), an effect of the divalent metal ions in a filter aid on the elution of acidic phospholipids through ion exchange seems possible. Furthermore, a considerable amount of iron is present in two of the filter aids. As this metal is known to catalyze the autoxidation of unsaturated lipids, its presence in the filter aids is still another reason to avoid their use in the column chromatography of lipids.

Silica gel column chromatography of cardiolipin applied on to columns as its sodium salt form

Fig. 1 shows the elution profiles for cardiolipin on (a) Merck silica gel, catalogue No. 7734; (b) Bio-Rad silica gel, catalogue No. 62380; (c) Mallinckrodt silica gel; and on these adsorbents extracted with hydrochloric acid before use (d, e and f, respectively). It is obvious that distinct differences exist between the elution profiles on the three adsorbents when used without extraction with hydrochloric acid (a, b, c). From the Merck silica gel the major part of the cardiolipin is eluted with chloroform–methanol (9:1), while from the Bio-Rad silica gel this elution occurs with the more polar solvent chloroform–methanol (8:2), and from the Mallinckrodt silica gel with the still more polar solvent chloroform–methanol (7:3). However, when the adsorbents have been extracted with hydrochloric acid in order to remove metal ions before use, the elution profiles (d, e and f) become similar to the profile obtained on non-extracted Mallinckrodt silica gel (c), except that elution from the Merck silica gel is slightly retarded. Thus, it appears that the metal ions contained in the Merck and the Bio-Rad silica gel cause cardiolipin to be eluted early from those adsorbents. The analyses of the metal ion composition of the eluted cardiolipin (Table II) confirm this view. At top of the table is listed the metal ion composition of cardiolipin applied on to the columns, then follows the metal ion composition of cardiolipin eluted from the columns. In analyzing the eluted cardiolipin, it appeared desirable to analyze separately the earlier and the later eluted part, as this might give information about the reason for the delayed elution of part of the phospholipid. In addition to these data, the cation compositions of the earlier and the later eluted portions of cardiolipin taken together have been calculated for each chromatography and are listed in Table II. By comparison with the metal ion composition of the applied cardiolipin, this gives the overall ion exchange.

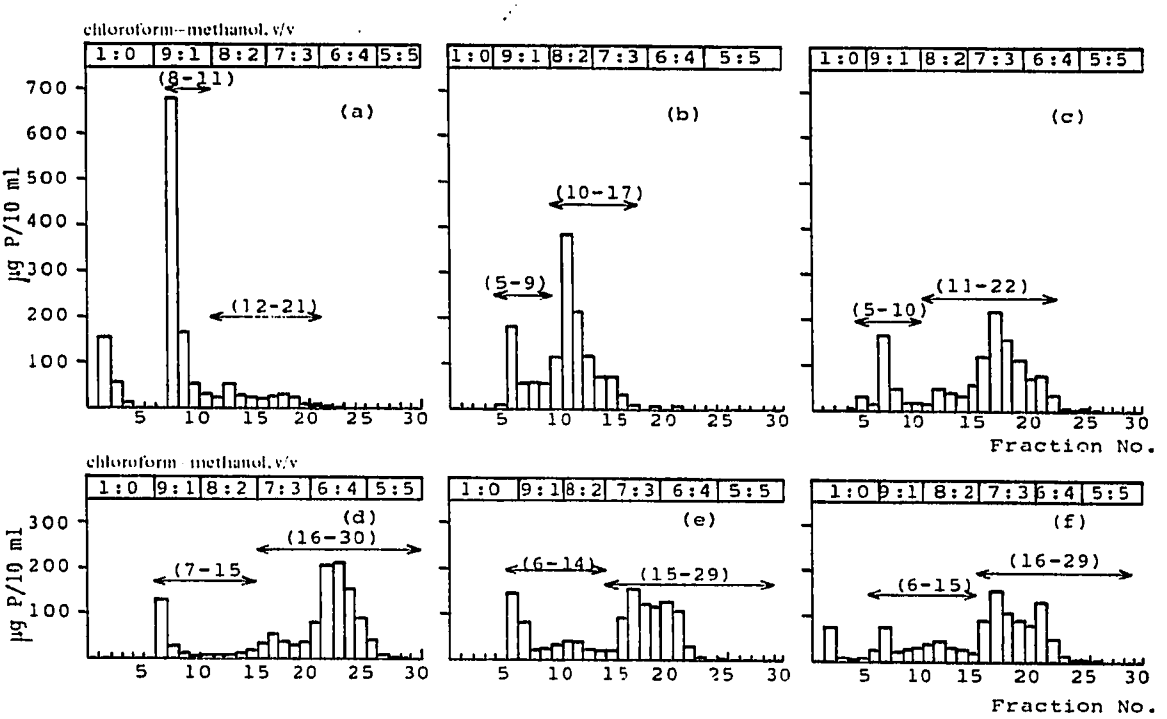

Fig. 1. Silica gel CC of cardiolipin (applied mainly as sodium salt form) on (a) Merck silica gel, catalogue No. 7734; (b) Bio-Rad silicic acid, catalogue No. 62380; (c) Mallinckrodt silicic acid; and on these adsorbents extracted with HCl before use (d, e and f, respectively).

Cardiolipin subjected to chromatography has a Na:P equivalence ratio of 0.91 and thus is mainly the sodium salt form. Only traces of magnesium, calcium and potassium were present. As the total metal ion:P equivalence ratio is 0.94, some 6% of the cardiolipin appears to be in the free acid form. Chromatography of cardiolipin on the Merck and Bio-Rad silica gel caused drastic changes in its cation composition. For Merck silica gel, the earlier eluted part, pool (8–11), had a metal ion composition of Na:P = 0.21, Ca:P = 0.90 and Mg:P = 0.158. The later eluted portion, pool (12–21), had the composition Na:P = 0.043, Ca:P = 0.57 and Mg:P = 0.283. These data show that sodium ions initially bound to cardiolipin are exchanged for calcium and magnesium ions of the adsorbent during chromatography. By this exchange, the sodium form of cardiolipin applied on to the column is converted mainly into the calcium–magnesium form.

The corresponding data for cardiolipin eluted from the Bio-Rad silica gel show that the earlier eluted portion of cardiolipin, pool (5–9), likewise contains a considerable amount of divalent cations (calcium and magnesium). However, the relative amounts of calcium and magnesium are different from those found for cardiolipin eluted readily from Merck silica gel. Another, more striking difference, is that with Bio-Rad silica gel, the earlier eluted portion constitutes only a minor part of the total, while with Merck silica gel it constitutes the major part. The later eluted portion, pool (10–17), has a cation composition essentially equal to the initial cation composi-

TABLE II

CATION EXCHANGE BETWEEN CARDIOLIPIN, APPLIED MAINLY AS THE SODIUM SALT, AND DIFFERENT SILICA ADSORBENTS DURING CC

The metal ion content of cardiolipin subjected to chromatography is listed at the top of the table. Then follows the metal ion content of cardiolipin eluted from the adsorbents. Comparison of the initial metal ion content of cardiolipin with its content after chromatography provides information about the cation exchange occurring during chromatography. In calculating the equivalence ratio (equiv. metal cation: equiv. P), 1 atom of P is considered as 1 equivalent, while 1 equivalent of metal ion equals 1 singly charged ion.

Adsorbent	*Pool*	*Equiv. metal cation:equiv. P*				
		Na	*K*	*Ca*	*Mg*	*Total metal ions:P*
Cardiolipin applied on to column		0.91	0.003	0.009	0.014	0.94
Merck silica gel (0.05–0.2 mm, für die Säulen-Chromatographie, catalogue No. 7734)	(8–11)	0.21	0.002	0.90	0.158	1.27
	(12–21)	0.043	0.001	0.57	0.283	0.90
	(8–11)+(12–21)*	0.176	0.002	0.84	0.183	1.20
Bio-Rad silicic acid (Bio-Sil HA, −325 mesh, catalogue No. 62380)	(5–9)	0.57	0.000	0.51	0.40	1.48
	(10–17)	0.95	0.001	0.012	0.017	0.98
	(5–9)+(10–17)*	0.85	0.001	0.14	0.118	1.11
Mallinckrodt silicic acid (100 mesh, A.R., marked "Suitable for chromatographic analysis by the method of Ramsey and Patterson")	(5–10)	0.81	0.005	0.109	0.119	1.04
	(11–22)	0.72	0.003	0.000	0.004	0.73
	(5–10)+(11–22)*	0.75	0.003	0.03	0.03	0.81
HCl-extracted Merck silica gel (0.05–0.2 mm, für die Säulen-Chromatographie, catalogue No. 7734)	(7–15)	0.83	0.002	0.217	0.046	1.10
	(16–30)	0.64	0.001	0.049	0.029	0.72
	(7–15)+(16–30)*	0.68	0.001	0.084	0.033	0.80
HCl-extracted Bio-Rad silicic acid (Bio-Sil HA, −325 mesh, catalogue No. 62380)	(6–14)	0.73	0.002	0.028	0.027	0.79
	(15–29)	0.83	0.002	0.001	0.000	0.83
	(6–14)+(15–29)*	0.80	0.002	0.011	0.010	0.82
HCl-extracted Mallinckrodt silicic acid (100 mesh, A.R., marked "Suitable for chromatographic analysis by the method of Ramsey and Patterson")	(6–15)	0.83	0.001	0.021	0.023	0.88
	(16–29)	0.86	0.002	0.002	0.001	0.87
	(6–15)+(16–29)*	0.85	0.002	0.008	0.008	0.87

* Calculated.

tion of cardiolipin, showing that on the Bio-Rad silica gel only the earlier eluted portion has been subjected to ion exchange during chromatography.

As for the data obtained with Mallinckrodt silica gel, the earlier eluted cardiolipin, pool (5–10), has a Na:P equivalence ratio of 0.81 and thus is mainly the sodium salt form. However, it also contains significant amounts of divalent metal ions (Ca:P = 0.109 and Mg:P = 0.119). The later eluted cardiolipin, pool (11–22), contains practically only sodium. The Na:P equivalence ratio of 0.72 indicates that 28% of this pool is in the free acid form. Thus, cation exchange has caused a limited amount of monovalent metal ions of cardiolipin to be exchanged for divalent metal ions of the adsorbent and, more significant, for acidic protons of the silica adsorbent. The overall cation exchange on Mallinckrodt silica is, however, relatively small.

The lower half of Table II gives the cation composition of cardiolipin eluted from the adsorbents extracted with hydrochloric acid before use. It is obvious that the extraction has greatly decreased the cation exchange, so that the cation composition of the eluted cardiolipin resembles that of the cardiolipin applied on to the columns. However, a slightly lowered metal ion:P equivalence ratio for most of the eluted cardiolipin fractions indicates that a small exchange of metal ions of cardiolipin for H^+ ions of the adsorbent has occurred.

It appears from the above results that divalent metal ions of an adsorbent influence the elution profile for cardiolipin subjected to chromatography as its monovalent salt. The divalent metal ions cause cardiolipin to be more readily eluted by converting it into the divalent salt form, which has a higher chromatographic mobility than the monovalent salt form. Accordingly, the higher the content of divalent metal ions in an adsorbent, the greater will be the proportion of the applied sodium cardiolipin readily eluted (*cf.*, the elution profiles obtained on Merck and Mallinckrodt silica gel not extracted with hydrochloric acid, *i.e.*, Fig. 1a and c). It also appears from the results that cation exchange is low on adsorbents freed from metal ions.

A more detailed inspection of the results raises some questions. For example, why is pool (12–21) eluted later than pool (8–11) from Merck silica gel not extracted with hydrochloric acid, both pools being principally the divalent salt (calcium–magnesium) of cardiolipin? The reason probably is that not all of the applied sodium cardiolipin is converted into the divalent salt form at the place of application (because of the local shortage of divalent metal ions in the adsorbent). The proportion of cardiolipin which is instantaneously converted into the calcium–magnesium salt will be readily eluted, while the remaining part will be eluted later because it is adsorbed as the sodium salt form, which has a relatively low chromatographic mobility. That portion of cardiolipin will, however, also be eluted as its calcium–magnesium salt, because as soon as it leaves the place of application, it comes into contact with new areas of adsorbent containing divalent metal ions available for ion exchange. The delayed elution of part of the applied sodium cardiolipin is analogous to the occurrence of streaking on thin-layer chromatograms of acidic phospholipids reported in the following paper[10]. Also, it might be asked why pool (10–17), being the sodium salt form of cardiolipin, is eluted with chloroform–methanol (8:2) and chloroform–methanol (7:3) from the Bio-Rad silica gel not extracted with hydrochloric acid (Fig. 1b and Table II) instead of with chloroform–methanol (7:3) and chloroform–methanol (6:4), which are the solvents that elute the main part of sodium cardiolipin from adsorbents free of metal ions (Fig. 1c, d, e and f). At present, it can only be said that the metal ions of the adsorbent are the cause, because when they are removed from the adsorbent, elution of sodium cardiolipin occurs at a late position with chloroform–methanol (7:3) and chloroform–methanol (6:4) (Fig. 1e). The mechanism of this effect is not clear.

Silica gel column chromatography of cardiolipin applied on to columns as its magnesium salt form

The results obtained in these studies were simple and can be given without presenting the elution profiles and the detailed metal ion:P equivalence ratios. All of the elution profiles were identical, and elution occurred very sharply and virtually completely with chloroform–methanol (9:1). Thus, it can be concluded that under the conditions used the metal ions of an adsorbent do not influence the elution profile of cardiolipin subjected to chromatography as its magnesium salt. The analysis of metal ion:P equivalence ratios showed that only on Merck and Bio-Rad silica gel and Merck silica gel extracted with hydrochloric acid ion exchange did occur. In each instance, exchange of Mg^{2+} of the applied magnesium cardiolipin (Mg:P equivalence ratio = 0.96) for Ca^{2+} of the adsorbent was the only type of ion exchange observed. It occurred to the greatest extent on Merck silica gel, the Mg:P and Ca:P equivalence ratios being 0.56 and 0.47, respectively, for the eluted cardiolipin. For cardiolipin eluted from Merck silica gel extracted with hydrochloric acid, the Mg:P and Ca:P equivalence ratios were 0.82 and 0.117, respectively. The extent of ion exchange was even smaller on the Bio-Rad silica gel adsorbent, the Mg:P and Ca:P equivalence ratios of the eluted cardiolipin being 0.88 and 0.05, respectively.

The corresponding studies with the monovalent and divalent salt forms of phosphatidylinositol revealed that similar ion-exchange reactions occurred with this phospholipid on adsorbents containing divalent metal ions. However, the effects of these reactions on the elution profiles were difficult to establish unequivocally, because other phenomena also influenced the elution profiles. The same effects of cation exchange on the elution profiles nevertheless seem to be operative in the chromatography of this phospholipid also.

Silica gel column chromatography of phosphatidylinositol applied on to columns mainly as its sodium salt form

Fig. 2 shows the elution profiles obtained when phosphatidylinositol, applied mainly as the sodium salt form, was chromatographed on (a) Merck silica gel, (b) Bio-Rad silica gel, (c) Mallinckrodt silica gel and on these adsorbents extracted with hydrochloric acid (d, e and f, respectively). Elution from the adsorbents used without extraction with hydrochloric acid occurred most readily from the Bio-Rad silica gel. From the Mallinckrodt silica adsorbent (c), elution occurred over a broad range of solvent compositions, beginning with chloroform–methanol (9:1) and ending with chloroform–methanol (6:4). Also, the elution from the Merck silica gel, which occurs with chloroform–methanol (7:3) and chloroform–methanol (6:4), took place relatively late. The elution profiles obtained on the adsorbents extracted with hydrochloric acid were, with the exception of the anomalous elution from Merck silica gel extracted with hydrochloric acid (Fig. 2d, solid line), identical with that obtained on Mallinckrodt silica gel not extracted with hydrochloric acid. The immediate elution with chloroform of phosphatidylinositol from Merck silica gel extracted with hydrochloric acid is noteworthy, because adsorption of a lipid sample on silica gel and elution with chloroform is a widely used method for the removal of neutral lipids. Probably, phosphatidylinositol was not adsorbed at all, because the micelles, which is assumed to be the form of sodium phosphatidylinositol in chloroform, were not broken down on the silica surfaces. The arrangement of sodium phosphatidylinositol molecules into micelles in chloroform presumably is such that the polar head groups are buried in the interior of the micelle, and the hydrocarbon chains are directed towards the outside. A structure of this type certainly would not be adsorbed on to silica gel from chloroform. However, when the sample was applied at room temperature as a 2.5-ml chloroform–methanol (7:3) solution, and chromatography performed at room temperature, no phosphatidylinositol was eluted with chloroform, and the elution profile obtained (Fig. 2d, broken line) resembled those obtained on the other adsorbents

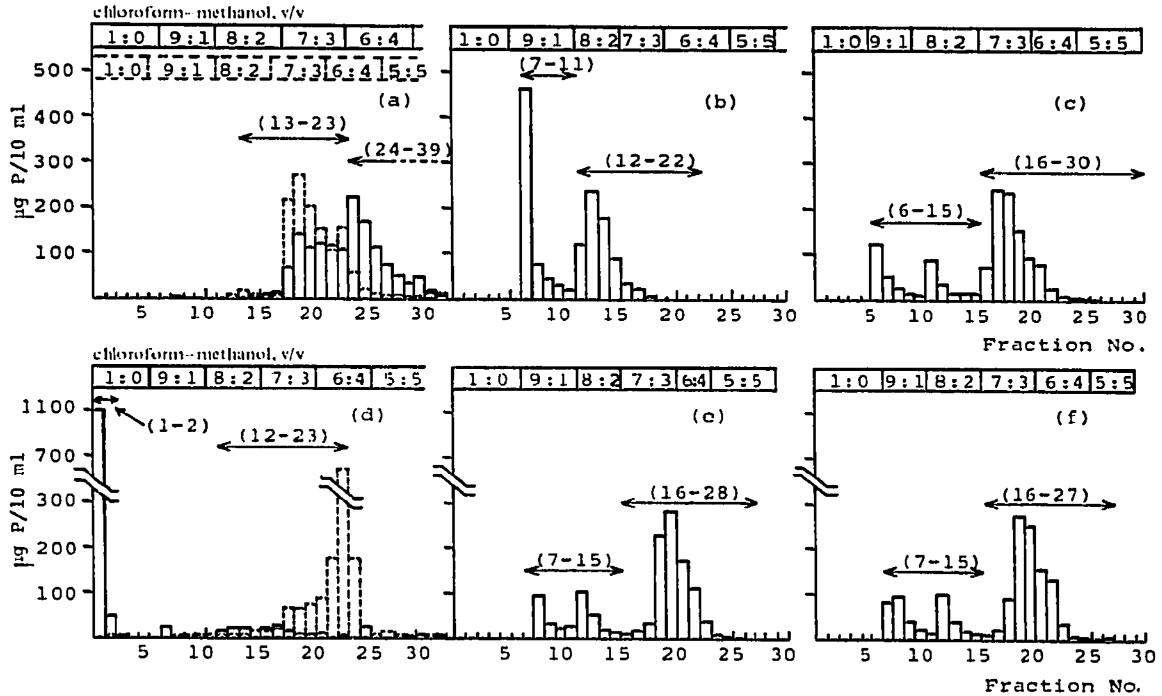

Fig. 2. Silica gel CC of phosphatidylinositol (applied mainly as sodium salt form) on (a) Merck silica gel, catalogue No. 7734; (b) Bio-Rad silicic acid, catalogue No. 62380; (c) Mallinckrodt silicic acid; and on these adsorbents extracted with HCl before use (d, e and f, respectively). The solid-line profiles were obtained when chromatography was performed under the conditions specified in the section *Conditions for the chromatographic studies*. The dotted-line profiles were obtained when the lipid sample was applied on to the column in 2.5 ml of chloroform–methanol (7:3) and chromatography performed at room temperature.

devoid of metal ions (Fig. 2c, e and f). Both alterations to the conditions of chromatography introduced are likely to decrease the stability of the micelles. Thus, provided the existence of stable micelles during chromatography is prevented, the removal of metal ions from different adsorbents results in similar elution profiles also being obtained for phosphatidylinositol applied as its monovalent salt. This result shows that the metal ions of the adsorbents are the cause of the differences.

Table III shows that phosphatidylinositol is subject to ion-exchange reactions similar to those reported for cardiolipin in Table II, *i.e.*, divalent metal ions of the adsorbents replace the monovalent metal ions of the phospholipid. The effect on elution profiles of this ion exchange was discussed in detail for cardiolipin[1] and a similar effect of ion exchange exists for phosphatidylinositol. However, another effect of metal ions of the adsorbent also seems to occur in the chromatography of phosphatidylinositol on Merck silica gel not extracted with hydrochloric acid (Fig. 2a and Table III). Here, one would expect phosphatidylinositol (or a considerable part of it) to be readily eluted because of its conversion into its calcium–magnesium salt form. However, elution does not start until chloroform–methanol (7:3) is used and needs both chloroform–methanol (6:4) and chloroform–methanol (5:5) to be completed.

TABLE III

CATION EXCHANGE BETWEEN PHOSPHATIDYLINOSITOL, APPLIED MAINLY AS THE SODIUM SALT, AND DIFFERENT SILICA ADSORBENTS DURING CC

The metal ion content of phosphatidylinositol subjected to chromatography is listed at the top of the table. Then follows the metal ion content of phosphatidylinositol eluted from the adsorbents. Comparison of the initial metal ion content of phosphatidylinositol with its content after chromatography provides information about the cation exchange occurring during chromatography. In calculating the equivalence ratio (equiv. metal cation: equiv. P), 1 atom of P is considered as 1 equivalent, while 1 equivalent of metal ion equals 1 singly charged ion.

Adsorbent	*Pool*	*Equiv. metal cation:equiv. P*				
		Na	*K*	*Ca*	*Mg*	*Total metal cations:P*
Phosphatidylinositol applied on to column		0.90	0.002	0.274	0.000	1.18
Merck silica gel (0.05–0.2 mm, für die Säulen-Chromatographie, catalogue No. 7734)	(13–23)	0.229	0.003	0.825	0.117	1.17
	(24–39)	0.372	0.006	0.542	0.155	1.08
	(13–23)+(24–39)*	0.30	0.005	0.68	0.136	1.12
Bio-Rad silicic acid (Bio-Sil HA, −325 mesh, catalogue No. 62380)	(7–11)	0.282	0.004	0.554	0.199	1.04
	(12–22)	0.60	0.011	0.255	0.039	0.91
	(7–11)+(12–22)*	0.45	0.008	0.40	0.115	0.97
Mallinckrodt silicic acid (100 mesh, A.R., marked "Suitable for chromatographic analysis by the method of Ramsey and Patterson")	(6–15)	0.281	0.004	0.691	0.125	1.10
	(16–30)	0.85	0.017	0.099	0.018	0.98
	(6–15)+(16–30)*	0.68	0.013	0.28	0.051	1.02
HCl-extracted Merck silica gel (0.05–0.2 mm, für die Säulen-Chromatographie, catalogue No. 7734)	(1–2)	0.70	0.014	0.293	0.008	1.02
	(12–23)	0.254	0.026	0.626	0.187	1.09
	(1–2)+(12–23)*	0.63	0.016	0.34	0.033	1.02
HCl-extracted Bio-Rad silicic acid (Bio-Sil HA, −325 mesh, catalogue No. 62380)	(7–15)	0.150	0.009	0.738	0.091	0.99
	(16–28)	0.76	0.015	0.053	0.012	0.84
	(7–15)+(16–28)*	0.58	0.013	0.25	0.035	0.88
HCl-extracted Mallinckrodt silicic acid (100 mesh, A.R., marked "Suitable for chromatographic analysis by the method of Ramsey and Patterson")	(7–15)	0.124	0.008	0.627	0.056	0.82
	(16–27)	0.71	0.014	0.118	0.014	0.86
	(7–15)+(16–27)*	0.53	0.012	0.28	0.027	0.85

* Calculated.

Rouser *et al.*[11] have discussed this retarding effect of metal ions of an adsorbent and suggested that it arises from hydrogen bonding between hydrated cations of the phospholipid and hydrated cations of the adsorbent. The elution profile on Bio-Rad silica gel not extracted with hydrochloric acid (Fig. 2b) seems to reflect only the ion-exchange effect of the divalent metal ions of the adsorbent. The early elution of pool (7–11) with chloroform–methanol (9:1) is due to the ready conversion of part of the applied sodium phosphatidylinositol into divalent salt (mainly the calcium–magnesium salt), while pool (12–22) is eluted later because it migrates mainly as its monovalent salt form.

Silica gel column chromatography of phosphatidylinositol applied on to columns as its magnesium salt form

Fig. 3 shows the elution profiles obtained when magnesium phosphatidylinositol was applied and eluted from columns made from (a) Merck silica gel, (b) Bio-Rad silica gel, (c) Mallinckrodt silica gel and from these adsorbents extracted with hydrochloric acid before use (d, e and f, respectively). The differences in the elution profiles obtained on the adsorbents not extracted with hydrochloric acid (a, b and c) are prominent. Phosphatidylinositol is eluted most readily from the Merck silica gel (a, solid line) and less readily from the Mallinckrodt silica gel (c). Elution from the Bio-Rad silica (b) is intermediate between the other two. The unexpected elution of almost all of the phosphatidylinositol with chloroform from the Merck silica gel is similar to that observed in the elution of sodium phosphatidylinositol from Merck silica gel extracted with hydrochloric acid (Fig. 2d). Probably, the stability of the phospholipid

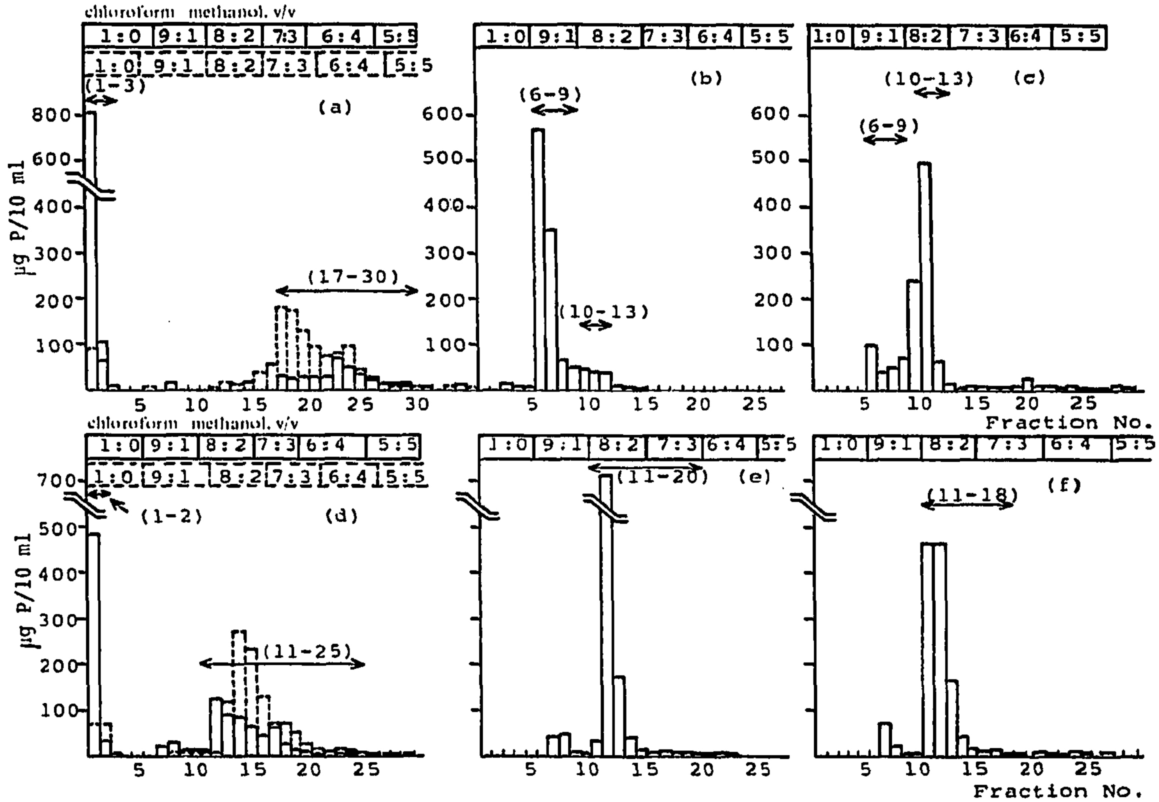

Fig. 3. Silica gel CC of phosphatidylinositol (applied as magnesium salt form) on (a) Merck silica gel, catalogue No. 7734; (b) Bio-Rad silicic acid, catalogue No. 62380; (c) Mallinckrodt silicic acid; and on these adsorbents extracted with HCl before use (d, e and f, respectively). The solid-line profiles were obtained when chromatography was performed under the conditions specified in the section *Conditions for the chromatographic studies*. The dotted-line profiles were obtained when the lipid sample was applied on to the column in 2.5 ml of chloroform–methanol (7:3) and chromatography performed at room temperature. The material eluted with chloroform–methanol (9:1) in (e) and (f) was a contaminant, probably dinitrophenylated phosphatidylserine.

micelles is also the cause in this instance. When the adsorbents were extracted with hydrochloric acid before use, the elution profiles obtained on Bio-Rad and Mallinckrodt silica gel (Fig. 3e and f, respectively) were similar to the elution profile on Mallinckrodt silica gel not extracted with hydrochloric acid (Fig. 3c). However, extraction of the Merck silica gel with hydrochloric acid affected the elution profile only to a limited extent, as the major part of the phosphatidylinositol continued to be eluted with chloroform (Fig. 3d). Part of the phosphatidylinositol was, however, eluted with chloroform–methanol (8:2) and chloroform–methanol (7:3) and thus obviously displayed a chromatographic behaviour similar to that observed on the other two adsorbents freed from metal ions.

When chromatography on the Merck silica gel was carried out under different conditions so as to favour the breakdown of phospholipid micelles as described above for sodium phosphatidylinositol, the immediate elution with chloroform was almost completely eliminated (Fig. 3a and d, broken lines). Thus, from the Merck silica gel not extracted with hydrochloric acid, elution now occurred with chloroform–methanol (7:3) and chloroform–methanol (6:4), and the eluted phosphatidylinositol was mainly the divalent salt form. The relatively late elution is probably caused by a retarding effect of metal ions of the adsorbent, similar to that observed for sodium phosphatidylinositol (Fig. 2a). From Merck silica gel extracted with hydrochloric acid, elution under the altered conditions occurred at the same position as from the other adsorbents devoid of metal ions, *i.e.*, mainly with chloroform–methanol (8:2). Hence, it can be concluded that also with magnesium phosphatidylinositol removal of metal ions present in the adsorbents will result in uniform elution profiles, provided that the occurrence of stable micelles during chromatography is prevented.

The analyses of metal ion:P equivalence ratios for the various pools indicated n Fig. 3 showed that exchange of Mg^{2+} of the applied magnesium phosphatidylinositol for Ca^{2+} of the adsorbent was the only type of ion exchange that occurred during chromatography. The phosphatidylinositol eluted later from Merck silica gel not extracted with hydrochloric acid (pool (17–30), Fig. 3a) had a Ca:P equivalence ratio of 0.63 and thus had been subject to considerable ion exchange. On the Merck silica gel extracted with hydrochloric acid, exchange of Mg^{2+} for Ca^{2+} of the adsorbent had also occurred to the later eluted phosphatidylinositol (pool (11–25), Fig. 3d), although to a lesser extent. Apparently, four extractions with 1 *N* hydrochloric acid will not completely remove metal ions accessible for ion exchange from the adsorbent. The phosphatidylinositol eluted immediately with chloroform both from Merck silica gel not extracted and extracted with hydrochloric acid was essentially the magnesium salt of phosphatidylinositol in both instances. This result confirms the assumption that these pools passed through the column without being adsorbed. Both pools eluted from Bio-Rad silica gel not extracted with hydrochloric acid (pools (6–9) and (10–13), Fig. 3b) had been subject to moderate ion exchange, having Ca:P equivalence ratios of 0.17 and 0.29, respectively. Ion exchange was negligible on Mallinckrodt silica gel (Fig. 3c), Bio-Rad silica gel extracted with hydrochloric acid (Fig. 3e) and Mallinckrodt silica gel extracted with hydrochloric acid (Fig. 3f).

CONCLUSION

The present work has shown that silica adsorbents used for the CC fractionation of lipids may have widely varying metal ion contents, and that this difference causes different elution profiles for an acidic phospholipid on the adsorbents. The metal ions of an adsorbent may influence the elution profile for an acidic phospholipid in two ways. One way is exchange of monovalent metal cations of the phospholipid for divalent metal ions of the adsorbent. As the monovalent and divalent salt forms of a phospholipid have different chromatographic mobilities, the elution profile of the phospholipid applied on to the column as its monovalent salt will be influenced by this ion exchange. The other possible effect of metal ions is a delayed elution of the phospholipid, which was observed only on the adsorbent with the highest content of metal ions. Probably this effect does not appear unless the adsorbent has a relatively high metal ion content.

It is obvious that the cation composition of both the adsorbent and the lipid sample must be controlled in order to obtain reproducible results in the column chromatography of acidic phospholipids. At present, more work is needed before the cation compositions of the adsorbent and a mixture of phospholipids can be recommended for the optimal separation of the components of the mixture. Nevertheless, the results presented in this paper allow a rational approach to be made to those problems of fractionation that require the separation of cardiolipin and phosphatidylinositol.

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