Some Observations on the Chromatographic Behaviour of Phosphatidylserine

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The lipid fraction (fraction III), consisting largely of phosphatidylserine, prepared from brain according to Folch (1942), usually gives several distinct peaks when chromatographed on silicic acid-Hyflo Super-Cel columns by the procedure of Hanahan, Dittmer & Warashina (1957). This essentially confirms results obtained by Marinetti, Erbland & Stotz (1958), who to account for the finding demonstrated that different ionic forms of phosphatidylserine possessed differing chromatographic mobilities. The present experiments extend this concept, showing that the behaviour of phosphatidylserine depends on the nature of the metal ion involved in the salt form and also on the ionic state of the column. An indication of the nature and origin of material corresponding to the various peaks is given.

EXPERIMENTAL

Materials

Chemicals. Silicic acid (Mallinckrodt, analytical reagent, 100 mesh, suitable for chromatography, lot no. s 108-80) was obtained from the Sigma Chemical Co.; Hyflo Super-Cel was from Hopkin and Williams Ltd. Solvents were as described by Rathbone, Magee & Thompson (1962). All other reagents were of AnalR grade wherever possible.

Methods

Preparation of fraction III from brain. A phosphatidylserine-rich fraction (fraction III) was prepared from post-mortem human brain according to Folch (1942).

Column chromatography. Columns composed of silicic acid and Hyflo Super-Cel were used essentially as described by Hanahan *et al.* (1957). Silicic acid and Hyflo Super-Cel were mixed in the proportion of 2:1 (w/w) and washed four times by the addition of chloroform-methanol (4:1, v/v) followed by filtration on a Buchner funnel. A slurry of the washed adsorbent was poured into a tube of suitable dimensions closed at one end by a small plug of cotton wool overlaid by glass wool. Unless otherwise stated, the columns, which were used within 24 hr. of preparation, contained 2.2 g. of silicic acid and 1.1 g. of Hyflo Super-Cel, and when settled were 0.95 cm. in diameter and 9-10 cm. in height. Lipid was applied to the surface of the column as a solution in chloroform-methanol (4:1, v/v) containing 470-630 μ g. of phosphorus. The column loading was 215-288 μ g. of phosphorus/g. of silicic acid and the flow rate of about ten fractions of 2.15 ml./hr. was maintained by pressure from a N₂ cylinder. Elution was carried out successively with chloroform-methanol (4:1, 3:2 and 1:4,

v/v). Ten fractions were collected with each solvent. The recovery of phosphorus was almost 100%.

Paper chromatography. This was carried out according to Lea, Rhodes & Stoll (1955) or Reed, Swisher, Marinetti & Eden (1960).

Preparation of the sodium form of the columns. Silicic acid and Hyflo Super-Cel were mixed in the proportion of 2:1 (w/w) and suspended in N-HCl. The suspension was filtered on a Buchner funnel, and the residue was resuspended in M-NaHCO₃ and again filtered. The adsorbent was then washed five times in a similar way with water and then three times with methanol, and was then left until needed for chromatography. Columns were prepared from the treated adsorbent as described above and used on the same day.

Preparation of various salts of phosphatidylserine. Fraction III (25 mg.) was dissolved in 10 ml. of chloroform-methanol-water (38:19:3, by vol.) and 0.2 vol. of 0.1N-HCl was added. After mixing and centrifuging the upper phase was removed and discarded. Then 2 ml. of chloroform-methanol-0.05M-NaHCO₃ [or with 0.05M-KHCO₃ or 0.012M-Ca(OH)₂ instead of 0.05M-NaHCO₃] (3:48:47, by vol.) was added to the lower phase. After mixing and centrifuging, the upper phase was removed and discarded. The remaining lower phase was then washed twice with 2 ml. portions of a solution similar to that described above, but containing the chloride of the metal ion concerned at a concentration of 0.05M.

The magnesium salt of phosphatidylserine was prepared by a similar procedure except that 0.1M-MgCl₂ replaced 0.1N-HCl and 0.05M-MgCl₂ replaced the bicarbonate or hydroxide in the aqueous solution containing chloroform and methanol. Under these conditions magnesium replaces the ion originally associated with the lipid as was first shown by Folch, Lees & Sloane-Stanley (1957). Methanol was added to the final lower phase, which was then evaporated to dryness, and the residue dissolved in a suitable volume, usually 0.7 ml., of chloroform-methanol (4:1, v/v).

Preparation of the acid form of phosphatidylserine. This was carried out according to the procedure described by Rathbone *et al.* (1962).

Extraction and estimation of metal ion in eluted fractions. Fractions were pooled in suitable groups and the solvent was removed. The residue was dissolved in 6 ml. of chloroform-methanol-water (38:19:3, by vol.). A sample (0.1 ml.) was taken for the estimation of phosphorus and 5.0 ml. was transferred to a stoppered tube. HCl (1 ml. of 0.1N) was added and the upper phase obtained by mixing and centrifuging was transferred to a 25 ml. round-bottomed flask. The lower phase was washed three times with 1 ml. portions of chloroform-methanol-0.05N-HCl (3:48:47, by vol.), the washings being added to the flask. Most of the solvent and HCl was then removed by main-

taining the flask at 80° under reduced pressure from a water pump (for approx. 3 min.). The residual solution was diluted to a suitable volume, and assayed for sodium and potassium at 589 and 768 m μ respectively with an EEL flame photometer (model A, Evans Electroselenium Ltd., Harlow, Essex), calcium at 622 m μ with a Baldwin calcium flame photometer (Baldwin Industrial Controls, Dartford, Kent) and magnesium at 285 m μ with a Zeiss flame photometer.

Phosphorus. This was estimated essentially as described by Tausky & Shorr (1953) after digestion as follows. The lipid samples containing 0.5–20 μ g. of phosphorus were introduced into small Pyrex tubes (1 cm. $\times$ 7 cm.). The solvent was removed by a jet of N₂, 0.1 ml. of a 10N-sulphuric acid–70% (w/w) perchloric acid mixture (1:1, v/v) was added and the tubes were placed on a sand bath at 120° and left for 15–24 hr. The tubes were then allowed to cool and water (1 ml.) was added followed by ferrous sulphate–ammonium molybdate reagent (1 ml.). After being mixed, the solution was left for 10 min. and the colour was then read against a water blank at 690 m μ . The colour was stable for at least 30 min.

Serine and ethanolamine. These were estimated by the method of Axelrod, Reichenthal & Brodie (1953).

Fatty acid. This was estimated as described by Rathbone *et al.* (1962).

Nitrogen. This was estimated as described by Long & Staples (1961).

RESULTS

The variability in the chromatographic behaviour of fraction III from brain was indicated by the work of Rathbone *et al.* (1962). The elution curve obtained with most preparations showed three distinct peaks (Fig. 1a). Since fraction III is known to consist almost entirely of phosphatidylserine (Folch, 1942), it was decided to determine the nature of the material corresponding to the peaks and consequently the cause of the variability in chromatographic behaviour of fraction III from one preparation to another. The omission of Hyflo Super-Cel from the column made little difference to the elution curve obtained with untreated fraction III.

Material corresponding to the three peaks obtained by chromatographing untreated fraction III (preparation i) on a column giving an elution curve similar to that shown in Fig. 1 (a) was rechromatographed on silicic acid-impregnated paper with chloroform–methanol (4:1, v/v) according to the procedure of Lea *et al.* (1955). Although the lipid streaked in this solvent, protoporphyrin- (Sulya & Smith, 1960) and ninhydrin-staining revealed that the material corresponding to peaks 2 and 3 gave only one spot with an R_f corresponding to that of phosphatidylserine, whereas peak 1 material produced a compound spot, presumably consisting of phosphatidylethanolamine and phosphatidylserine. Chemical analysis (Table 1) confirmed this interpretation.

In the experiments of Marinetti *et al.* (1958), the phosphatidylserine which was eluted in two successive peaks was shown to be the acid form and the sodium salt of the lipid respectively. An indication of the nature and also the origin of phosphatidylserine corresponding to the various peaks observed in the present work was obtained as follows.

Fig. 1. Chromatographic behaviour of untreated and the sodium form of fraction III on untreated and treated columns. The fractions on the left of each diagram were eluted with chloroform–methanol (4:1, v/v), those in the middle with chloroform–methanol (3:2, v/v), and those on the right with chloroform–methanol (1:4, v/v). (a) Chromatography of fraction III (preparation i) in the untreated (●) and in the Na⁺ (▲) form. (b) Chromatography of fraction III (preparation ii) in the Na⁺ form on untreated columns (▲) and on columns made from adsorbent treated with HCl and NaHCO₃ (○).

Composition and origin of peak 1 material. Chromatography of fraction III after its conversion into the acid form resulted in the elution of almost 90 % of the applied lipid with chloroform-methanol (4:1, v/v) (Table 2, Expt. 2), thus confirming the chromatographic characteristics of acid phosphatidylserine noted by Marinetti *et al.* (1958). When acid phosphatidylserine was converted into the sodium or potassium salt, however, by equilibration with bicarbonate, an elution curve very similar to that of the untreated fraction III was obtained on chromatography (Fig. 1a). The occurrence of peak 1 under these conditions suggested the presence either of a serine-containing lipid with similar chromatographic properties in both the acid and salt form, or alternatively that acidic lipid or lipid with similar mobility was produced by ion exchange on the column. Conversion of this peak 1 material into the potassium form followed by rechromatography showed that the proportion of applied phosphorus eluted with chloroform-methanol (4:1, v/v) (Table 2, Expt. 7) was similar to that obtained by chromatographing the potassium form of the original fraction III (Table 2, Expt. 4). It is therefore clear that the species of phosphatidylserine eluted with chloroform-methanol (4:1, v/v) is produced from the sodium or potassium salt by ion exchange on the column. The extent of this conversion in the present experiments was 20–36 % of the applied phosphorus.

Further proof of the influence of the ionic state of the column on the chromatographic mobility of phosphatidylserine was given by the elution curve obtained by chromatographing the sodium form of fraction III (preparation ii) on a silicic acid-Hyflo Super-Cel column made from adsorbents treated with *n*-hydrochloric acid, followed by *m*-sodium hydrogen carbonate. By this treatment the ionizable groups, presumably H^+ ions, which appear to exist on the column are replaced by Na^+ ions. Under these conditions, applied sodium phosphatidylserine is eluted almost completely with chloroform-methanol (3:2, v/v) (Fig. 1b; Table 2, Expt. 10). Paper chromatography showed that

material eluted with chloroform-methanol (4:1, v/v) represented mainly phosphatidylethanolamine present as a contaminant in the original fraction III and contained no phosphatidylserine. Similarly material eluted with chloroform-methanol (3:2, v/v) contained no phosphatidylethanolamine. The sample of fraction III used in this experiment did not give an appreciable third peak under any of the conditions used. When a sample of fraction III which gave a third peak in the untreated state was converted into the sodium form and chromatographed on a column in the sodium form, however, the third peak was still apparent in the elution curve, although diminished.

Nature of phosphatidylserine corresponding to peak 2. The material corresponding to peak 2 obtained by chromatographing untreated fraction III gave a $Na^+ : P$ ratio of less than unity in every experiment (see, for example, Table 2, Expt. 8). When fraction III was converted into the sodium form ($Na^+ : P$ ratio approximately unity) before chromatography, however, the $Na^+ : P$ ratio in the eluate corresponding to peak 2 was much nearer unity, e.g. 0.89 and 0.87 in Expts. 3 and 9 (Table 2). When the potassium form of fraction III was applied to a similar column (Table 2, Expt. 4), no potassium was found in the eluate but only sodium, the $Na^+ : P$ ratio of peak 2 material being 0.56. There is thus a complete exchange of lipid potassium for sodium and possibly other ions on the columns used in the present work. This is again apparent from Table 2 (Expt. 8) when untreated fraction III which contains mainly potassium was chromatographed. In this case the $Na^+ : P$ ratio of peak 2 material was 0.71. The eluate corresponding to peaks 1 and 3 in most experiments contained small and variable amounts of sodium which appeared to bear no constant relationship to the amount of phosphorus present. The small amount of sodium which was eluted from the columns in the absence of applied lipid (Table 2, Expt. 11) was taken into account in the calculation of the ratios.

It is concluded from these experiments that lipid eluted with chloroform-methanol (3:2, v/v) during chromatography of fraction III consists mainly of the sodium salt of phosphatidylserine, almost entirely so when the sodium salt of the lipid is applied to the column. The position of sodium phosphatidylserine in the elution sequence was confirmed by the finding that all the phosphatidylserine in fraction III, with the exception of peak 3 material when present, was eluted with this solvent when the sodium salt of fraction III was chromatographed on columns converted into the sodium form. The amount of sodium present in the eluate from these columns, however, was much greater than the amount of lipid (Table 2, Expt. 10). It

Table 1. *Composition of material corresponding to the three peaks obtained by chromatographing fraction III on silicic acid-Hyflo Super-Cel columns*

The molar ratios given are all relative to phosphorus (1.00).

Peak no.	Molar ratios			
	Fatty acid	Serine	Ethanol-amine	Nitrogen
1	1.84	0.83	0.20	1.35
2	2.03	0.98	0	1.08
3	2.02	0.97	0	1.00

appears that the treatment of the adsorbents with sodium hydrogen carbonate introduces a considerable amount of sodium which is easily removed by the solvents.

Nature of phosphatidylserine corresponding to peak 3. The material eluted with chloroform-methanol (1:4, v/v) during the chromatography of untreated fraction III gave the same analysis

Table 2. *Distribution of phosphorus and metal ion in the eluate obtained during the chromatography of various forms of fraction III*

Fraction III (usually 25 mg.), either untreated or after conversion into the acid or salt form, was dissolved in 0.7 ml. of chloroform-methanol (4:1, v/v), and 0.5-0.6 ml. of the solution was transferred to the column. This contained 2.2 g. of silicic acid and 1.1 g. of Hyflo Super-Cel. Peak 1 material was eluted with chloroform-methanol (4:1, v/v), peak 2 material with chloroform-methanol (3:2, v/v), and peak 3 material with chloroform-methanol (1:4, v/v). Ten fractions of 2.15 ml. each were collected with each solvent.

Expt. no.	Fraction III prep. no.	Form of fraction III applied	Treatment of adsorbent	Material analysed	Amount present (μ moles)					Percentage of total eluted P
					P	Na ⁺	K ⁺	Mg ²⁺	Ca ²⁺	
1	(i)	Untreated	Untreated	Peak 1	4.40	—	—	—	—	27.2
				Peak 2	9.35	—	—	—	—	57.8
				Peak 3	2.41	—	—	—	—	14.8
				Applied lipid	19.10	—	—	—	—	—
2	(i)	H ⁺ form	Untreated	Peak 1	19.25	—	—	—	—	88.5
				Peak 2	1.62	—	—	—	—	7.4
				Peak 3	0.96	—	—	—	—	4.4
				Applied lipid	21.83	—	—	—	—	—
3	(i)	Na ⁺ form	Untreated	Peak 1	4.06	1.82	0.15	—	—	22.6
				Peak 2	12.20	12.50	0.26	—	—	67.8
				Peak 3	1.76	4.02	0.13	—	—	9.8
				Applied lipid	18.02	18.34	0.54	—	—	—
4	(i)	K ⁺ form	Untreated	Peak 1	3.37	1.36	0	—	—	20.6
				Peak 2	9.98	7.20	0	—	—	61.0
				Peak 3	3.03	3.40	0	—	—	18.5
				Applied lipid	16.40	11.96	0	—	—	—
5	(i)	Mg ²⁺ form	Untreated	Peak 1	20.20	—	—	13.25	—	96.4
				Peak 2	0.53	—	—	1.50	—	2.5
				Peak 3	0.22	—	—	0	—	1.1
				Applied lipid	20.95	—	—	14.75	—	—
6	(i)	Ca ²⁺ form	Untreated	Peak 1	15.70	—	—	—	9.95	94.0
				Peak 2	0.53	—	—	—	0.72	3.0
				Peak 3	0.55	—	—	—	0.30	3.2
				Applied lipid	16.78	—	—	—	10.97	—
7	(i)	Peak 1 material from Expt. similar to 4 re-converted into K ⁺ form	Untreated	Peak 1	1.62	—	—	—	—	29.4
				Peak 2	3.47	—	—	—	—	60.0
				Peak 3	0.56	—	—	—	—	9.9
				Applied lipid	5.65	—	—	—	—	—
8	(ii)	Untreated	Untreated	Peak 1	4.06	2.70	0.03	—	—	25.4
				Peak 2	10.65	9.12	0.12	—	—	67.4
				Peak 3	1.13	2.90	0	—	—	7.2
				Applied lipid	15.84	14.72	0.15	—	—	—
9	(ii)	Na ⁺ form	Untreated	Peak 1	4.90	3.15	0.09	—	—	25.2
				Peak 2	12.45	12.40	0.25	—	—	70.0
				Peak 3	0.92	2.28	0	—	—	5.0
				Applied lipid	18.27	17.83	0.34	—	—	—
10	(ii)	Na ⁺ form	Washed with N-HCl followed by M-NaHCO ₃	Peak 1	0.99	2.38	0	—	—	6.3
				Peak 2	14.50	33.90	0.07	—	—	91.5
				Peak 3	0.39	18.00	0.10	—	—	2.4
				Applied lipid	15.78	54.28	0.17	—	—	—
11	—	No lipid applied	Untreated	Peak 1	—	0.48	0.05	0	0.63	—
				Peak 2	—	1.61	0.03	0	0.05	—
				Peak 3	—	1.04	0	0	0	—

results as pure phosphatidylserine (Table 1). The elution curves obtained with untreated fraction III and fraction III in the sodium form were similar (Fig. 1*a*). Some preparations of fraction III (e.g., preparation ii) did not give an appreciable third peak either in the untreated state or after conversion into the sodium or potassium form (Fig. 1*b*). It is clear therefore that the phosphatidylserine corresponding to peak 3 is not in equilibrium with the lipid in the peak 2 eluate, and represents a different substance. The yellow colour of the material supports this view. Whether the differences between the lipid corresponding to peaks 2 and 3 reflect different species of phosphatidylserine occurring *in vivo* or whether the occurrence of a third peak is an artifact produced during extraction remains to be discovered.

Effect of different cations on the chromatographic behaviour of phosphatidylserine. The behaviour of the sodium and potassium forms of fraction III has already been described. When fraction III was converted into the calcium or magnesium form before chromatography (Table 2, Expts. 5 and 6), 89–96% of the phosphorus was eluted with chloroform–methanol (4:1, v/v), the elution curve being very similar to that obtained by chromatographing the acid form of phosphatidylserine (Rathbone *et al.* 1962). The $\text{Ca}^{2+}:\text{P}$ ratio in the eluate corresponding to peak 1 was 0.59 whereas the $\text{Mg}^{2+}:\text{P}$ ratio was 0.66 in the experiments quoted.

Of the four metal cations investigated, interaction of fraction III with the bivalent ions before chromatography resulted in the elution of almost all the lipid with chloroform–methanol (4:1, v/v), whereas interaction with the univalent ions caused the bulk of the lipid to be eluted with chloroform–methanol (3:2, v/v).

DISCUSSION

The two main features of interest arising from the present work concern, first, the ion exchange which occurs on the columns, and, secondly, the dependence of the chromatographic mobility of phosphatidylserine on the type of cation associated with it. The exchange involves two cations. Univalent metal cations combined with phosphatidylserine exchange presumably with H^+ ions from the column, resulting in the elution of a considerable proportion of the lipid with chloroform–methanol (4:1, v/v). In addition K^+ ions in combination with phosphatidylserine applied to the column are completely removed and are replaced to a great extent by Na^+ ions, thus revealing the considerable reservoir of Na^+ ions in the adsorbents. Analysis of the solutions obtained by extracting silicic acid and Hyflo Super-Cel with 0.1*N*-hydrochloric acid showed that the silicic acid yielded 0.031 mg. of

Na^+ and 0.003 mg. of K^+ ions/g., and Hyflo Super-Cel 0.830 mg. of Na^+ and 0.054 mg. of K^+ ions/g. Thus in the columns described, the silicic acid contributed approximately 3 μmoles of Na^+ ions and the Hyflo Super-Cel approximately 40 μmoles of Na^+ ions. Hyflo Super-Cel is therefore responsible for the exchange of lipid K^+ for Na^+ ions. The exchange which brings about the elution of phosphatidylserine presumably in the acid form, with chloroform–methanol (4:1, v/v) during chromatography of the sodium or potassium form, however, is a property of the silicic acid, since a considerable proportion (35%) of the applied phosphatidylserine is eluted with this initial solvent in comparable experiments in which Hyflo Super-Cel was omitted from the columns.

This type of exchange, which converts one ionic species into another having a different mobility, implies that it is not possible to separate quantitatively the acid from the salt forms of phosphatidylserine present in mixtures applied to the column.

A similar type of exchange might also explain the differences between the behaviour of phosphatidylserine on silicic acid–Hyflo Super-Cel columns and silicic acid-impregnated paper. The material eluted from silicic acid columns with chloroform–methanol (4:1, v/v) and (1:1, v/v) respectively in the experiments of Marinetti *et al.* (1958) gave single spots having the same R_F on silicic acid-impregnated paper. These authors explained their finding by assuming that the phosphatidylserine corresponding to each peak was converted into the acidic form by the acidic chromatographic solvents used. In the present work, however, phosphatidylserine corresponding to the three peaks obtained during the chromatography of untreated fraction III gave spots on silicic acid-impregnated paper having the same R_F even when a neutral solvent [chloroform–methanol (4:1, v/v)] was used. It is tentatively suggested that, in this case, exchange between H^+ ions associated with silicic acid on the paper and Na^+ ions present in the applied lipid was complete. The observed R_F in this system would in this event be characteristic of acid phosphatidylserine.

The findings imply that, to achieve reproducible chromatographic behaviour of phosphatidylserine involving the elution of the lipid with a single solvent, it is necessary to control both the cations in combination with the lipid and also those associated with the column. Some ways of achieving this are suggested.

SUMMARY

1. An indication is given of the nature of the material corresponding to the three peaks occurring

when most samples of phosphatidylserine (fraction III, Folch, 1942) are chromatographed on silicic acid-Hyflo Super-Cel columns.

2. Exchange of lipid sodium or potassium for another cation, presumably H^+ ion, occurs on the columns causing the elution of lipid with chloroform-methanol (4:1, v/v). This can be prevented by treatment of the adsorbent with hydrochloric acid followed by sodium hydrogen carbonate.

3. Exchange of lipid potassium for sodium occurs on the columns.

4. The chromatographic mobility of phosphatidylserine depends upon the metal ion combined with the lipid. Application of the calcium and magnesium forms to the column results in the elution of the lipid with chloroform-methanol (4:1, v/v). When the sodium or potassium forms are applied, the bulk of the lipid is eluted with chloroform-methanol (3:2, v/v).

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The Kinetics of the Reaction between the Blood-Group Antibody Anti-c and Erythrocytes

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It has been realized for some time that antigen-antibody reactions are reversible, but it is only recently that precise physicochemical studies have been made and kinetic and thermodynamic constants reported. The subject has been reviewed by Talmage & Cann (1961).

The antibodies so far investigated have been mainly those produced in rabbits after the injection of various hapten-protein compounds. Only a few observations have been made on human red-cell antibodies. Filitti-Wurmser, Jacquot-Armand, Aubel-Lesure & Wurmser (1954) computed thermodynamic constants for anti-B antibody against group B erythrocytes; antibody concentration was measured indirectly, in terms of the maximum number of erythrocytes that the anti-serum could agglutinate. Boursnell, Coombs & Rizk (1953) used ^{131}I as a protein label to investigate the uptake by red cells of the antibody anti-D.

They found, however, that the red cells not only took up ^{131}I -labelled antibody but also considerable amounts of bound ^{131}I that was not associated with antibody, and which were large enough to mask the uptake of ^{131}I -labelled antibody. They termed this uptake of ^{131}I 'non-specific' and found that it was considerably decreased in the presence of unlabelled serum. Masouredis (1959) also used ^{131}I as a label for anti-D antibody. He overcame the problem of non-specific uptake by using antibody purified by elution from red cells. It has been shown that the non-specific uptake of ^{131}I by red cells from ^{131}I -labelled serum is due to three factors, namely, the absorption of ^{131}I -labelled serum proteins, the exchange of ^{131}I -labelled lipids between serum lipoproteins and red cells and the uptake of contaminating [^{131}I]iodide (Hughes-Jones & Gardner, 1962). Non-specific uptake of ^{131}I -labelled protein and lipid can be reduced by com-