Chromatographic Separation of Brain Lipids

3. SERINE-CONTAINING PHOSPHOLIPIDS*

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The chromatographic behaviour on silicic acid columns of phosphatidylserine has been previously reported (Hanahan, Dittmer & Warashina, 1957; Marinetti, Erbland & Stotz, 1958; Macfarlane, Gray & Wheeldon, 1960). From these and other studies it may be concluded that phosphatidylserine is eluted together with the ethanolamine-containing phospholipids from silicic acid by a solvent containing 10–20% (by vol.) of methanol in chloroform, and that there is no satisfactory separation of these two types of phospholipid. A statement on the elution pattern of brain phosphatidylserine on an alumina column has also appeared (Barkhan, Flute & Rhodes, 1957). Long, Shapiro & Staples (1960) have reported that the phosphatidylserine of rat brain is very strongly adsorbed on alumina, whereas the whole of the ethanolamine-containing phospholipid is readily eluted by a chloroform–methanol solvent mixture containing 8–14% (by vol.) of water (Long & Staples, 1961*b*). Elution of the phosphatidylserine could only be effected by the use of an alkaline solvent, albeit with some decomposition. Final purification was achieved by subsequent chromatography of the eluted material on silicic acid.

The present paper describes the detailed procedure for isolating about 70% of the serine-containing phospholipids present in a brain extract. The material is free from other contaminating lipids.

EXPERIMENTAL

Procedures for the extraction of rat-brain lipids by means of CHCl_3 –methanol (2:1, v/v), and for the removal of ganglioside and other water-soluble contaminants and of proteolipid protein, were as described by Long & Staples (1959). Column-chromatographic techniques and the determination of total nitrogen and total phosphorus were according to Long & Staples (1961*a*); analytical methods for ethanolamine, acyl ester, aldehyde and potassium were as given by Long & Staples (1961*b*). Inositol was deter-

mined on 48 hr. hydrolysates (5.5*N*-HCl at 100°) by the microbiological assay with *Schizosaccharomyces pombe*, of Norris & Darbre (1956).

Silicic acid (analytical reagent 100-mesh powder, Mallinckrodt Chemical Works; purchased from Bell and Croyden Ltd., 50 Wigmore Street, London, W. 1) was sieved, and the material passing a 240 B.S. sieve was discarded. The silicic acid was then activated by heating at 120° for 24 hr. Alumina (Brockmann, grade II) was obtained from Savory and Moore Ltd., London. It was similar to 'batch B' used by Long & Staples (1961*a*).

Determination of serine. Serine was determined by the dinitrophenyl method of Axelrod, Reichenthal & Brodie (1953), as described by Long & Staples (1961*b*). This method would give too high an apparent value for the serine content of a lipid sample if the latter were contaminated by traces of protein, since protein would be partially hydrolysed to give amino acids under the conditions of analysis and the amino acids would be estimated as serine. As it is difficult to remove the last traces of proteolipid protein from a brain-lipid extract, a comparison was made between the serine content as determined by the principle of Axelrod *et al.* (1953) with that found for a larger sample after application to an ion-exchange resin column under the conditions described by Moore & Stein (1951). In a single experiment, only 80.2% of the apparent serine content could be eluted from the ion-exchange resin in the position known to be occupied by serine. Thus it appears probable that about 20% of the 'serine' estimated by the method of Axelrod *et al.* (1953) is due to amino acids present in contaminating proteolipid. Some variation between lipid samples would be expected. The ion-exchange chromatography was performed by Dr J. E. Eastoe, Department of Dental Science.

Paper chromatography of decomposition products of phosphatidylserine. Whatman no. 1 filter paper was used and the solvents were either (a) ethanol–0.1*M*-sodium acetate buffer, pH 4.63 (2:1, v/v) (Kennedy & Smith, 1954) or (b) phenol saturated with water–acetic acid–ethanol (50:5:6, by vol.) (Dawson, 1960). After development overnight at room temperature, the papers were dried and sprayed for phosphorus (Hanes & Isherwood, 1949) or for amino nitrogen with ninhydrin. Serine and *O*-phosphorylserine were used as markers.

RESULTS

Chromatography of rat-brain-lipid extracts on alumina columns

In studies on alumina chromatography Long & Staples (1961*a*, *b*) showed that, by increasing

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gradually the water content of a chloroform-methanol (1:1, v/v) solvent system, successive elution of cerebroside, sulphatide and ethanolamine-based phospholipids takes place. However, even with the highest possible water content of this system [chloroform-methanol-water (43:43:14, by vol.)], no serine-containing lipids could be eluted. This indicates that the serine-based lipids are tenaciously retained by the alumina.

In an attempt to find a suitable eluent for phosphatidylserine, pilot experiments were carried out in which 2 ml. samples of rat-brain-lipid extract, dissolved in chloroform-methanol-water (43:43:14, by vol.) and containing about 0.2 μ mole of serine-based phospholipid, were treated with 100 mg. quantities of alumina in a series of tapered 10 ml. centrifuge tubes. After 5 min., during which the tubes were intermittently shaken, the alumina was centrifuged down and washed twice with 2 ml. of the same solvent to remove any ethanolamine-containing phospholipid. The tubes were then treated with 2 ml. of one of several possible eluting solvents. The suspensions were centrifuged and the supernatants were tested for amino nitrogen by the ninhydrin method of Moore & Stein (1948). The only solvent capable of extracting any appreciable quantity of amino nitrogen was diethyl ether-ethanol-aq. 0.04N-potassium hydroxide (10:7:5, by vol.).

When the lipid from two rat brains was applied to an alumina column (13 g.; 1 cm. diam. $\times$ 17 cm.) and the column was eluted with increasing content of water in chloroform-methanol (1:1, v/v), so as first to remove the bulk of the lipids, subsequent elution with the alkaline solvent system gave fractions which contained a small proportion of the serine applied to the column. However, the analytical values for ester, phosphorus, serine and total nitrogen on the individual fractions did not suggest that intact phosphatidylserine was being eluted. There were strong indications that the phosphatidylserine had been largely decomposed on prolonged contact with alumina.

It was clear that, in order to elute phosphatidylserine with minimum decomposition, it would be necessary to carry out the chromatography as rapidly as possible. Preliminary experiments showed that alumina would adsorb phosphatidylserine almost completely, provided that (a) not more than 30 μ moles of this lipid/g. of alumina were used and (b) the concentration of phosphatidylserine in the solution applied to the column did not exceed 3 μ moles/ml. The lipid-bound serine of rat brain amounts to about 9 μ moles/g. fresh wt., i.e. about 15 μ moles/brain.

The total lipid from four rat brains, washed and deproteinized, was dissolved in 25 ml. of chloroform-methanol-water (43:43:14, by vol.); 20 ml.

was applied to an alumina column (2 g.; 1 cm. diam. $\times$ 2.5 cm.) equilibrated with the same solvent mixture. The column was washed through with this solvent and four 50 ml. fractions were collected in about 45 min. The solvent was then changed to diethyl ether-ethanol-aq. 0.04 N-potassium hydroxide (10:7:5, by vol.) and eight 15 ml. fractions were collected. After the change of solvent, the first fraction to show alkalinity towards phenolphthalein was the fifth. This and subsequent fractions were immediately made just acid to phenolphthalein by the addition of the minimum quantity of acetic acid, and all fractions were kept at 2°. They were analysed for total nitrogen, amino nitrogen, ethanolamine, serine, inositol, total phosphorus, acyl ester and aldehyde. Results of a typical experiment are given in Fig. 1.

The first 200 ml. of eluate, obtained with chloroform-methanol-water (43:43:14, by vol.), contained the neutral lipids, choline-containing phospholipids, galactose-containing lipids and ethanolamine-containing phospholipids. The detailed elution of the latter is shown in Fig. 1. The first 100 ml. of eluate also contained 81% of the total

Fig. 1. Elution curve of rat-brain lipid applied to an alumina column (2 g.; 1 cm. diam. $\times$ 2.5 cm.). The first solvent was chloroform-methanol-water (43:43:14, by vol.). At the time indicated by the arrow, the solvent was changed to diethyl ether-ethanol-aq. 0.04N-KOH (10:7:5, by vol.). The lipid was derived from 6.78 g. wet wt. of rat brain. $\circ$, Ethanolamine; $\bullet$, serine; $\square$, total phosphorus; $\blacksquare$, acyl ester.

inositol; the second 100 ml. was inositol-free. After the change to the alkaline solvent, amino nitrogen was again eluted, and analysis showed that this was almost completely due to serine.

Summation of the analytical values for fractions covering the 200–305 ml. of effluent from the column gave the following results (in $\mu\text{g. atoms}$ or μmoles): serine, 50.4; ethanolamine, 2.5; total nitrogen, 58.6; inositol, 3.0; total phosphorus, 68.8; acyl ester, 116.2; aldehyde, 3.7. The inositol was eluted only in the first fraction to show alkalinity to phenolphthalein. These results indicate that the main product eluted from the alumina column with the alkaline solvent is phosphatidylserine. Contamination by ethanolamine- and inositol-containing phospholipids is very slight, but the relatively high value for total phosphorus shows that another unidentified phospholipid is also present. The aldehyde content was very low throughout the elution (mean aldehyde:phosphorus ratio, 0.05). Based on serine analyses by the dinitrophenyl method, the average total recovery from the column was 88 %, with 83 % in the main peak. Since, however, as mentioned in the Experimental section, only about 80 % of the apparent serine content of the crude lipids could be identified as true serine by ion-exchange chromatography in the one case examined, it is possible that the total recovery of serine approaches 100 %. The assumption has been made here that alumina would retain any protein present in the lipid extract.

Silicic acid chromatography of the crude phosphatidylserine eluted from an alumina column

The phosphatidylserine at this stage was present in an aq. ethanol–diethyl ether solvent mixture. It seemed desirable to avoid evaporation of this solution to dryness in view of possible decomposition during such a lengthy process. The following procedure was therefore used. The combined fractions were concentrated under reduced pressure at 40° to remove the ether and most of the ethanol. When the concentrate began to froth, it was cooled and treated with 0.1 vol. of *m*-potassium chloride, and then extracted with 4 vol. of chloroform–methanol (2:1, v/v). The chloroform-rich phase was removed and set aside, and the aqueous layer was extracted twice with an equal volume of chloroform–methanol (17:3, v/v). The combined chloroform-rich phases were evaporated to dryness *in vacuo* and the residue was dissolved in chloroform–methanol (49:1, v/v). After a trace of insoluble material had been filtered off, the solution was diluted to 25 ml. Amino-nitrogen analyses showed that about 97 % of the lipid was recovered under these conditions, and that it had the same composition as the combined fractions eluted from the alumina, except that (a) the total nitrogen value was slightly lower and corresponded more

nearly to the serine content and (b) it was now free from inositol. It was inferred that the inositol and excess of nitrogen were partitioned into the aqueous phase because they were in fact present in water-soluble form and were decomposition products resulting from chromatography on alumina.

The crude phosphatidylserine, dissolved in 20 ml. of chloroform–methanol (49:1, v/v) was applied to a column of silicic acid (8 g.; 1 cm. diam. $\times$ 17 cm.) and eluted with the same solvent. Two 25 ml. fractions were collected, and were found to contain acyl ester but no serine and virtually no phosphorus (Fig. 2). The solvent was then replaced by chloroform–methanol (9:1, v/v), and six more 25 ml. fractions were collected. The first two fractions eluted by this second solvent contained material having an acyl ester:phosphorus ratio of 2.0, but were free from serine. The next four fractions contained material having acyl ester:phosphorus:serine proportions closely approximating to 2:1:1, suggesting that phosphatidylserine was now being eluted. The remainder of the phosphatidylserine was eluted from the column by gradient elution. The mixing vessel contained 125 ml. of chloroform–methanol (9:1, v/v) and 150 ml. of chloroform–methanol (17:8, v/v) was placed in the reservoir. Ten more 25 ml. fractions were collected. As shown in Fig. 2, the phosphorus contents of the

Fig. 2. Silicic acid chromatography of lipid material obtained by combining the serine-containing fractions from an alumina column of the type shown in Fig. 1. Stepwise, followed by gradient, elution was used whereby the methanol content of the chloroform–methanol solvent was increased from 2 to 25% (by vol.) (broken line). At the time indicated by the arrow, the solvent was changed to methanol–chloroform–water (14:5:1, by vol.). The dimensions of the silicic acid column were: 8 g.; 1 cm. diam. $\times$ 17 cm. ●, Serine; □, total phosphorus; ■, acyl ester.

individual fractions closely followed the serine values, whereas the ester contents were about double these values. Finally, the solvent was changed to chloroform-methanol-water (5:14:1, by vol.), a system used by Rhodes & Lea (1957) for the elution of lyso compounds. A sharp peak appeared immediately after the solvent change, having acyl ester:phosphorus:serine proportions of 1.04:1.00:0.89, which is in fair agreement with the expected value for lysophosphatidylserine.

In the experiment described in Fig. 2, the lipid applied to the column contained 16.2 μ moles of serine, and 14.6 μ moles were eluted altogether. When fractions representing the first serine-containing peak (125–400 ml.) were combined, evaporated to dryness and dissolved in chloroform, the acyl ester:phosphorus:serine:aldehyde proportions were 1.91:1.00:1.05:0.06, indicating a mixture of phosphatidylserine (94%) and serine plasmalogen (6%).

Instability of phosphatidylserine on alumina

In an attempt to prepare phosphatidylserine on a large scale, the crude mixed lipids from 810 g. of ox brain, free from protein and water-soluble contaminants, were dissolved in 3 l. of chloroform-methanol-water (43:43:14, by vol.) and chromatographed on an alumina column (300 g.; 5.7 cm.

diam. $\times$ 12.5 cm.). Seven 2.5 l. fractions were collected with the same solvent, by which time the concentrations of ethanolamine, acyl ester and phosphorus had fallen to very low levels. The solvent was then changed to diethyl ether-ethanol-aq. 0.04N-potassium hydroxide (10:7:5, by vol.) and nine more 2.5 l. fractions were taken. The duration of the experiment was 12.5 hr. for the collection of 40 l. of eluate. The curves for the elution of ethanolamine, serine, acyl ester and total phosphorus were similar to those shown in Fig. 1, except that the scale was increased 150-fold.

To eliminate the need of evaporating the water-containing eluates, fractions 9–14, which together contained 94% of the total serine, were separately treated as follows. Light petroleum (b.p. 40–60°, 250 ml.) was added to each and, after mixing, two phases separated. The upper phase (1660 ml.) usually contained an appreciable amount of acyl ester, but considerably less amino nitrogen. This ester was thought to result mainly from decomposition on the alumina column. The lower phase (1090 ml.) was extracted three times with 570 ml. portions of chloroform. The combined lower chloroform-rich phases contained most of the phosphatidylserine and lysophosphatidylserine. Almost no acyl ester was present in the upper aqueous phase, although there was much amino

Table 1. *Large-scale chromatography of ox-brain lipids on alumina*

Lipids from 810 g. of ox brain were chromatographed on an alumina column (300 g.; 5.7 cm. diam. $\times$ 12.5 cm.). Fractions 9–14 each had a volume of 2.5 l., and were eluted by diethyl ether-ethanol-aq. 0.04N-KOH (10:7:5, by vol.). Extractions with light petroleum (b.p. 40–60°) and chloroform are described in the text. In (b) the results are given in μ moles or μ equiv./fraction. Abbreviations: GPS, glycerylphosphorylserine; LysoPS, lysophosphatidylserine; PS, phosphatidylserine.

(a) Analytical values for individual fractions

Fraction no.	Light-petroleum-rich phase		Chloroform-rich phase		Aqueous phase	
	Amino N (μ g.atoms)	Acyl ester (μ equiv.)	Amino N (μ g.atoms)	Acyl ester (μ equiv.)	Amino N (μ g.atoms)	Acyl ester (μ equiv.)
9	119	283	273	558	23	15
10	250	615	650	1388	94	15
11	185	604	1045	1956	340	84
12	122	1124	487	798	945	0
13	28	1167	142	81	1064	0
14	60	419	48	23	464	0

(b) Calculated composition of individual fractions

Fraction no.	Light-petroleum-rich phase		Chloroform-rich phase				Aqueous phase		Total				Calc. decomposition (%)
	PS	Excess of ester	GPS	LysoPS	PS	Excess of ester	GPS	LysoPS	GPS	LysoPS	PS	Excess of ester	
9	119	45	0	0	273	12	8	15	8	15	391	45	6
10	250	115	0	0	650	88	79	15	79	15	900	203	10
11	185	234	0	134	911	0	256	84	256	218	1096	234	30
12	122	880	0	176	311	0	945	0	945	176	333	880	77
13	28	1111	61	81	0	0	1064	0	1125	81	28	1111	98
14	60	299	25	23	0	0	464	0	489	23	60	399	90

nitrogen, particularly in the later fractions. The complete results are shown in Table 1(a).

In Table 1(b) the probable composition of the individual fractions has been calculated. The following assumptions have been made. (a) The light-petroleum-rich phase contains some phosphatidylserine, which is calculated from the amino nitrogen value, and nearly all the excess of acyl ester, calculated from the amount of ester in excess of the 2 μ equiv. required/ μ g. atom of amino nitrogen. (b) The aqueous phase contains lysophosphatidylserine, calculated from the acyl ester content, and glycerylphosphorylserine, given by the difference between the acyl ester and amino nitrogen contents; any free serine or phosphorylserine would be included in the value assigned to glycerylphosphorylserine. (c) The chloroform-rich phases may contain any two of the four components mentioned above. The acyl ester:amino nitrogen ratios suggest that fractions 9 and 10 contain excess of acyl ester and phosphatidylserine, that fractions 11 and 12 contain phosphatidylserine and lysophosphatidylserine, and that fractions 13 and 14 contain lysophosphatidylserine and glycerylphosphorylserine. The last column of Table 1(b) indicates the extent of decomposition of the fractions, and is given by the percentage of the total amino nitrogen which is not present as phosphatidylserine.

During the process of elution of the column with the alkaline solvent system the percentage of material in the form of lysophosphatidylserine and especially of glycerylphosphorylserine increases, and these increases are accompanied by corresponding rises in the elution of excess of acyl ester.

The chloroform-rich phases from fractions 9–12 were combined and evaporated to dryness. The residue was dissolved in chloroform-methanol (49:1, v/v), and gave on analysis acyl ester:phosphorus:serine proportions of 1.84:1.00:0.79.

The combined aqueous phases from fractions 12 and 13 gave a phosphorus:serine ratio of 1.00:0.93, and were free from acyl ester. A 5 ml. sample was evaporated to dryness, and the residue was dissolved in 1 ml. of water (solution A). A portion (0.5 ml.) was treated with 0.05 ml. of concentrated hydrochloric acid, the mixture was heated on a boiling-water bath for 20 min., then evaporated to dryness in a current of nitrogen. Finally the dry residue was dissolved in 0.5 ml. of water (solution B). Solutions A and B (10 μ l.), together with serine and phosphorylserine markers, were examined by paper chromatography. Solution A gave a single spot which was positive for both amino nitrogen and phosphate, but was well separated from the phosphorylserine marker. Solution B gave a ninhydrin-reacting spot, identical in position with that given by serine. Thus the analytical values, coupled with chromatographic behaviour, indicate

the presence in solution A of an acid-labile substance containing both serine and phosphate, but distinct from phosphorylserine. Its properties suggest its probable identity with glycerylphosphorylserine.

DISCUSSION

Since its discovery by Folch & Schneider (1941; see also Folch, 1942) and by Schuwirth (1941, 1942), brain phosphatidylserine has proved extremely difficult to separate from other lipids by solvent-fractionation techniques (see Folch, 1948). Further, silicic acid chromatography of lipids by the technique of Hanahan *et al.* (1957) did not lead to a satisfactory separation.

Our past experience has been that alumina chromatography has one distinct advantage over silicic acid, namely that the separations are governed to a greater extent by the nature of the polar constituents of the lipids. Thus all the choline-containing lipids (lecithin, lysolecithin and sphingomyelin) are eluted together, as are the galactose-containing lipids, cerebroside and sulphatide (Long & Staples, 1961a). It was reasonable to expect, therefore, that the presence of a carboxyl group in phosphatidylserine would permit its separation from the ethanolamine-containing lipids. As shown in this paper, these two classes of phospholipid are in fact well separated on an alumina column, and C. Long & K. Owens (unpublished work) have found that phosphatidylinositol of liver has chromatographic properties on alumina which are intermediate between those of the ethanolamine- and serine-containing lipids, and can readily be separated from both.

Although the alumina used in this work is slightly alkaline to phenolphthalein in aqueous suspension, its amphoteric properties cause it to act as a powerful cation exchanger, so that acidic lipids such as sulphatide, when applied as the potassium salt, are eluted in the free-acid form (Long & Staples, 1961b). With phosphatidylserine, an alkaline solvent containing potassium hydroxide is required to elute it from the alumina. Even under these conditions, however, the bulk of the phosphatidylserine is eluted as the free acid. Not until about two-thirds of the phosphatidylserine has been eluted does the eluate contain potassium. It follows, therefore, that when the potassium salt of phosphatidylserine is applied to an alumina column in a neutral solvent the K^+ ions are adsorbed on acidic sites. When a solvent containing 0.009N-potassium hydroxide is used for elution, it is necessary to pass about 50 hold-up volumes through the column before potassium can be detected in the eluate. During this period it is presumed that the acidic sites are continuing to take up K^+ ions and that this capacity is very

considerable. It is not at all clear, however, why this process should lead to the simultaneous elution of the free-acid form of phosphatidylserine.

Alkaline solvents are not required to elute certain other acidic lipids from alumina. Thus sulphatide can be eluted by a chloroform-methanol-water (23:23:4, by vol.) solvent system (Long & Staples, 1961*a*, *b*). Similarly phosphatidylinositol is eluted by a similar solvent of higher water content (C. Long & K. Owens, unpublished work).

Chromatography on alumina suffers from the disadvantage that certain phospholipids undergo decomposition on contact with it. With the ethanolamine-containing phospholipids (Long & Staples, 1961*b*), the extent of decomposition is not very serious (about 3% in 24 hr. at room temperature). Phosphatidylserine is many times more labile, however, and it is necessary to conduct the chromatography very rapidly in order to isolate the intact lipid. In an experiment lasting 2-3 hr., about 20% decomposition occurred; in a 12 hr. experiment, about 85% decomposed.

Though the results presented are not absolutely conclusive, the evidence strongly suggests that the course of the decomposition is best explained by eqns. (1) and (2). The presence of excess of acyl ester, the ester:amino nitrogen ratios of the various fractions and the acid-lability of the water-soluble decomposition product are consistent with this scheme.

The nature of the phosphatidylserine eluted from silicic acid columns, i.e. whether as the free acid or as the potassium salt, has not been determined. The elution peak (Fig. 2), which occurs with chloroform-methanol (83:17, v/v), is very close to the position found by Marinetti *et al.* (1958) for the free acid form, however. Thus it may well be that silicic acid removes cations from phosphatidylserine, and this would be consistent with observations with another acidic lipid, namely sulphatide (Long & Staples, 1961*b*).

SUMMARY

1. Rat-brain lipid, free from water-soluble contaminants and protein, was applied to a short alumina column equilibrated with chloroform-

methanol-water (43:43:14, by vol.). In this solvent mixture the neutral lipids, together with the choline-, galactose-, ethanolamine- and inositol-containing lipids, passed through the column.

2. Phosphatidylserine was then eluted with an alkaline solvent system. Partial decomposition of the phosphatidylserine occurred and the eluted material also contained a non-nitrogenous phospholipid.

3. The analytical evidence was consistent with the view that the decomposition involved the deacylation of phosphatidylserine.

4. Final purification of the phosphatidylserine was effected on a silicic acid column. The product contained about 6% of serine plasmalogen.

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