Chromatographic Behaviour of Rat-Liver Monophosphoinositide

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1. Chromatography of rat-liver lipids on a column of silicic acid or a mixture of silicic acid and Hyflo Super-Cel, with chloroform-methanol mixtures, gave monophosphoinositide-containing fractions which were invariably contaminated by the presence of nitrogen-containing phospholipids. The behaviour of the inositide was extremely sensitive to column loading and the results with different batches of silicic acid were not reproducible. 2. However, when chromatography on an alumina column was used, the solvent system chloroform-methanol-water (23:23:4, by vol.) completely eluted the neutral lipids, choline-containing phospholipids and phosphatidylethanolamine. An increase of the water content of the solvent to 14% (by vol.) then led to the elution of the monophosphoinositide component, now free from nitrogen-containing phospholipids, but still contaminated by the presence of a phospholipid, which from its properties was taken to be polyglycerophosphatide. 3. Most of the polyglycerophosphatide could be removed from a rat-liver lipid extract by silicic acid chromatography with chloroform-methanol (19:1, v/v). The other phospholipids were then eluted and applied to an alumina column, whereby a monophosphoinositide fraction of much greater purity was obtained. 4. Further purification of the monophosphoinositide was achieved by chromatography on a mixture of silicic acid and cellulose powder. The final product was virtually pure by thin-layer chromatography and gave the expected analysis for monophosphoinositide.

Several groups of workers have described the chromatographic behaviour of the monophosphoinositide (phosphatidylinositol) component of rat-liver lipids on columns of silicic acid. Solvent systems containing chloroform and methanol have normally been used, and for the elution of this substance the percentage by volume of methanol required has been variously reported within the range from 5 to 50 (Dawson, 1958; Macfarlane, Gray & Wheeldon, 1960; Getz, Bartley, Stirpe, Notton & Renshaw, 1961; see also review by Wren, 1960). In some cases, a filter aid (Hyflo Super-Cel) has been added to the silicic acid (Hanahan, Dittmer & Warashina, 1957; Hanahan & Olley, 1958; Morgan, Tinker & Hanahan, 1963), but it has not been established whether this modification affects the chromatographic behaviour of monophosphoinositide and other phospholipids. It is generally agreed that silicic acid chromatography of crude lipid extracts never yields pure monophosphoinositide; the product is always grossly contaminated with nitrogen-containing lipids.

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Less information is available concerning the behaviour of monophosphoinositide on columns of alumina. However, Dawson (1958) reported that this phospholipid could be eluted from alumina by means of the solvent mixture chloroform-ethanol-water (2:5:2, by vol.). When this enriched material was subsequently chromatographed on a silicic acid column, an eluate was obtained from which it was possible to crystallize a pure sample of monophosphoinositide.

Hanahan (1960) has described the separation of phosphatidylethanolamine from monophosphoinositide on an alumina column, also using solvent mixtures containing ethanol, chloroform and water. However, no inositol determinations were reported, so that it is not possible to assess the purity of the monophosphoinositide eluted. On some occasions the latter was free from nitrogen, whereas on others the nitrogen-containing contaminant varied between 5 and 20%.

Previous experience with alumina columns (Long & Staples, 1961*a,b*; Long, Shapiro & Staples, 1962) has suggested that this adsorbent tends to separate lipids almost exclusively according to the nature of their major polar groups. Thus cerebroside and

sulphatide are eluted close together, as are the choline-containing phospholipids, lecithin, lysolecithin and sphingomyelin. In the present work, the chromatographic behaviour of the monophosphoinositide contained in a rat-liver lipid extract was examined on alumina. As a result, it was found possible to elute a fraction nearly 50% of which consisted of monophosphoinositide, and which was free from contamination by nitrogen-containing phospholipids. This material was subsequently purified by chromatography on a column composed of a mixture of silicic acid and cellulose.

A preliminary report on this work has already appeared (Long & Owens, 1962).

EXPERIMENTAL

Materials

Rat-liver lipid extract. The methods of extraction and removal of water-soluble contaminants were similar to those described for rat-brain lipids by Long & Staples (1961a). The homogenization of the tissue and filtration of the resulting homogenate were carried out under N_2 . Usually the livers from four rats (40–50 g. moist wt.) were taken and the extracted lipids were finally dissolved in an appropriate solvent. These extracts contained, on average, 36 μ g. atoms of lipid P/g. of fresh rat liver.

Column adsorbents. Silicic acid (A.R. 100-mesh powder; Mallinckrodt Chemical Works) was mechanically sieved (Endrock Sieve Shaker; Endecotts Ltd., London) and only those particles which passed an 85 but were retained by a 240 British Standards sieve were used. This material was washed by sedimentation in methanol and finally activated by heating at 120° for 24 hr. Alumina (Brockmann grade II) was obtained from Savory and Moore Ltd., London; Whatman no. 1 cellulose powder and Hyflo Super-Cel were purchased from L. Light and Co. Ltd., Colnbrook, Bucks. The last-named materials were used without further treatment.

Solvents. Methanol and $CHCl_3$ were purified according to Long & Staples (1961a).

Chemicals. The *meso*-inositol used as a standard was obtained from George T. Gurr Ltd., London. All other chemicals were the best available commercially.

Methods

General chromatographic procedures and analytical methods for the determination of total phosphorus, total nitrogen, amino nitrogen, ethanolamine and acyl ester were those described by Long & Staples (1959; 1961a,b).

Determination of inositol. The microbiological method of Norris & Darbre (1956) was used. The lipid, containing not more than 0.015 μ mole (2.7 μ g.) of inositol, was evaporated to dryness in a glass-stoppered 10 ml. tube. To the residue 1 ml. of constant-boiling HCl (about 5.5N) was added. The stoppered tube was heated on a boiling-water bath for 48 hr. After cooling, the hydrolysate was treated with 4 vol. of $CHCl_3$ -methanol (2:1, v/v). The contents were mixed thoroughly with a teat pipette and the lower $CHCl_3$ -rich phase, containing lipid-soluble material, was

withdrawn and discarded. The upper phase was washed twice in the same way with $CHCl_3$ -methanol (17:3, v/v). The final aqueous methanolic phase was evaporated to dryness in a current of air on a warm-water bath, and the unstoppered tube was placed in a vacuum desiccator over solid KOH for at least 1 hr. The residue was dissolved in 3.5 ml. of water, and 3 ml. of this solution was taken for inositol determination by using *Schizosaccharomyces pombe*. Inositol standards submitted to the hydrolytic and extraction procedures gave recoveries in the range 98–100% of the calculated values.

Thin-layer chromatography. Hot-air dried films (250 μ thick) of Kieselgel G or H (Merck) were washed by the ascending technique with the solvent mixture $CHCl_3$ -methanol-water (60:35:8, by vol.) before activation at 105° for 30 min. The bulk of an unidentified yellow substance which normally travelled with the solvent front in water-containing solvents and which charred strongly after spraying and heating with H_2SO_4 was removed by this means. The solvent mixtures used for chromatography were $CHCl_3$ -methanol-water (65:25:4 or 65:35:8, by vol.) as recommended by Wagner, Hörhammer & Wolff (1961), and $CHCl_3$ -methanol-water-acetic acid (65:40:3:1, by vol.), which is a modification of the system described by Skipski, Peterson, Sanders & Barclay (1962).

Phospholipids were detected by means of the molybdate-hydrazine- H_2SO_4 spray used by Long, Odavić & Sargent (1962). Amino nitrogen was detected after spraying with the reagent of Moore & Stein (1948), from which the $SnCl_2$ had been omitted. Chromatograms which had been sprayed with either of the above reagents could subsequently be sprayed with 50% (v/v) H_2SO_4 and charred at 180° for detection of organic substances.

RESULTS AND DISCUSSION

Elution of rat-liver monophosphoinositide from columns of silicic acid with and without addition of Hyflo Super-Cel

Equal quantities of mixed rat-liver lipids, dissolved in 4.5 ml. of chloroform-methanol (99:1, v/v), containing 16.4 μ moles of lipid inositol and 250 μ g. atoms (7.75 mg.) of lipid phosphorus, were applied to three 1 cm.-diameter columns, consisting of (a) 8 g. of silicic acid, (b) 8 g. of silicic acid plus 4 g. of Hyflo Super-Cel, and (c) 12 g. of silicic acid. The loadings were therefore 0.97, 0.97, and 0.65 mg. of lipid phosphorus/g. of silicic acid respectively. Fractions (25 ml.) were collected, as follows: nos. 1–3 with chloroform-methanol (99:1, v/v), 4–9 with chloroform-methanol (4:1, v/v), and 10–13 with chloroform-methanol (3:2, v/v). All fractions were examined by thin-layer chromatography. Fractions 1–3, 4–9 and 10–13 were then combined and analysed for phosphorus, nitrogen and inositol. The results are shown in Table 1.

When columns (a) and (c) were compared, silicic acid alone being used, no differences were noted in the elution of monophosphoinositide, which was present in both the 4–9 and 10–13 fractions, but the higher phosphorus loading in column (a)

Table 1. *Elution of rat-liver lipids from silicic acid columns*

Rat-liver lipid, containing 250 μ g.atoms of lipid P and 16.4 μ moles of inositol, was applied to each column. All fractions were of 25 ml. Abbreviations: NL, neutral lipids; PE, phosphatidylethanolamine; MPI, monophosphoinositide; Lec, lecithin; PGP, polyglycerophosphatide; Sph, sphingomyelin.

Column ...	(a)			(b)			(c)		
Silicic acid (g.) ...	8			8			12		
Hyflo Super-Cel (g.) ...	0			4			0		
Fractions ...	1-3	4-9	10-13	1-3	4-9	10-13	1-3	4-9	10-13
CHCl ₃ -methanol (v/v) ratio	99:1	4:1	3:2	99:1	4:1	3:2	99:1	4:1	3:2
Thin-layer chromatography	NL	PGP PE MPI Lec	MPI Lec Sph	NL	PGP PE MPI Lec	MPI Lec Sph	NL	PGP PE MPI	MPI Lec Sph
Phosphorus (μ g.atoms)	—	116.2	101.1	—	140.0	53.2	—	76.6	123.0
Nitrogen (μ g.atoms)	—	104.7	102.2	—	120.6	54.1	—	70.1	123.2
Inositol (μ moles)	0	6.91	9.28	0	11.17	3.64	0	6.43	9.60
N/P ratio	—	0.90	1.01	—	0.86	1.02	—	0.91	1.00
Inositol/P ratio	—	0.060	0.092	—	0.080	0.068	—	0.084	0.078
Inositol recovery (%)	98.9			90.6			98.0		

resulted in elution of an appreciable amount of lecithin in fractions 6-9, as seen by thin-layer chromatography, suggesting that this column was overloaded even though less than 1mg. of lipid phosphorus/g. of silicic acid had been applied. The addition of Hyflo Super-Cel (column b) resulted in an increased elution of lecithin in fractions 4-9, as shown both by thin-layer chromatography and determination of phosphorus and nitrogen; more monophosphoinositide was also present in these combined fractions.

It is not clear why the addition of Hyflo Super-Cel should have diminished the capacity of silicic acid to retain both lecithin and monophosphoinositide in the presence of chloroform-methanol (4:1, v/v). Further, the elution of both these lipids by this solvent system is contrary to the observations of Hanahan *et al.* (1957) and of Hanahan & Olley (1958). This apparent discrepancy may be due to the fact that the silicic acid used here had been sieved so as to eliminate the finest particles, and it may be that these have the greatest capacity for the retention of lipids.

It seems, however, that the chromatographic behaviour of monophosphoinositide can also vary according to the batch of silicic acid used. In an earlier experiment (C. Long & K. Owens, unpublished work) a result was obtained which was more in accordance with the experience of Hanahan *et al.* (1957). With a column composed of 8g. of silicic acid and 4g. of Hyflo Super-Cel (i.e. similar to column b or Table 1) and a loading of 0.83mg. of lipid phosphorus/g. of silicic acid, chloroform-methanol (4:1, v/v) eluted the bulk of the aminophospholipid; the monophosphoinositide, however, was only eluted with chloroform-methanol (3:2, v/v).

In all three experiments, shown in Table 1, the

monophosphoinositide was present in fractions which also contained much larger quantities of nitrogen-containing lipids. In fractions 4-9 from all three columns, thin-layer chromatography showed also the presence of a fast-running phospholipid, which was presumed to be polyglycerophosphatide (Macfarlane, 1961). It gave no reaction with the ninhydrin reagent.

The bulk of the polyglycerophosphatide could be separated from the monophosphoinositide in the following way. With a column of silicic acid alone, after elution of the neutral lipids with chloroform-methanol (99:1, v/v), the solvent was changed to chloroform-methanol (19:1, v/v) as described by Macfarlane (1961). Under these conditions most of the polyglycerophosphatide was readily eluted and was not accompanied by any monophosphoinositide. A further change of solvent to chloroform-methanol (1:1, v/v) eluted all the monophosphoinositide and most of the other phospholipids, and the contamination by polyglycerophosphatide was minimal.

Chromatography on alumina

Dawson (1958) reported that monophosphoinositide could be eluted from an alumina column by means of the solvent system chloroform-ethanol-water (2:5:2, by vol.). We have confirmed this finding. In one experiment, rat-liver lipids dissolved in chloroform-methanol (1:1, v/v) were applied to an alumina column; continued passage of this solvent caused elution of the neutral lipids and choline-containing phospholipids. When the solvent was changed to chloroform-ethanol-water (2:5:2, by vol.) the monophosphoinositide was eluted together with much aminophospholipid, so that the mean molar ratio, inositol:phosphorus, was only 0.24. Since Dawson (1958) and Hanahan

(1960) had already shown that phosphatidylethanolamine was somewhat more easily eluted than was monophosphoinositide from alumina, less polar solvent mixtures were tested to discover whether a satisfactory separation could be achieved. For this purpose, several experiments of the following type were carried out.

Rat-liver lipids, equivalent to about 5g. of fresh tissue, were dissolved in chloroform-methanol (1:1, v/v) containing between 6 and 10% (by vol.) of water, and the solution (about 10ml.) was applied to an alumina column (13g.; 17cm. $\times$ 1cm. diam.) equilibrated with the same solvent. Several 25ml. fractions were collected, and the eluting solvent was then changed to chloroform-methanol-water (43:43:14, by vol.). More fractions were collected, and all were analysed for amino nitrogen, phosphorus and inositol. It was found that when the first solvent contained 8% (by vol.) of water, i.e. had the composition chloroform-methanol-water (23:23:4, by vol.), all the phosphatidylethanolamine was eluted from the column while the monophosphoinositide remained adsorbed. When the water content of the solvent was subsequently increased to 14% (by vol.), the monophosphoinositide was eluted free from nitrogen-containing contaminants. The trace of phosphatidylserine present in rat-liver lipid would remain firmly adsorbed upon the column (Long *et al.* 1962).

If the first solvent had contained only 6 or 7% (by vol.) of water, incomplete elution of phosphatidylethanolamine was seen; if, however, this solvent contained 9 or 10% (by vol.) of water, then some monophosphoinositide was also eluted. It appears therefore that a water content very close to 8% (by vol.) in the first solvent is quite critical for separating the phosphatidylethanolamine from the monophosphoinositide.

The following experiment illustrates the preparation of a monophosphoinositide-rich fraction, free from nitrogen-containing phospholipids. Rat-liver lipid, derived from 4.73g. of fresh tissue, was dissolved in 5ml. of chloroform-methanol-water (23:23:4, by vol.). It was applied to an alumina column of similar dimensions to those already described, equilibrated with the same solvent. This corresponded with a column loading of 0.34mg. of lipid phosphorus/g. of alumina. The column was developed with this solvent and fractions were collected until the amino nitrogen value had fallen to less than 0.06 $\mu\text{g. atom}/25\text{ml.}$; this occurred after 475ml. of solvent had been collected.

A water gradient was then applied, as described by Long & Staples (1961a). The mixing vessel contained 125ml. of chloroform-methanol-water (23:23:4, by vol.) and chloroform-methanol-water (43:43:14, by vol.) was placed in the reservoir. Sixteen 25ml. fractions were collected. Ethanol-

Fig. 1. Chromatography of mixed rat-liver lipids on a column of alumina (13g.; 17cm. $\times$ 1cm. diam.), with gradient elution whereby the water content (broken line) of the chloroform-methanol (1:1, v/v) solvent was increased from 8 to 13.5% (by vol.). The lipid was derived from 4.73g. of rat liver (moist wt.). $\circ$, Inositol; $\bullet$, phosphorus; $\blacksquare$, ethanolamine. For clarity, some of the experimental points have been omitted.

amine determinations were made on all the early fractions and until just after the gradient was applied; total phosphorus and inositol determinations were made on all fractions. The result is shown in Fig. 1. It will be seen that the skewed peak, eluted after applying the gradient, contains both phosphorus and inositol. However, the inositol:phosphorus ratio was always less than unity; the highest value in any fraction was 0.72, and the mean was only 0.40. Thin-layer chromatography with neutral solvent mixtures indicated the presence of other phosphorus-containing compounds, most of which had R_f values higher than that of monophosphoinositide, and were judged to consist of polyglycerophosphatide and its breakdown products. One phosphorus-containing contaminant had a lower R_f value, however. The inositol-containing fractions were free from phosphatidylethanolamine, as shown also by thin-layer chromatography. When the inositol-containing fractions were combined and analysed, nitrogen could not be detected in the product. The recovery of inositol from the column was 86%.

To minimize contamination of monophosphoinositide by polyglycerophosphatide or its breakdown products, the alumina chromatography experiment was repeated with a rat-liver lipid preparation from which the bulk of the polyglycerophosphatide had previously been removed on a silicic acid column. This was effected by eluting the silicic acid column with chloroform-methanol (19:1, v/v) as already described,

Fig. 2. Chromatography of rat-liver lipids on a column of alumina (13g.; 17cm. $\times$ 1cm. diam.). The bulk of the polyglycerophosphatide had previously been removed by chromatography on a column of silicic acid, as described in the text. After 500ml. of chloroform-methanol-water (23:23:4, by vol.), gradient elution was applied whereby the water content (broken line) of the solvent was increased to 12.5% (by vol.). $\circ$, Inositol; $\bullet$, phosphorus; $\blacksquare$, amino nitrogen. For clarity some of the experimental points have been omitted.

before using chloroform-methanol (1:1 v/v), to recover the remaining phospholipids in a relatively small volume of eluate. This latter was then applied to an alumina column.

The result of this experiment is shown in Fig. 2, from which it will be seen that the monophosphoinositide elution peak is now almost symmetrical. The fractions which contained monophosphoinositide were also examined by thin-layer chromatography. Spots corresponding to polyglycerophosphatide or its breakdown products were now much fainter; the slow-running phospholipid spot was also just visible. The combined monophosphoinositide peak gave an average inositol:phosphorus ratio of 0.76, compared with 0.40 when crude rat-liver lipids were chromatographed directly on alumina. The recovery of inositol from the column was 93%.

Further purification of monophosphoinositide

The material used for further purification was that obtained from rat-liver lipid after (a) preliminary removal of most of the polyglycerophosphatide on a silicic acid column, followed by (b) gradient elution from an alumina column, as described in the last section. It was 60–80% pure, as judged from the inositol:phosphorus ratio, and was free from nitrogen-containing contaminants. This material was rechromatographed on silicic

Fig. 3. Rat-liver lipids were first enriched in monophosphoinositide by chromatography on an alumina column, under the conditions shown in Fig. 2. This material was chromatographed on a column of silicic acid-cellulose powder (2:1, w/w). First solvent (0–200ml.), chloroform-methanol (23:2, v/v); second solvent (at arrow A), chloroform-methanol (3:1, v/v) containing 2% (by vol.) of water; third solvent (at arrow B), chloroform-methanol-water (23:23:4, by vol.). Volumes of collected fractions: 1–4, 10ml.; 5–10, 25ml.; 11, 10ml.; 12–21, 4ml.; 22–25, 10ml.; 26–30, 25ml. For further details, see text. $\blacksquare$, Acyl ester; $\bullet$, phosphorus; $\circ$, inositol. For clarity, some of the overlapping inositol and phosphorus points have been omitted. Fractions 2, 3, 4, 5, 15, 18 and 21–30 were examined by thin-layer chromatography (see Fig. 4.).

acid alone, and on silicic acid mixed with Hyflo Super-Cel, but no substantial purification was effected in either case.

Marinetti & Stotz (1956) had shown that model mixtures of phospholipids could be separated successfully from one another upon paper impregnated with silicic acid, using chloroform-methanol (3:1, v/v) containing 2% (by vol.) of water. By analogy, it seemed possible that a separation of monophosphoinositide from its contaminants might be obtained by chromatography on a column composed of silicic acid and cellulose powder. For this purpose, a column was prepared consisting of 8g. of silicic acid and 4g. of cellulose powder (36cm. $\times$ 1cm. diam.), equilibrated with chloroform-methanol (23:2, v/v). Partially purified monophosphoinositide, containing 21.6 μ moles of inositol and 34.4 μ g. atoms of phosphorus, dissolved in 10ml. of the same solvent mixture, was applied to the column, which was eluted with 200ml. of the same solvent. The solvent was then changed to chloroform-methanol (3:1, v/v) containing 2% (by vol.) of water. Finally the column was eluted with chloroform-methanol-water (23:23:4, by vol.). The result is shown in Fig. 3. Total recoveries were:

Fig. 4. Thin-layer chromatogram of selected fractions taken from the experiment described in Fig. 3. The volumes of the various fractions were such as to contain about $0.03 \mu\text{g. atom}$ of total phosphorus. These were evaporated to dryness, dissolved in a few drops of chloroform-methanol (2:1, v/v) and transferred to the Kieselgel G plate. The chromatogram was developed with chloroform-methanol-water (65:25:4, by vol.). The plate was sprayed with 50% (v/v) H_2SO_4 and heated at 180° .

phosphorus, $35.1 \mu\text{g. atoms}$ (102%); inositol, $21.1 \mu\text{moles}$ (98%). Selected fractions were examined by thin-layer chromatography (Fig. 4). It will be seen from Figs. 3 and 4 that the early fractions eluted with chloroform-methanol (23:2, v/v) contained phosphorus but no inositol. After the change of solvent (at point A, Fig. 3) the eluted material consisted of two peaks, the first containing phosphorus but no inositol, the second containing both phosphorus and inositol, and giving only one major spot on thin-layer chromatography; the latter appears to be substantially pure monophosphoinositide. When these fractions (nos. 23-28) were combined, representing 89% of the lipid inositol applied to the column, the overall inositol:phosphorus ratio was found to be 0.96. It is not certain whether the divergence from unity is due to residual contamination or experimental error. It is possible that during the acid hydrolysis of the monophosphoinositide before microbiological assay, some of the inositol may have undergone decomposition while still in combined form. Free inositol is not destroyed under the conditions of hydrolysis.

The average ester:phosphorus ratio was 2.24, a sample of pure ethyl palmitate being used as the standard for acyl ester. This agrees closely with an apparent average value of 2.25 for the

ester:phosphorus ratio of pure ovolecithin, also with ethyl palmitate as the standard.

When the solvent was finally changed at B (Fig. 3) to chloroform-methanol-water (23:23:4, by vol.), other phospholipids were eluted (Fig. 4) including the slow-running spot already referred to.

The overall recovery of lipid inositol as a result of the three successive column-chromatographic procedures, namely silicic acid alone, alumina and silicic acid plus cellulose powder, was 91%.

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