

A COLUMN CHROMATOGRAPHIC SEPARATION OF CLASSES OF PHOSPHOLIPIDES*

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The separation and purification of the individual components of mixed phospholipides from tissues have long posed a difficult problem. In addition to the possible oxidative changes and enzymatic alterations that can occur during the isolation procedure, one is confronted with the presence of a variety of possible contaminants, such as free sterols, steroid glycosides, free amino acids, glycerides, sugars, salts, etc., which complicate the fractionation scheme. Several possible routes of fractionation of the mixed lipides have been utilized, i.e. salt fractionation, low temperature separation, and adsorption chromatography. The classical cadmium chloride procedure of Levene and Rolf (1) has provided a useful route to a fairly pure lecithin. Through the use of solvents at a low temperature Sinclair (2) was able to separate, though in low yields, a reasonably pure lecithin. Taurog *et al.* (3) were able to fractionate the choline-containing phospholipides from the non-choline-containing phospholipides of liver by differential adsorption on magnesium oxide. More recently, a column chromatographic technique which employs aluminum oxide (4) has allowed a facile approach to the preparation in good yield of highly purified lecithin. However, none of these procedures mentioned above has permitted the subsequent isolation of the other components, "the cephalins," and hence has presented a limitation. Within the past few years, the availability of pure silicic acid has made possible its application to the chromatographic separation of the phospholipides. McKibbin, by employing essentially a gradient elution scheme with silicic acid as the adsorbent, was able to separate a polyglycerophosphatide (5) and an inositide (6) from dog and horse liver, respectively. Lea *et al.* (7) applied the silicic acid technique only to the separation of the two major components of egg-mixed phospholipides.

In the present communication, a procedure is described wherein the various classes of compounds in the mixed phospholipides from rat liver, beef liver, and bakers' yeast can be separated in a reasonable state of purity on a single column of silicic acid.

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EXPERIMENTAL

Materials and Methods

The silicic acid was Mallinckrodt's analytical reagent, suitable for chromatographic analysis. Whenever possible, fresh bottles of the acid were used. Otherwise, before use, the silicic acid was dried for 12 hours at 105°. As an aid in the flow of solvent through the columns, Hyflo Super-Cel (Johns-Manville) was mixed with the silicic acid (2 parts silicic acid to 1 part Super-Cel). Reagent grade solvents were used.

Phosphorus was assayed by King's method (8), nitrogen by the micro-Kjeldahl procedure, with selenium oxychloride as catalyst, and choline by Glick's method (9). Sphingosine nitrogen was determined by Carter's¹ modification of the method of McKibbin and Taylor (10); this modification employed a 6 hour reflux of the lipid with saturated Ba(OH)₂, cooling, and acidification with acid in the cold. The mixture is then extracted with chloroform and the CHCl₃-soluble fraction is analyzed for total nitrogen and phosphorus. Total fatty acids were isolated by ether extraction of an acidified hydrolysate of a 5 hour 0.5 N KOH reflux, washed well with water, and processed in the usual manner. Total glycerophosphate was determined by Burmaster's periodate method (11) on the water-soluble fraction from the above hydrolysate and also identified by paper chromatography (12). Unsaturation was assayed either by the Wijs iodine absorption method (13) or by catalytic hydrogenation over PtO₂. Inositol was assayed by the microbiological technique with use of *Kloeckera apiculata* as the test organism.² The lipid sample for inositol and glycerol assay was refluxed for 48 hours in 2 N HCl, cooled, and filtered. If the lipid sample was refluxed in 6 N HCl for 48 hours, as much as 70 per cent of the glycerol was destroyed. Also, it was not advisable to remove any excess acid from a glycerol sample by evaporation on a steam bath, as a considerable loss of glycerol, but not inositol, occurred. Removal of mineral acid and any hydroxy amines can be achieved with no loss of inositol or glycerol, by passage of the sample through Amberlite IR-45 and IRC-50 exchange resins. Glycerol can be assayed quantitatively in a hydrolysate by a modified periodate oxidation procedure in which the liberated formaldehyde is detected by reaction with chromotropic acid.³ Glycerol and inositol can also be identified qualitatively by chromatography on Whatman No. 3 paper with propanol-water (4:1) and by spraying with AgNO₃ reagent (14). In addition, glycerol may be assayed quantitatively

¹ The authors are indebted to Dr. Herbert E. Carter for the details of his unpublished method.

² We are indebted to Dr. Howard Douglas for his aid in establishing this procedure. This particular organism has an absolute requirement for *myo*-inositol.

³ Olley, J., unpublished observations.

by first being separated by chromatography on borax-impregnated paper (Whatman No. 3) with 0.5 per cent borax-*n*-propanol (1:1) as the solvent, and by location of the spot with lead tetraacetate reagent. Subsequently the spot can be eluted with water and the formaldehyde determined by the chromotropic acid method.³

The detection of bases such as choline was also made by the paper chromatographic procedure of Levine and Chargaff (15), but with 80 per cent propanol as the solvent in an ascending system. Acetals were detected by a modified Feulgen reaction (16), carbohydrates by the Molisch reaction, and sterols by the Liebermann-Burchard test. Free amino acids were detected by paper chromatography of the samples with chloroform-methanol (4:1)-water (0.5 per cent) as the solvent and by spraying with ninhydrin reagent. Under these conditions the amino acids and ethanolamine do not migrate.

Amino acids and other ninhydrin-reactive components such as ethanolamine were detected qualitatively by a paper chromatographic technique which utilized the methods of Moore and Stein (17) and Connell *et al.* (18). The most effective medium for the hydrolysis of the phospholipides was anhydrous methanolic 6 *N* HCl, which caused the least formation of free ammonia from either serine or ethanolamine.

Isolation of Phospholipides

In all instances the lipides were isolated from a tissue by repeated ethanol-diethyl ether extractions at room temperature. The particular tissue (except yeast)⁴ was homogenized for 2 minutes in a Waring blender with 95 per cent ethanol and then allowed to stand at room temperature for 4 hours. The mixture was filtered as rapidly as possible, leaving the residue slightly moist with solvent each time. This material was then reextracted with a 95 per cent ethanol-diethyl ether (3:1) mixture for an additional 3 hours and then filtered. The residue was reextracted for a third time with the same solvent system and the filtrate from all the extractions then concentrated at 37° *in vacuo* to remove the solvent. The concentrates were combined and extracted with petroleum ether, except with yeast extracts in which diethyl ether is the preferred extractant at this stage. The ether extracts were washed five to six times with small volumes of water and then concentrated to a small volume and 10 volumes of acetone were added. The mixture was then placed at -25° for several hours to insure a satisfactory separation of the phospholipides. The acetone-insoluble material, representing 90 to 95 per cent of the total

⁴ Fresh bakers' yeast is extracted for 8 hour periods each time with these solvent systems. We wish to thank Standard Brands, Inc., Sumner, Washington, for generous supplies of the yeast.

phospholipides, was washed several times with acetone, redissolved in ether, and reprecipitated with acetone. This procedure was repeated three times. If this material were not chromatographed immediately, it was stored under acetone at -25° in the dark. When this fraction was to be used as the total mixed phospholipides, it was dissolved in a chloroform-methanol mixture (usually 4:1 or in certain instances, 7:1) and then applied directly to a column. When the cephalin fraction was desired, the above acetone-insoluble fraction was warmed at 35° *in vacuo* to remove traces of solvent. The residue was treated with 95 per cent ethanol and mixed as well as possible and allowed to stand at room temperature for 3 to 4 hours. The ethanol-soluble fraction, which contained the bulk of the lecithin fraction and some phosphatidylethanolamine, was removed usually by decantation, and the residue reextracted with 95 per cent ethanol for an additional 4 hours at room temperature. The ethanol-insoluble fraction was then dissolved in CHCl_3 -MeOH, 4:1 or 7:1, and used directly for the chromatographic separation. An alternative procedure was to dissolve the acetone-insoluble fraction in a small volume of chloroform and add excess 95 per cent ethanol. This mixture was then placed at -25° for several hours. However, the amounts of cephalin fraction recovered by this procedure were often much lower than those recovered by the direct ethanol extraction of the acetone-insoluble fraction.

Chromatographic Separation

The following technique has been adopted as a general procedure for the fractionation of mixed phospholipides on silicic acid.

Preparation of Columns—Essentially the columns were prepared by the procedure outlined by Lea *et al.* (7). The silicic acid and Hyflo Super-Cel⁶ were mixed in a ratio of 2 parts to 1, respectively, and suspended in the initial solvent system (i.e. chloroform-methanol, 4:1) and filtered with suction on a sintered glass Büchner funnel. The residue was resuspended in the same solvent and the washing and filtration procedure were repeated for three additional times. Finally the washed silicic acid was suspended in the same solvent system and poured into the columns described below.

Two different sized columns have been used, the characteristics of which are as follows:

Type A Column—This column measured 30×400 mm. and was tapered at the lower end to approximately 8 mm. It was fitted at the top with a ball and socket joint to which was attached a solvent reservoir, approximately 1 liter in capacity, and this reservoir was coupled to a cylinder of

⁶ If the Hyflo Super-Cel was heated to 110° before use, there was an almost complete reversal in the elution pattern of the compounds and a considerable overlapping of the eluted components.

nitrogen. This sized column could accommodate 60 to 80 gm. of silicic acid (plus Super-Cel). The usual load was 0.8 to 1.0 mg. of phospholipide phosphorus per gm. of silicic acid and flow rates were 1.5 to 2.0 ml. per minute, maintained if necessary by slight nitrogen pressure. A glass wool plug, approximately 25 mm. thick, was used as a support for the silicic acid.⁶

Type B Column—This column measured 42 X 890 mm. and had a standard taper perforated glass disk (Scientific Glass Apparatus Company, interjoint T 35/45) at the lower end of the column, and on this was placed a thin layer of glass wool (~10 mm. thick) for support of the silicic acid. The remainder of the column, reservoir, etc., was constructed in essentially the same manner as Type A column. This sized column could hold 300 gm. of silicic acid (plus Super-Cel) and again the normal loading range was 0.8 to 1.0 mg. of phospholipide P per gm. of silicic acid. The flow rate was usually 1.5 to 2.0 ml. per minute, maintained, if necessary, by a slight nitrogen pressure.

Chromatographic Procedure—After the silicic acid had reached a constant level, the solvent was then allowed to drain nearly to the surface of the silicic acid and the sample in 10 ml. of the initial solvent for Type A column (and in 30 ml. for the Type B column) was allowed to flow slowly on to the column so as not to disturb the surface. Gentle tapping of the column at this stage aided in maintaining a level surface to the silicic acid. The solvent was allowed to drain nearly to the surface, and additional small quantities of solvents were added slowly until the sample was completely washed on to the column. Then additional solvent was added carefully, and the column was attached to a fraction collector. The size of the fractions collected was 8 ml. with the small and 16 ml. with the large columns. In each experiment, the progress of the fractionation was followed by phosphorus analyses on each individual tube and, when it was necessary to change to a new solvent system, the new solvent was added after the previous solvent had reached the surface of the silicic acid.

Solvent System—To date, the solvent system most satisfactory for the separation of mixed phospholipides or cephalins on silicic acid has been mixtures (v/v) of chloroform and methanol applied in the following dilutions: (1) 7:1 or (2) 4:1, (3) 3:2, and (4) 1:4. These solvent combinations permit recovery of at least 88 to 95 per cent of the phospholipide phosphorus applied to the columns. The composition of the initial eluting solvent will depend upon the nature of the phospholipide mix-

⁶ When a plug of cotton was used in addition to the glass wool plug for a support of the silicic acid, approximately 20 per cent of the phospholipides, particularly the phosphoinositides, was adsorbed by the cotton and could not be easily removed.

ture. In general an exploratory experiment with Solvent 2 as the first solvent will indicate the ease with which the fast moving fractions can be separated by this system. The use of a gradient elution technique, wherein subtle changes in the polarity of the solvents are made, was not successful with these materials. Specifically it was found that, unless sharp substantial changes were made in the ratio of the solvents, there was a tendency towards general "smearing" of all the fractions. For example, a change from a chloroform-methanol mixture of 4:1 (Solvent 2) to 3:1 or 2:1 completely obscured the inositol lipides peak, and as a result it was eluted with the lecithin fraction. In addition, the length of time for elution of the various components by this approach (up to 2 to 3 days for a 60 gm. silicic acid column) was not justified by the inadequate fractionation results.

When egg-mixed phospholipides were separated on a silicic acid column by use of the solvent system recommended by Lea *et al.* (7), namely Solvent 2, the phosphatidylethanolamine was removed readily, but the subsequent elution of the lecithin in the same solvent was extremely slow. However, the lecithins can be removed at a much more rapid rate with Solvent 3.

General Comments on Column Performance—The reproducibility of the columns has been excellent. Over twenty different columns have been run on the phospholipides from each of the sources described and there has been good agreement as to elution pattern, composition of the fractions, and application of this technique to large as well as small scale preparation of individual components. No untoward effects of the silicic acid, light, or temperature (25–28°) upon the elution pattern or composition have been noted. This strongly supports the conclusion that these components are present in the original tissues and have not arisen as artifacts of the isolation procedure.

The time involved in the complete fractionation of a particular mixture varies with the type of sample, size of column, and the flow rate. In general, with the mixed phospholipides a complete fractionation on a 60 gm. column can be accomplished in 20 hours and with a 250 gm. column in 48 hours at a flow rate of 1.5 to 2.0 ml. per minute. Through the use of the cephalin fractions, this time period can be reduced by a minimum of 4 hours in each case.

While it might be desirable to use higher flow rates than described here, the increased nitrogen pressure required to maintain a high flow rate (4 to 5 ml. per minute) will cause ultimately a tight packing of the column and a substantial decrease in the flow of solvent. Except for solvent changes, it is best not to interrupt the fractionation procedure once it has been started.

Chemical Nature of Eluted Fractions—In all the sources described here, the mixed phospholipides have been separated into five distinct fractions. The graphic results of experiments on the separation of the mixed phospholipides of rat liver, beef liver, and bakers' yeast are shown in Figs. 1, 2, and 3, and the data on the composition of the individual fractions, without further purification or treatment, are recorded in Tables I, II, and III. The results may be separated into categories as follows, according to the components removed by each solvent system.

Solvent 2, Fraction A (or Solvent 1)—The initial phosphorus-containing fraction from beef liver- and yeast-mixed phospholipides was composed of

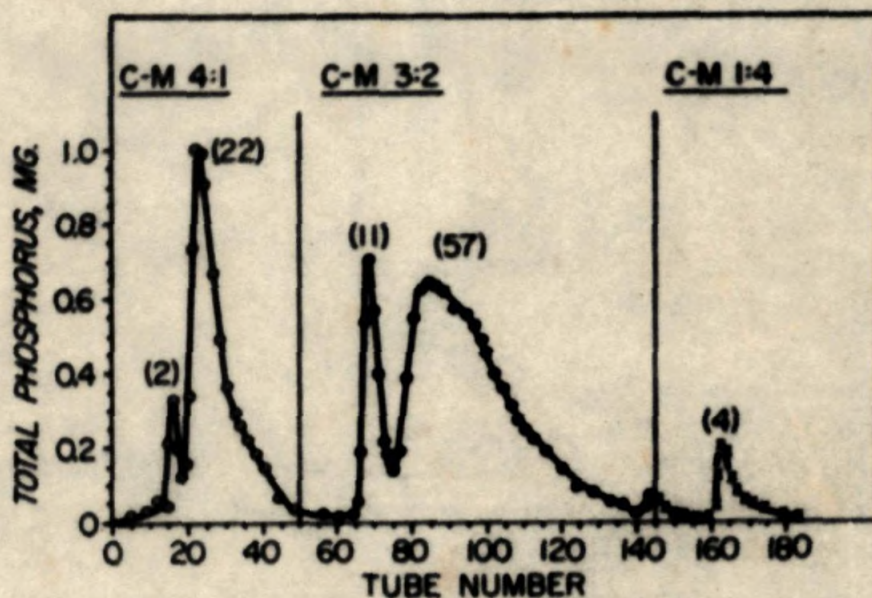


FIG. 1. Chromatogram of rat liver-mixed phospholipides (40 mg. of P) on a 60 gm. silicic acid-30 gm. Hyflo Super-Cel column. The eluting solvents were mixtures of chloroform and methanol (v/v). The numbers above the various fractions represent the per cent of the total applied phosphorus eluted. The composition of each of the fractions is recorded in Table I.

a low nitrogen-containing component, the bulk of the pigment of the original sample and traces of glycerides, sterols, and sterol glycosides. The phosphorus-containing component may be comparable to the polyglycerophosphatide described by McKibbin and Taylor (5), but insufficient data are available for a suitable comparison. In the particular experiments cited here, the initial fraction from rat liver phospholipides contained mainly phosphatidylserine, but it represents only a small percentage of the total phosphatidylserine which is found in the next fraction.

Solvent 2, Fraction B (or Solvent 2)—In all the sources examined here, this eluent had, as its main components, phosphatidylserine and phosphatidylethanolamine. While there have been many methods described in the literature for the quantitative estimation of ethanolamine and

serine in phospholipides, these techniques have not worked to our satisfaction. Although this problem is under active investigation at present

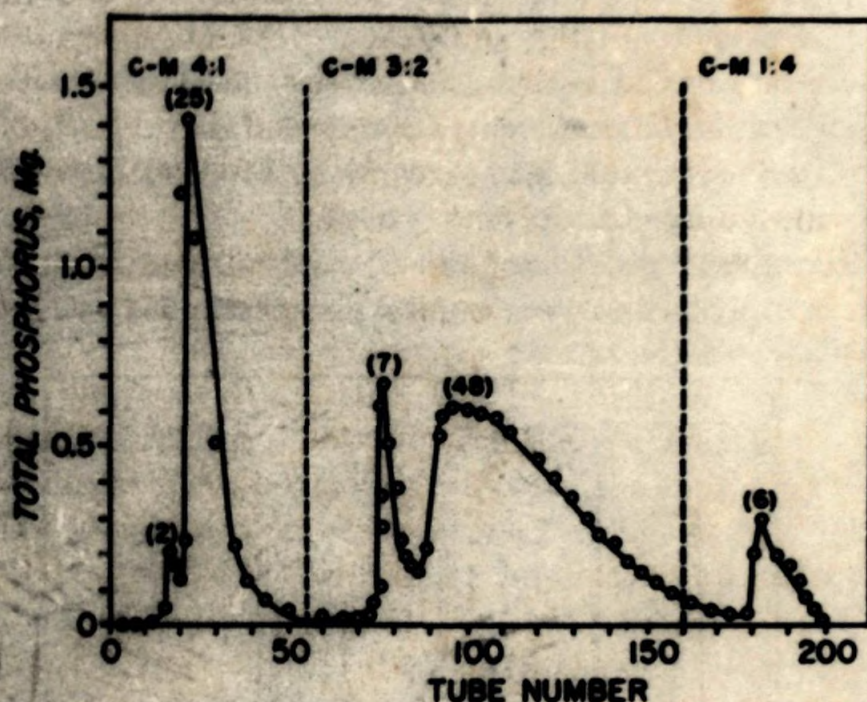


FIG. 2. Chromatogram of beef liver-mixed phospholipides (53 mg. of P) on a 60 gm. silicic acid-30 gm. Hyflo Super-Cel column. The eluting solvents were mixtures of chloroform and methanol (v/v). The numbers above the various fractions represent the per cent of the total applied phosphorus eluted. The composition of each of the fractions is recorded in Table II.

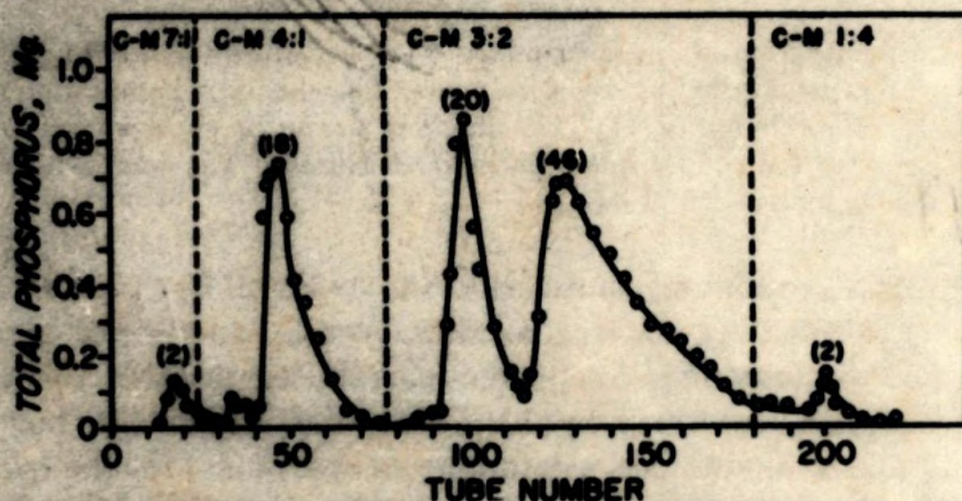


FIG. 3. Chromatogram of yeast-mixed phospholipides (47 mg. of P) on a 60 gm. silicic acid-30 gm. Hyflo Super-Cel column. The eluting solvents were mixtures of chloroform and methanol (v/v). The numbers above the various fractions represent the per cent of the total applied phosphorus eluted. The composition of each of the fractions is recorded in Table III.

in this Laboratory,⁷ there are two main considerations of concern; namely, the mode of hydrolysis, wherein the least amount of ammonia from degrada-

⁷ Unpublished observations.

tion of the ethanolamine and serine occurs, and the subsequent unequivocal assay of these nitrogen components. In view of our evidence to date with ion exchange resins for separation of ethanolamine and serine, the

TABLE I
Composition of Fractions Obtained from Chromatogram of Rat Liver-Mixed Phospholipides on Silicic Acid-Hyflo Super-Cel (3:1)* (Fig. 1)

	Solvent 2, Fraction A†	Solvent 2, Fraction B†	Solvent 3, Fraction A†	Solvent 3, Fraction B†	Solvent 4 fraction
P, %	3.91	4.01	3.34	3.82	3.80
N, %	1.82	1.79	0.27	1.71	2.93‡
Choline, %	None present	None present	None present	14.70	10.3
N:P, molar ratio	1.03	0.98	0.18	0.98	1.70
Choline:P, molar ratio				0.99	0.70
Inositol, %	None present	None present	11.6	0.2	None present
Inositol:P, molar ratio			0.60	0.01	
Glycero-P:P, molar ratio	0.95	0.97	Not run	0.99	Not run
Fatty acid, %	67.0	70.4	52.5	68.2	" "
Neutral equivalent	265	281	308	285	" "
Fatty acid:P, molar ratio	1.92	1.97	1.58	1.95	" "
Qualitative tests					
Ninhydrin	+	+	+	-	+
Free amino acids	-	-	-	-	++
Molisch	-	-	+	-	-
Liebermann-Burchard	+ (slight)	-	-	-	-
Acetal	-	-	-	-	-
Principal N components	Serine	Ethanol- amine, serine	Serine, ethanol- amine	Cho- line	Sphingo- sine, cho- line, free amino acids

* The composition of the original lipid mixture is 3.78 per cent P, 1.81 per cent N, 8.26 per cent choline. N:P, molar ratio, 1.06; choline:P, molar ratio, 0.56.

† A and B refer to the first and second components removed, respectively, with this particular solvent mixture.

‡ Analysis showed at least 85 per cent of the N to be sphingosine-like.

yeast Solvent 2 fraction contains approximately 50 per cent of its lipid nitrogen as serine and the remainder as ethanolamine. In the rat liver, it has been observed that there is approximately 55 per cent phosphatidylethanolamine and 45 per cent phosphatidylserine. However, in the beef liver fraction, only 55 per cent of the total nitrogen may be obtained

as serine and ethanolamine. The remaining nitrogen apparently represents a new nitrogen component, which does not react with periodate at

TABLE II

Composition of Fractions Obtained from Chromatogram of Beef Liver-Mixed Phospholipides on Silicic Acid-Hyflo Super Cel (2:1) (Fig. 2)*

	Solvent 2, Fraction A†	Solvent 2, Fraction B†	Solvent 3, Fraction A†	Solvent 3, Fraction B†	Solvent 4 fraction
P, %	3.37	3.71	3.36	3.61	3.68
N, %	0.21	1.63	0.42	1.69	3.39‡
Choline, %	None present	None present	None present	13.30	11.2
N:P, molar ratio	0.14	0.98	0.28	1.02	2.04
Choline:P, molar ratio				0.94	0.78
Inositol, %	None present	None present	15.6	0.08	None present
Inositol:P, molar ratio			0.80	0.004	
Inositol-glycerol, molar ratio			0.80		
Glycero-P:P, molar ratio	1.0	0.96	Not run	0.97	Not run
Fatty acid, %	56.4	74.1	60.4	70.0	" "
Neutral equivalent	355.0	322.0	290.0	322.0	" "
Fatty acid:P, molar ratio	1.45	1.91	1.90	1.95	" "
Qualitative tests					
Ninhydrin	—	+	+	—	+++
Free amino acids	—	—	—	—	++
Molisch	—	—	+	—	—
Liebermann-Burchard	++	—	—	—	—
Acetal	—	—	—	—	—
Principal N components	Serine	Ethanol- amine, serine	Serine, ethanol- amine	Cho- line	Sphingo- sine, cho- line, free amino acids

* The composition of the original lipid mixture: 3.83 per cent P, 1.83 per cent N, 7.50 per cent choline; N:P, molar ratio, 1.05; choline:P, molar ratio, 0.50.

† A and B refer to the first and second components removed, respectively, with this particular solvent mixture.

‡ Analysis showed 80 to 85 per cent of the total N to be sphingosine-like.

room temperature (ethanolamine and serine react readily) or the phosphomolybdic acid reagent of Levine and Chargaff (15), but does form a dinitrophenyl derivative upon reaction with dinitrofluorobenzene, and is apparently ninhydrin-reactive. The chemical nature of this component is under active investigation at the present time.

These fractions all showed the typical infrared pattern for phosphoglycerides and had optical activity in the expected range, $[\alpha]_D^{25} +6.0^\circ$ to

TABLE III
Composition of Fractions Obtained from Chromatogram of Yeast-Mixed Phospholipides on Silicic Acid-Hyflo Super Cel (2:1)* (Fig. 3)

	Solvent 1 fraction	Solvent 2 fraction	Solvent 3, Fraction A†	Solvent 3, Fraction B†	Solvent 4 fraction
P, %	3.17	3.80	3.44	3.72	2.96
N, %	0.24	1.63	0.68	1.60	2.20‡
Choline, %	None present	None present	None present	14.3	10.0
N:P, molar ratio	0.17	0.95	0.45	1.00	1.65
Choline:P, molar ratio				0.99	0.87
Inositol, %	None present	0.18	20.7	None present	None present
Inositol:P, molar ratio		0.008	1.04		
Inositol:glycerol, molar ratio			1.00		
Glycerol:P, molar ratio	0.90	0.98	Not run	1.04	Not run
Fatty acid, %	52.0	66.7	50.0	60.1	" "
Neutral equivalent	285	280	267	254	" "
Fatty acid:P, molar ratio	1.79	1.95	1.70	1.97	" "
Qualitative tests					
Ninhydrin	—	+	+	—	+++
Free amino acids	—	—	—	—	+++
Molisch	—	—	+	—	—
Liebermann-Burchard	+++	—	—	—	—
Acetal	—	—	—	—	—
Principal N components	Not identified	Ethanol-amine, serine	Serine, ethanol-amine	Choline	Sphingosine, choline, free amino acids

* The composition of the original lipid mixture: 3.88 per cent P, 1.77 per cent N, 7.1 per cent choline. N:P, molar ratio, 1.01; choline:P, molar ratio, 0.47.

† A and B refer to the first and second components removed, respectively, with this solvent mixture.

‡ Analysis showed 80 per cent of the N to be sphingosine-like.

6.4°. In addition, they contained no choline, sphingosine, or acetals, and less than 0.1 per cent inositol.

Solvent 3, Fraction A—The analytical data on this eluent revealed it to

be uniquely the major phosphoinositide fraction. A minimum of 95 per cent of the inositol-containing phospholipides placed on the column was recovered in this fraction. When the solvent pattern described here was used, similar results were obtained with the phospholipides from rat liver, beef liver, and yeast. These components contained inositol, glycerol, fatty acids, and small (contaminant) amounts of nitrogen (as serine and ethanolamine). No choline, sphingosine, or acetals were present. The phosphoinositides are soluble in chloroform, dry diethyl ether (99 per cent), and glacial acetic acid, are partially soluble in wet diethyl ether (95 per cent) and warm ethyl acetate (45°), and are insoluble in acetone, methanol, and ethanol.

When it is desired to obtain this component in bulk amounts, it is preferable to utilize the ethanol-insoluble fraction from the original phospholipide preparation, which contains the major amount of the phosphoinositide (in two to three times the original concentration). However, one should be cognizant of the fact that the differentiation between the phosphoinositide and lecithin elution peaks is not as clear cut as with the original phospholipide mixture. Hence, one must take more frequent samples near the expected change from one component to the other.

Solvent 3, Fraction B—This fraction contained only the lecithins. As is evident from Figs. 1, 2, and 3, the lecithins are removed from the column at a slow rate and with considerable skewing of the elution curve. A closer examination of the composition of the fore and back portions of this curve showed it to have an almost identical composition, the only difference being in the degree of unsaturation. The front half has a higher amount of unsaturated fatty acids present and the back half a much lesser amount. The results on the composition of the two sections of the lecithin elution curve (from rat liver phospholipides) are shown in Table IV. However, the degree of unsaturation is not the only factor involved, as the yeast lecithin tends to have the same degree of unsaturation in both portions of the elution curve. Hydrolysis studies showed only choline, fatty acids, and glycerophosphate to be present. An infrared spectrum of the preparations shows a typical curve for a phosphoglyceride and the optical activity of these fractions was in the expected range $[\alpha]_D^{25} +6.0^\circ$ to 6.3° .

In agreement with the observations of Long and Penney (19), it was found that the lecithin fraction obtained via silicic acid columns is not attacked by lecithinase A. However, if this fraction is passed through aluminum oxide, or through adjustment of the pH to 7.0, the attack by this enzyme proceeds in a normal fashion.

Solvent 4 Fraction—The final phosphorus-containing lipid removed from the column appeared to contain 80 to 85 per cent of its nitrogen as a

sphingosine-like material. It also has solubility characteristics similar to those of other sphingolipides. However, more definitive exact information on the chemical nature of this fraction has not been obtained. In all the sources examined here, this fraction contained all the free amino acids present in the original mixture applied to the column. Prior removal of the amino acids of the original sample by passage through cellulose columns or ion exchange resins worked well, but there was approximately a 10 to 20 per cent loss of phospholipide (particularly the phosphoinositides) in this operation. Although there was only a trace amount of lysolecithin in these samples, this material was usually found in the eluent between the tail of the lecithin curve and the sphingolipide fraction.

TABLE IV
*Chemical Nature of Lecithin Fractions from Chromatography of
Rat Liver-Mixed Phospholipides*

The material obtained in the Fraction B elution indicated in Fig. 1 was divided into two parts, a fast moving component (Tubes 85 to 105) and a slow moving component (Tubes 106 to 190), and was analyzed.

	Tubes 85-105	Tubes 106-190
P, %	3.82	3.80
N, %	1.77	1.75
Choline, %	14.5	14.4
N:P, molar ratio	1.02	1.01
Choline:P, molar ratio	0.99	0.99
Fatty acid:P, molar ratio	1.98	1.95
Hydrogen uptake, fatty acid, mm per mm	1.4	0.38

DISCUSSION

In the present investigation it has been shown that a satisfactory separation of many of the components of the mixed phospholipides from rat liver, beef liver, and yeast can be achieved through chromatography on a single column of silicic acid. In effect with varying mixtures of chloroform-methanol (4:1, 3:2, and 1:4), a combined phosphatidyl ethanolamine-phosphatidyl serine fraction, the phosphoinositides, the lecithins, and sphingolipides can be separated from each other and obtained in good yields. In the elution pattern described here, there is an initial fraction, containing approximately 1 to 2 per cent of the total phosphorus, which may contain traces of glycerides, pigments, sterols, and sterol glycosides. The last eluted component (in Solvent 4 fraction) contained all the free amino acids present in the original sample, the entire phosphosphingoside fraction, and upon occasion traces of a compound thought to be lysolecithin.

Although only traces of acetal phospholipides were present in the original phospholipide mixture used here, preliminary evidence in this Laboratory on the separation of beef heart mitochondrial phospholipides with these solvent systems indicated that the ethanolamine and the choline derivatives would distribute themselves with their counterparts, phosphatidylethanolamine and phosphatidylcholine (lecithin), respectively.

88 to 95 per cent of the lipid phosphorus applied to a column can be recovered in the fractions mentioned above. It is important to stress at this point that the presently described procedure has worked well with the mixed phospholipides from beef liver, rat liver, and yeast, but that it is not a guarantee that it will work satisfactorily with every source of phospholipides. As in any chromatographic procedure with compounds as labile and perhaps as equivocal in composition as the phospholipides, this presently described procedure is no panacea. However, it does allow a facile approach to the separation in a reasonable state of purity of the major components of these three tissues. Thus this can represent an initial phase in the final purification of certain of the fractions.

Inasmuch as the results from these diverse sources showed an almost identical elution pattern and rather similar composition, a justifiable suspicion was cast on the possibility that artifacts had arisen during the isolation and chromatographic procedures. However, since the extraction procedure is performed under mild conditions and the chromatograms are reproducible with different loading factors, it seems improbable that artifact production was of any significance. Moreover, a chromatogram of egg-mixed phospholipides on silicic acid with the same solvents used above showed the presence of only two major components (*cf.* Rhodes and Lea (20)). Although these two components were contaminated with small amounts of other substances, these results supported the proposal that no significant degradation of the mixed phospholipides occurred on silicic acid chromatography.

Of considerable interest was the finding that 90 to 95 per cent of the inositol-containing phospholipides from all these sources could be obtained in a single fraction; namely, the initial component in the Solvent 3 fraction. In view of this evidence, it would appear that this chromatographic procedure offers a decided advantage over the classical solvent method of Folch (21) for the isolation of phosphoinositides. McKibbin (6), using silicic acid chromatography, has isolated phosphoinositides from liver, but, in contrast to the findings reported here, has observed that inositides are present in significant amounts in several different fractions. One possible explanation is that McKibbin used the equivalent of 1.6 mg. of P per gm. of silicic acid in his procedure compared to the 0.8 to 1.0 mg. of P per gm. of silicic acid used in our experiments. It has been our experience that a

loading factor higher than 1.0 mg. of P per gm. of silicic acid resulted in the overloading of the column with the consequent finding of the phosphoinositides in several different fractions. In addition it has been our experience that the use of the more subtle changes in polar solvent concentrations as practiced in a strict gradient elution technique resulted in considerable overlapping of the various fractions. Hence the use of substantial changes in solvent concentrations appears necessary to insure a suitable separation of the individual components of a phospholipide mixture.

As has been indicated previously (see under "Results"), the methods reported in the literature for the quantitative estimation of ethanolamine and serine in lipid hydrolysates were not as satisfactory as expected. Perhaps one of the major difficulties involved in a suitable analysis of lipides for their nitrogenous components resides in the procedure chosen for the hydrolysis of the lipides. Although little attention has been directed toward this aspect of the problem, it was found that anhydrous methanolic 6 N HCl, as suggested by Artom (22), was the most effective hydrolytic agent at reflux temperatures and caused the least formation of free ammonia from ethanolamine or serine. The use of aqueous acids for the hydrolytic decomposition of the lipides caused high production of free ammonia (10 to 15 per cent). Subsequently the use of ion exchange resins (Amberlite IRC-50) has proved unequivocally to be the most effective procedure for the quantitative separation of ethanolamine and serine. Through the use of this method and by checks on the total nitrogen of each of the separated fractions, it has been found that in addition to the ethanolamine and serine of Solvent 2 fraction of beef liver phospholipides there was an additional, previously unidentified nitrogenous component.⁷ This compound represented approximately 50 per cent of the total lipid nitrogen. The details of this investigation will be reported in a forthcoming publication.

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SUMMARY

The chromatography of the mixed phospholipides from rat liver, beef liver, and yeast on a single silicic acid column is described. Through the use of various mixtures (v/v) of chloroform-methanol, namely 4:1; 3:2, and 1:4, a satisfactory separation of the phospholipides into five different fractions is possible. A phosphatidylethanolamine, phosphatidylserine fraction, the phosphoinositides, and lecithins comprise the major fraction of the lipides, although sphingolipides and an unidentified low nitrogen-

containing phospholipide are present to a small extent. 90 to 95 per cent of the inositol-containing phospholipides is eluted in a single fraction and thus introduces an improved procedure for the isolation of phosphoinositides.

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