The Separation of Phosphatidyl Ethanolamine and Phosphatidyl Serine by Column Chromatography

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A method for the complete separation and apparent quantitative recovery of both phosphatidyl ethanolamine and phosphatidyl serine from lipid extracts of beef brain is presented. Evidence for the purity, quantitative recovery, and the suitability of the technique for the subsequent analysis of the fatty acid and fatty aldehyde composition of the phospholipids is described.

THE COMPLETE SEPARATION and quantitative recovery of phosphatidyl ethanolamine and phosphatidyl serine by column chromatography does not appear to have been described. Partial separation of these phosphatides on silicic acid columns, using the technique described by Lea, Rhodes, and Stoll (1), has been reported (2,3). Because of our continued interest in the effects of lipids on blood coagulation and a desire to determine the fatty acid composition of these lipids as they exist in the tissues, we have studied paper and column chromatographic techniques that would make possible the complete separation and quantitative recovery of these phosphatides. The present report describes several paper chromatographic systems for the separation of phosphatidyl ethanolamine and phosphatidyl serine and a column chromatographic procedure, using a combination of silicic acid and silicic acid-silicate-water columns for the complete separation and quantitative recovery of these two phospholipids. The development of rapid paper chromatographic methods for the separation of phospholipids has been instrumental in the development of column chromatographic techniques. All fractions from columns have been monitored by paper chromatography, and the successful analysis of the basis for separations on paper has led to the development of suitable column techniques.

Dieckert and Reiser (4), Lea, Rhodes, and Stoll (1), and Marinetti *et al.* (5) have described paper chromatographic methods for the separation of phospholipids on silicic acid-impregnated glass fiber and cellulose papers. Dieckert and Reiser did not describe the behavior of phosphatidyl serine, and the technique of Lea *et al.* did not give satisfactory separation of phosphatidyl ethanolamine from phosphatidyl serine. Although the Marinetti technique separates phosphatidyl ethanolamine and phosphatidyl serine, the development time is long and the solvent mixture cannot be used on thick paper. Furthermore the extension of such acid solvents to column chromatography results in the rapid breakdown of the plasmalogen forms of phosphatidyl ethanolamine and phosphatidyl serine.

Materials and Methods

Solvents and Nitrogen. All solvents were reagent grade, and prepurified nitrogen (less than 6 parts per million oxygen) was used throughout. All solvent proportions of mixtures are expressed as volume/volume ratios.

Preparation of Crude Beef Brain Phosphatide. Beef brain has proved to be a particularly good source of lipid since it contains large amounts of phosphatidyl ethanolamine and phosphatidyl serine and is readily available in quantity. Fresh beef brain was obtained from the slaughter house within a few minutes after removal from the animal, freed of blood, and transported to the laboratory over solid carbon dioxide. The brain was weighed, chiselled into relatively small pieces when frozen, transferred to a large Waring Blendor, and homogenized with chloroform/methanol, 2/1, in a nitrogen atmosphere. For the homogenization a volume in milliliters equal to 10 times the weight in grams of the wet brain was used. The homogenate was then filtered through a coarse-grade, fritted-glass filter, and the residue was extracted twice more in the same manner. The chloroform/methanol extracts were pooled, and suitable small aliquots of this original mixture were withdrawn and evaporated to dryness under a stream of nitrogen and reduced pressure from a water pump. The crude solid obtained by evaporation of the original extract was used for chromatography. Prior to chromatography the crude extracts were stored in desiccators that had been alternately evacuated with a vacuum pump and filled with nitrogen three times. Such a crude extract contains material that will not dissolve in the relatively small volume of chloroform/methanol used for application of the sample to the column; however no difficulties were encountered when a solution containing dispersed solid was applied to the column.

Formation of Lysophosphatides by the Action of Phospholipase A. During the course of this work it was necessary to compare the chromatographic migration of lysophosphatides with components detected in some preparations of phosphatidyl ethanolamine and phosphatidyl serine. We have used a modification of the enzymatic procedure introduced by Hanahan (6), who carried out the reaction in diethyl ether. Rattlesnake venom *(Crotalus adamanteus)* obtained from the Ross Allen Reptile Institute, Silver Springs, Fla., was used as the source of phospholipase A. A solution of the venom (4 mg./ml.) in distilled water was prepared immediately before use, and 0.2 ml. of 0.025 *M* calcium chloride was added to each 5 ml. of venom solution. A solution of the lipid to be degraded was prepared in peroxide-free diethyl ether (Mallinckrodt reagent grade obtained in cans) at a concentration of 10 to 20 mg./ml. In the case of brain lipids any insoluble residue was filtered off on Whatman No. 4 filter paper. Occasionally when hydrogenated phosphatides were examined, their relatively low solubility in ether made it necessary to conduct the enzyme reaction with a great deal of undissolved solid. The degradation of the phosphatides was accomplished by adding 0.2 ml. of the venom solution to 5 ml. of the ether solution of the phosphatide, followed by incubation at room temperature. Ordinarily within a few minutes (1 to 10) the solution became turbid, and a precipitate of lyso-

phosphatide was formed very quickly. If the turbidity did not develop quickly, 1 to 5 drops of 0.1 N potassium hydroxide were added, and the mixture was again observed for several minutes. The addition of base was continued until the enzyme reaction proceeded at a very rapid rate. Some pure phosphatides that contain a great deal of unsaturated fatty acids may not give a precipitate as the lysocompound containing unsaturated fatty acids is relatively soluble in ether. Evidence of reaction in this case is obtained by paper chromatography of the ether solution (for rapid determination the phosphate paper technique with chloroform/methanol, 4/1, as solvent is used). To be sure that reaction is complete the mixture can be left for 1 to 4 hrs. at room temperature. This technique is much more rapid than the procedures described in the literature as very high enzyme and substrate concentrations are used. It is interesting to note that phosphatidyl ethanolamine and phosphatidyl serine of beef brain are quantitatively degraded under these conditions to lysophosphatide.

Preparation of Paper for Chromatography. Whatman Nos. 3 and 3 MM papers were used. The papers were washed by capillary descent with 2 N acetic acid, followed by 95% ethanol. The washing was conducted in chromatocabs, and each trough was filled three times with each solvent. Although this washing procedure is not absolutely essential for each of the applications to be described, it has been found convenient to use one procedure for all purposes.

Phosphate paper was prepared as follows. Washed Whatman No. 3 or 3 MM paper was impregnated with 0.5 M disodium hydrogen phosphate or 0.5 M dipotassium hydrogen phosphate and allowed to dry (papers should not be heated and then stored in a dry atmosphere as some water is required for successful chromatography). The papers were cut to 7 x 9 in. for chromatography. The use of this paper is a modification of our previous technique (2).

Whatman No. 3 or 3 MM paper was impregnated with silicic acid as follows. The washed paper was immersed in a solution of sodium silicate, prepared by diluting Mallinckrodt sodium silicate (40–42°Bé) 1/1 with distilled water. Each paper was allowed to stand in the sodium silicate for approximately 2 min., removed, and allowed to drain for 1 to 2 min. (papers must not be allowed to dry). The wet paper was immersed in 6 N hydrochloric acid saturated with sodium chloride (salt not required with some lots of silicate) for 5 to 10 min., then washed with distilled water until the pH of the wash water was approximately 5 (to give silicic acid-silicate paper). The number of washes that were required varied and depended upon the number of papers and the volume of wash water used. The well-washed paper was hung to dry at room temperature (drying may be completed at 50 to 60°C. although the heating step is not essential). The paper was cut to 7 x 9 in. for chromatography. All impregnated papers were stored in sealed polyethylene bags.

Paper Chromatography. Descending chromatography was carried out in chambers 18 in. high and 12 in. in diameter with all solvents except the acetic acid mixture. The chambers were lined with Whatman No. 3 paper saturated with solvent and contained 1½ to 2 in. of the solvent mixture in the bottom of the chamber. Before the papers were inserted in the chambers, the paper liners were saturated with solvent poured from a beaker, and the chamber was quickly closed. Chromatograms were developed with acetic acid solvent (see below) in unlined chambers, using the ascending technique.

Phosphate-impregnated papers were developed in a mixture of chloroform/methanol, 4/1. The development time varied between 15 and 40 min. The papers were inserted into the chamber rapidly (10 to 20 sec.) to avoid disturbing the atmosphere of the chamber greatly.

Silicic acid-impregnated papers were developed in a mixture of chloroform/methanol, 4/1, to which 8 ml. per liter of concentrated aqueous ammonia were added (occasionally 1 g. of ammonium acetate per liter was added) or in a mixture of chloroform/acetone/acetic acid/water, prepared by mixing 4 volumes of chloroform/acetone, 4/1, and one volume of glacial acetic acid/water, 9/1. The development time varied between 1½ and 4 hrs.

The Detection of Compounds on Paper Chromatograms. Two techniques were used for the location of all lipid materials on paper chromatograms. The first of these involved the use of 0.001% Rhodamine 6G made up in 0.25 M dipotassium hydrogen phosphate. The potassium phosphate has been found to intensify the colors observed when the wet chromatograms are viewed under ultraviolet light. Dry paper chromatograms were dipped in this dye solution for 5 sec. to 2 min., excess dye was rinsed off under a stream of distilled water, and the chromatograms were viewed wet under ultraviolet light from a Mineralite lamp. The spots were marked with a red pencil that did not smear on the wet paper (Cosmos red marking pencil).

It was found that the p-rosanilin reagent originally prepared for a Schiff's test gave a nonspecific staining of lipids when freshly prepared. The reagent was prepared by adding to 150 ml. of distilled water 0.5 g. of p-rosanilin (National Aniline, New York), 8 ml. of concentrated hydrochloric acid, and 5 g. of sodium bisulfite. The solution was then diluted to 1 liter and decolorized by the addition of at least 6 g. of Norite activated charcoal, which was then removed by filtration through Whatman No. 4 filter paper. A sulfurous acid wash solution was prepared that contained 8 ml. of concentrated hydrochloric acid and 5 g. of sodium bisulfite per liter. Chromatograms were dipped in freshly-prepared dye solution until the spots appeared. Each chromatogram was then washed at least three times with sulfurous acid solution, finally with distilled water, and dried. Purple or pink-purple spots were observed with most lipids against a white background. If upon exposure to light or on standing for several days a background appeared, it was reduced by another wash with the sulfurous acid solution.

The p-rosanilin reagent described above may be used as a relatively specific Schiff's test for aldehyde-containing phospholipids if it has been allowed to stand in an open tray in the laboratory for a few minutes. As the reagent is variable, control samples should be run whenever it is used.

Unsaturated fatty acids or phospholipids containing unsaturated fatty acids were visualized on chromatograms dipped in 1% potassium permanganate. Ninhydrin-positive lipids were located as follows. A stock solution of ninhydrin in n-butanol (1 mg./ml.) was diluted with 2,4-lutidine (20% by volume) immediately before use. The reagent was sprayed on chro-

matograms, and the color was developed at 120°C. for 5 min. Very intense ninhydrin colors were obtained if the phosphate or silicic acid papers were first dipped in 2 N hydrochloric acid for 2 min., washed in water, and dried before spraying with this reagent.

Preparation of Silicic Acid for Column Chromatography. Mallinckrodt 100 mesh silicic acid for chromatography in the desired quantity was placed in a coarse-grade, sintered-glass filter and washed first with three volumes of methanol. The first wash was begun by allowing the methanol to pass down the dry bed of silicic acid in order to force off a yellow impurity. These washes were followed by three volumes of chloroform/methanol, 1/1, three volumes of chloroform, and one volume of methanol. The washing was accomplished under gentle suction from a water pump, and, after the final wash, the silicic acid was left on the filter until reasonably dry. The relatively dry material was then transferred to a three-necked flask and heated at 115 to 140°C. under suction (water pump) and a stream of nitrogen to dehydrate and remove oxygen. A mixture of chloroform/methanol, 4/1, that had been previously thoroughly deoxygenated by removing dissolved gases under reduced pressure (water pump) and passing nitrogen through the mixture was poured into the flask, and the contents were then transferred to a glass-stoppered bottle.

Column Chromatography. Column chromatography was carried out in tubes 2.5 x 40 cm. equipped with a sintered-glass disk at the bottom. Enough of the washed silicic acid (about 60 g.) in chloroform/ methanol, 4/1, was transferred to the chromatography tube to give a height of 20 centimeters. The columns were run under a nitrogen pressure of approximately 1 to 2 lbs./sq. in. at a flow rate of 2 ml./min.

The sample, consisting of no more than 0.4 g. of crude beef brain lipid, was applied to columns in 5 ml. of the solvent to be used for chromatography.

Fractions from a column were followed during the course of development with a rapid ninhydrin test, carried out as follows. Where chloroform/methanol alone was used as the eluting solvent, 0.1 ml. of the fraction from the column was mixed with 0.1 ml. of the ninhydrin reagent and 0.1 ml. of 2,4-lutidine in a 2-ml. tapered centrifuge tube and was heated in a sand bath maintained at 150 to 190°C. The tube was rotated rapidly, removed periodically, and tapped with the finger to prevent bumping. Color development required from 1 to 3 min. This rapid test makes possible a change of solvent when a given component has been eluted without collection of a large number of intermediate fractions. It is particularly useful when new elution techniques are under study. When the column effluent contained ammonia the aliquot was first evaporated to dryness in a sand bath, the ninhydrin and lutidine were added, and color was developed as before. After completion of a run, the quantitative hydroxamic acid procedure for the determination of esters described by Rapport and Alonzo (7) was carried out on each of the fractions, using 0.5 to 1.0 ml. aliquots.

Results and Discussion

The separation of phosphatidyl ethanolamine and phosphatidyl serine was readily accomplished with all paper chromatographic systems. The phosphate paper system was particularly useful for the identification of amino phosphatides as phosphatidyl ethanolamine migrated nearly to the solvent front, lysophosphatidyl ethanolamine usually migrated with an R_f value of approximately 0.5, phosphatidyl serine migrated with an R_f of approximately 0.15, and lysophosphatidyl serine either remained at the origin or moved a very small distance from the origin. These phosphatides were located with the ninhydrin reagent.

As the paper chromatographic techniques were capable of yielding separation of phosphatidyl ethanolamine and phosphatidyl serine, attempts were made to extend them to column procedures. Attempts to use Baker's anhydrous disodium hydrogen phosphate were not successful, and this was the first indication that hydrated salts were required for successful separations on paper and columns. Although phosphatidyl ethanolamine and phosphatidyl serine were separable on hydrated sodium or potassium phosphate columns, the use of hydrated silicic acid-silicate columns was found to be simpler.

After various trials with hydrated silicic acid-silicate columns alone and in combination with silicic acid columns, the following procedure was judged the most suitable for the isolation of pure phosphatidyl ethanolamine and phosphatidyl serine from crude beef brain lipid. A silicic acid column 20 cm. high in the 2.5-cm. tube was prepared in chloroform/methanol, 4/1; the sample (no more than 0.4 g.) was applied in the same solvent; and the column was eluted with the same solvent according to the technique of Lea, Rhodes, and Stoll (1). The amino phosphatide fraction recovered from this column was a mixture of phosphatidyl ethanolamine and phosphatidyl serine. It was absolutely essential that the entire fraction be collected without loss. This was necessary for quantitative recovery, also because the ratio of phosphatidyl ethanolamine to phosphatidyl serine was found to vary in different portions of the peak. Ten-ml. fractions were collected in glass-stoppered centrifuge tubes, and immediately after collection a 0.1-ml. aliquot was withdrawn for the ninhydrin test and a 0.5 or 1.0-ml. aliquot for the hydroxamic acid ester test; 0.1 ml. of concentrated ammonia was added to the fraction; and each tube was bubbled with nitrogen, stoppered, and stored at 0°C. The addition of ammonia was found to prevent decomposition of the ethanolamine plasmalogen. The pooled fractions were evaporated immediately after collection at 0° to 10°C. under a gentle stream of nitrogen and reduced pressure from a water pump. The dry solid was transferred to a desiccator that was flushed at least three times with nitrogen and stored at −20°C. prior to the next column separation. For the separation of the amino phosphatides a second column 20-cm. high was prepared in chloroform/methanol, 4/1, as before, the solvent was washed out with exactly 70 ml. of a chloroform/methanol/ammonia mixture that was prepared by adding 10 ml. of concentrated aqueous ammonia to 1 liter of the chloroform/methanol, 4/1, mixture. The mixture of phosphatidyl ethanolamine and phosphatidyl serine was then dissolved in 5 ml. of chloroform/methanol, 4/1, applied to the column, and elution of phosphatidyl ethanolamine was accomplished with chloroform/methanol, 4/1. The phosphatidyl ethanolamine peak emerged sharply as the first fraction, and, when complete collection was assured by the fact that the ninhydrin test was negative for

several fractions, absolute methanol was added to the column for the elution of phosphatidyl serine. The results of such a run are shown in Figure 1.

The hydrated silicic acid-silicate column run was carried out with a mixture of the two amino phosphatides previously freed of other lipids because, when the total crude lipid was applied to the hydrated columns, phosphatidyl serine, although separable from phosphatidyl ethanolamine, was not completely separated from other phosphatides.

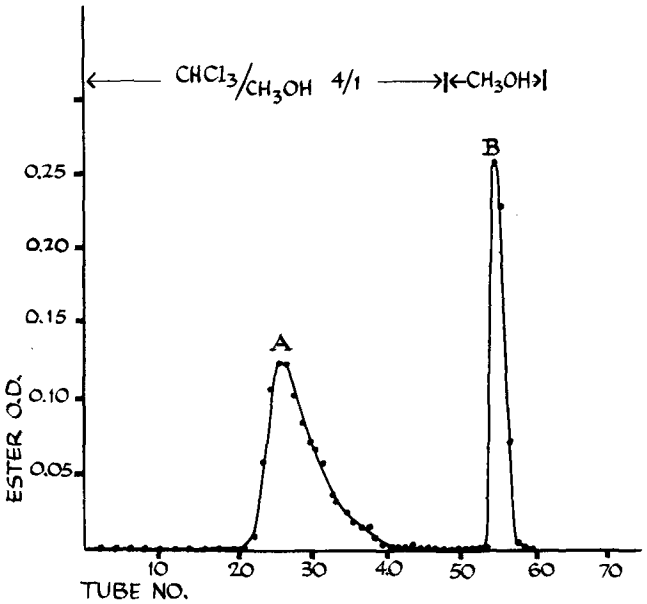

FIG. 1. Separation of a mixture of phosphatidyl ethanolamine (A) and phosphatidyl serine (B) on a silicic acid-silicate-water column. Each fraction was 10 ml., and the collection of the first fraction was begun when the sample was applied to the column. Ester O.D. refers to the optical density determined by using the hydroxamic acid color reaction.

The fact that the fractions were indeed phosphatidyl ethanolamine and phosphatidyl serine and that each fraction was entirely free of contamination by the other phosphatides was established as follows. Chromatographic migration characteristic of phosphatidyl ethanolamine and phosphatidyl serine was established on silicic acid- and phosphate-impregnated papers by comparison with synthetic compounds obtained from Erich Baer. The characteristic migration on silicic acid-silicate and phosphate papers is shown in Figure 2. These chromatograms demonstrate the high degree of purity of the phosphatidyl ethanolamine and phosphatidyl serine in fractions from the hydrated silicic acid-silicate columns. No trace of phosphatidyl serine was found in the phosphatidyl ethanolamine fraction, and no phosphatidyl ethanolamine was found in the phosphatidyl serine fraction. The complete absence of lysophosphatides in the two fractions was also demonstrated. A trace of an oxidation product in phosphatidyl serine can be observed in Figure 2A. This developed on standing prior to paper chromatography.

The exposure of preparations to air brings about several changes in the chromatographic migration on phosphate paper. In the early stages of oxidation a spot migrating just behind phosphatidyl ethanolamine was observed to appear in the phosphatidyl ethanolamine fraction while more extensive oxidation was

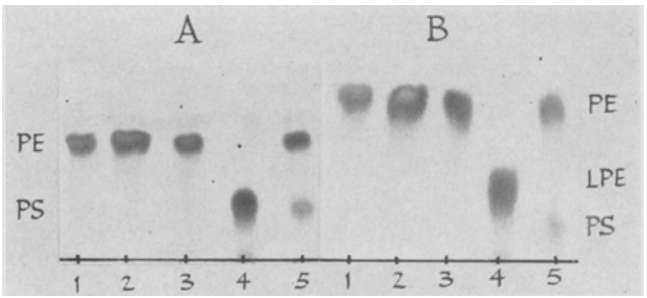

FIG. 2. Demonstration of purity of phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS) from a silicic acid-silicate column. (A) Silicic acid-silicate paper, developed in chloroform/methanol, 4/1, containing 10 ml. of concentrated aqueous ammonia and 1 g. of ammonium acetate per liter (running time about 2 hrs.). (B) 0.5 M disodium hydrogen phosphate paper with chloroform/methanol, 4/1, as solvent (running time about 20 min.). Both chromatograms sprayed with ninhydrin. (1)-(3) 150, 300, 150 μgm. PE, respectively. (4A) 50 μgm. PS, (4B) 50 μgm. lysophosphatidyl ethanolamine (LPE), (5) starting mixture of beef brain PE + PS applied to the column. In (A) the absence of PS in PE and PE in PS preparations is shown. In (B) the absence of both LPE and PS in the PE is demonstrated. LPE migration was not as far as was commonly obtained on similar chromatograms.

indicated by the presence of progressively larger amounts of material that streaked back or did not migrate from the origin. Similarly the phosphatidyl serine fraction upon oxidation showed a spot migrating behind phosphatidyl serine, and with more extensive oxidation, material that did not migrate from the origin. On silicic acid-silicate papers one of the oxidation products of phosphatidyl ethanolamine migrated to very nearly the same position as lysophosphatidyl ethanolamine while some of the oxidation products migrated with or overlapped with phosphatidyl ethanolamine. The fact that these substances were oxidation artifacts was demonstrated by the failure to find such substances when oxygen was rigidly excluded and by the finding that the products could be distinguished chromatographically from the parent phosphatide and the lysophosphatide produced from the parent compounds by degradation with snake venom phospholipase A.

Further proof of the purity of the phosphatidyl ethanolamine and phosphatidyl serine fractions was obtained by examination of hydrolysis products. Acid hydrolysis was carried out by placing 20 to 50 mg. of the phosphatide and 3 ml. of 3 N HCl in a sealed tube, followed by heating to 100°C. for 6 to 12 hrs. This procedure is similar to that used by Levine and Chargaff (8). After hydrolysis the free fatty acids were extracted into petroleum ether, and the water-soluble hydrolysis products were recovered after evaporation of the aqueous phase to remove acid, dissolved in water, and applied to Whatman No. 1 paper. For the demonstration of the absence of serine in hydrolysates of phosphatidyl ethanolamine an amount of hydrolysate calculated to contain from 100 to 500 μgm. of ethanolamine was applied to a single spot while for the demonstration of the absence of ethanolamine in the phosphatidyl serine hydrolysate an amount of hydrolysate equivalent to 100 to 200 μgm. of serine was applied. Chromatograms were developed in phenol saturated with water or in lutidine saturated with water. Figure 3 indicates the type of result obtained by such chromatographic examinations. Figure 3A demonstrates the absence of free

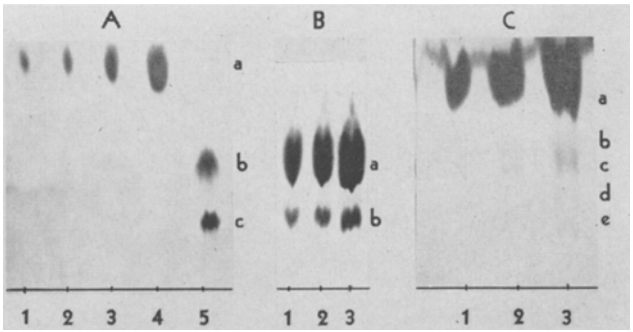

FIG. 3. Acid hydrolysis products of phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS). (A) Phospholipid before hydrolysis, chromatograms developed with lutidine saturated with water and sprayed with ninhydrin. (1)-(4) 50, 100, 200, 400 μgm. of PE plus PS (a). (5) 10 μgm. each of ethanolamine (b) and serine (c). Note the absence of free amino acids in the phospholipid sample. (B) Water-soluble acid hydrolysis products of a mixture of PE plus PS. 1, 2, 3, from 0.7, 1.25, and 2.5 mgs. of PE + PS, showing ethanolamine (a) and serine (b). Chromatogram developed in phenol saturated with water and sprayed with ninhydrin. (C) Water-soluble hydrolysis products from brain PE. 1, 2, 3 from 1.25, 2.5, and 5.0 mg. of PE, showing ethanolamine (a) and unknown products b, c, d, and e. Chromatography as for B.

amino compounds in the fractions prior to hydrolysis. Figure 3B demonstrates the presence of both ethanolamine and serine in phosphatidyl ethanolamine-phosphatidyl serine mixtures isolated from silicic acid columns and applied to the hydrated silicic acid-silicate columns. Figure 3C demonstrates the absence of serine in the acid hydrolysate from the phosphatidyl ethanolamine that was isolated from silicic acid-silicate-water columns. It is interesting to note that traces of unidentified ninhydrin positive compounds were invariably found in the phosphatidyl ethanolamine hydrolysate when amounts of hydrolysate corresponding to 2.5 to 5.0 mg. of phospholipid were applied to paper. These substances are present in amounts below 1% if we assume that the average limit of detectibility of slightly less than 1 μgm. pertains to these substances. It is not known whether these ninhydrin positive compounds represent artifacts or are present as a result of the hydrolysis of some very minor lipid present in beef brain.

We have previously reported that phosphatidyl ethanolamine and phosphatidyl serine are rapidly destroyed by exposure to air. The extent of oxidation may be related to the molar extinction coefficients at 235 and 275 mμ as suggested by Lea (9). For this determination to be meaningful the lipid must dissolve completely in cyclohexane. As oxidation increases, insoluble material appears and absorption in the ultraviolet may not be reliable. Our best preparations of phosphatidyl ethanolamine had molar extinction coefficients of 486 and 109 at 235 and 275 mμ, respectively. Our best preparations of phosphatidyl serine had molar extinction coefficients of 308 and 84 at 235 and 275 mμ, respectively. The values for phosphatidyl ethanolamine were very close to those reported by Lea (550, 250) for egg yolk phosphatidyl ethanolamine that was obtained under oxygen-free conditions by silicic acid column chromatography and after storage under nitrogen for one day. Lea did not report values for phosphatidyl serine as the egg phospholipid did not contain this phosphatide.

Table I demonstrates the reproducibility of the recovery of both phosphatidyl ethanolamine and phosphatidyl serine from two crude beef brain lipid preparations. Table II shows the recovery of crude lipid and the percentage of phosphatidyl ethanolamine and phosphatidyl serine. The close correspondence of replicate runs on the same lipid extract, as well as the close correspondence of extracts prepared from different beef brains, demonstrated the reproducibility of the procedure and indicated that it was probable that the isolation was essentially quantitative. Another indication of the quantitative nature of the isolation of the two phosphatides was obtained by the study of the material eluted from silicic acid columns after the collection of phosphatidyl ethanolamine and phosphatidyl serine. After phosphatidyl serine and phosphatidyl ethanolamine had been eluted with a mixture of chloroform/methanol, 4/1, residual lipids were removed from the column with methanol. Recoveries of 98–102% of the weight of material applied to the columns was obtained by adding the weights of the neutral lipid fraction that appeared ahead of the amino phosphatides, the phosphatidyl ethanolamine plus phosphatidyl serine fraction, and the methanol eluate when the figures were corrected for insoluble matter that remained at the top of the column. Paper chromatographic examination of the methanol eluate for phosphatidyl ethanolamine and phosphatidyl serine failed to disclose the presence of even traces of these substances, and quantitative isolation was thus indicated. Unfortunately the amounts of phosphatidyl ethanolamine and phosphatidyl serine in beef brain have not been determined by other methods. Chargaff *et al.* (10) have however determined the amount of ethanolamine present in beef brain lipid by isotope dilution, and it is possible to calculate from their data that the phosphatidyl ethanolamine to phosphatidyl serine molar ratio was 2.75 for their preparation of beef brain lipid. This is in good agreement with our values of 2.60 and 2.64 which were obtained by isolation. It is also to be noted that the values obtained in the present study are very close to those calculated from the data of Collins and Wheeldon (11), who determined

TABLE I
Recoveries of Amino Phospholipids from Silicic Acid and Silicic Acid-Silicate-Water Columns

	Silicic Acid		Silicic Acid-Silicate-Water			
	Wt. sample (mg.)	Recovered[a] (mg.)	Wt. sample (mg.)	PE recovered (mg.)	PS recovered[a] (mg.)	Recovery (%)
Brain 1	264.3	72.5	71.8	50.3	19.5	97.2
	400.0	106.0	105.0	76.5	29.2	100.7
Brain 2	307.0	75.3	74.5	54.0	20.3	99.7
	322.0	76.5	74.5	54.6	20.8	101.2

[a] Corrected for amount removed for ninhydrin and hydroxamic acid ester tests.
PE = phosphatidyl ethanolamine, PS = phosphatidyl serine.

TABLE II
Comparison of Recoveries from Amino Phospholipids from Two Beef Brains

	Total wet wt. (g.)	Total wt. crude lipid (g.)	% PE (wet wt.)	% PS (wet wt.)	PE/PS
Brain 1	370	45.1[a]	2.42	0.93	2.60
Brain 2	379	57.0	2.67	1.01	2.64

[a] Total crude lipid, lower in Brain 1 as a methanol-water layer that developed during extraction, was discarded after it was shown that it did not contain amino phospholipid.
PE = phosphatidyl ethanolamine, PS = phosphatidyl serine.

ethanolamine and serine in brain lipids by a dinitrofluorobenzene technique. When their data were calculated as a phosphatidyl ethanolamine/phosphatidyl serine molar ratio, values of 2.90 for rat brain and 2.70 for sheep brain were obtained.

The technique described above appears to be suitable for the quantitative isolation of phosphatidyl ethanolamine and phosphatidyl serine for the subsequent determination of the amounts of fatty acids and fatty aldehydes present in these phospholipids. It is to be noted that the crude solid obtained by evaporation of the original extract was used for chromatography. Such a sample will allow an accurate determination of the amount of phosphatidyl ethanolamine and phosphatidyl serine in the original brain specimen, and the exact ratio of the two substances can be determined. The use of acetone precipitation or other forms of preliminary purification of the crude extract may result in variable losses.

Addendum. Since this work was completed, we have obtained values for phosphatidyl ethanolamine in beef brain by an independent method, using diethylaminoethyl cellulose for column chromatography. The fresh brain was found to contain 2.36% phosphatidyl ethanolamine by ion exchange cellulose chromatography compared to 2.42 and 2.67% determined for two other beef brains as described above. The close agreement of the values obtained by two independent methods on different beef brain samples is a further indication that the techniques of extraction and chromatographic separation are reproducible and quantitative.

The experience of several investigators with our method has demonstrated that a common difficulty is fragmentation of silicic acid during preliminary washing and drying. Gentle handling will assure minimum mechanical trauma. Unnecessary stirring and shaking can cause the silicic acid bed to pack so firmly that a suitable flow-rate cannot be obtained.

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Properties of Cottonseed Meals Prepared with Acetone-Petroleum Ether-Water Azeotrope [1]

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EVIDENCE from the most recent studies on the nutritive quality of cottonseed meals indicates that the most important factors affecting the nutritive quality of the meals as protein supplements for nonruminant feedings are the total gossypol and the lysine contents of the meals (1). The two effects are additive; the nutritive quality is directly proportional to the lysine content and inversely proportional to the total gossypol content. It is possible, on the basis of the lysine and gossypol contents of cottonseed meals, to predict, with a reasonable degree of accuracy the growth response of broilers, perhaps also of swine, receiving cottonseed meals of known total gossypol and lysine contents.

Research to test further the hypothesis that cottonseed meals may be graded in this way is continuing, and there has been included in this research effort the preparation of cottonseed meals with low total gossypol and high lysine contents.

Whether oil is pressed out of cottonseed or removed by extraction with an oil solvent, cottonseed meats are usually subjected to pretreatments to enhance the extractability of the oil, to reduce the "fines" problem, and to bind gossypol. In most of the pretreatments of cottonseed meats the moisture content is at least that present in the natural seed, and the temperatures are increased to 212°F. or more. The meats are usually held at these elevated temperatures for at least 30 min. While this type of pretreatment usually brings about an improvement in the extractability of the oil, it causes a part of the gossypol to become bound to the meal and it also brings about the destruction of a part of the lysine in the meal protein.

Lysine in cottonseed is heat-labile (2,3,5), and the quantities found in cottonseed meal proteins are invariably less than those found in the proteins of the raw seed. The wide variation of the lysine content (*ca.* 2–3.8 g. per 16 g. of nitrogen, as determined through the use of dinitrofluorobenzene) of the proteins in commercial cottonseed meals occurs because of the wide variation from mill to mill in the quantity of heat applied to, the moisture content of, and the temperatures reached in the seed while being processed for oil. Furthermore gossypol becomes bound in the meal while the cottonseed is being processed for

[1] Presented at the 51st Annual Meeting, American Oil Chemists' Society, Dallas, Tex., April 4–6, 1960.
[2] One of the laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.