Phospholipids

3. ON THE CHROMATOGRAPHIC SEPARATION OF GLYCEROPHOSPHOLIPIDS*

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Chromatographic methods of identification, separation and preparation have been applied only comparatively recently to the phospholipids. One source of difficulty, in addition to the general one of insolubility in water, has been the fact that lipids occur naturally as mixtures of classes, e.g. triglycerides, cholesterol esters, phosphatidylcholines, phosphatidylethanolamines, ethanolamine plasmalogens, inositol lipids, etc., each of which is liable to contain a complex mixture of saturated and unsaturated fatty acids with consequent variation in the physical and chemical properties of the parent compounds. The solubilities of individual lipids are also influenced greatly by the presence of other lipids. Lysophospholipids, for example, which are normally insoluble in ether remain in solution in the presence of ether-soluble phospholipids (Fairbairn, 1945), and plasmalogens, insoluble in ether when pure, can nevertheless be extracted by ether during the preparation (Thannhauser, Boncoddio & Schmidt, 1951). Anomalous behaviour of this kind has an adverse effect on methods of separation which depend on differential solubility or partition between solvents, and modern methods of examination have been tending to throw doubt on the purity of products obtained, even in comparatively recent work, by the use of isolation procedures of the older type.

Phosphatidylcholine (lecithin), the best known of the phospholipids, has usually been separated and purified via its sparingly soluble complex with cadmium chloride. This method, however, is rather tedious, gives a far from quantitative yield and tends to fractionate the material with loss of the more unsaturated portion. Moreover, Hack (1953) has found an ox-heart lecithin prepared by this method to contain a major proportion of acetal phospholipids (plasmalogens) and specimens of egg lecithin prepared in the present work have been found to be contaminated, apparently by lysophospholipids. Phosphatidylethanolamine and phosphatidylserine prepared from ox brain and spinal cord by the solvent fractionation procedures

of Folch (1942, 1948, 1949) have been found by Lovern (1952) to be still far from pure.

Adsorption chromatography was first applied to the separation of mixed lipids into their main constituent classes by Trappe (1940), who showed by the use of a variety of adsorbents and solvents that lipids are more strongly adsorbed as their polarity increases from hydrocarbons, through sterol esters, triglycerides, sterols and fatty acids to phospholipids. More recently, Fillerup & Mead (1953) have defined conditions for the separation of these same classes of lipids on silicic acid columns by use of a series of solvents of increasing polarity. Borgström (1952) has shown that phospholipids can be quantitatively adsorbed on silicic acid from mixed lipids in chloroform solution and quantitatively eluted (without fractionation) with methanol.

For separation of the phospholipids themselves Taurog, Entenman, Fries & Chaikoff (1944) adsorbed liver phospholipids from light petroleum on to magnesia and eluted with methanol to obtain a choline-containing fraction free from amino N. By the use of alumina, with 95% (v/v) ethanol as solvent, Hanahan, Turner & Jayko (1951) similarly separated a lecithin fraction from egg phospholipid in fairly good yield and achieved the notable isolation of dipalmitoleoyl lecithin from yeast (Hanahan & Jayko, 1952). Fauré (1950) used alumina to remove amino-N-containing contaminants from egg lecithin prepared by the cadmium chloride procedure. In none of these cases was the adsorbed 'kephalin' fraction recovered.

Rice & Osler (1951) fractionated lecithins on magnesium silicate, finding that only about one-fifth of ox-heart lecithin preparations made by the cadmium chloride method was active in serological tests, but no chemical data on the composition of the fractions were given. McKibbin & Taylor (1952) isolated an acidic, nitrogen-free polyglycerol phospholipid from dog's liver by fractional elution with chloroform-methanol from silicic acid, but did not examine the nitrogen-containing fractions.

Diemair & Poetsch (1949) examined the behaviour of commercial egg lecithin on silica columns and claimed that individual phospholipids could be

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recognized as they separated by characteristic fluorescence in ultraviolet light. We find that neither synthetic glycerophospholipids nor purified fractions from egg yolk show more than a very weak fluorescence, and the results of Diemair & Poetsch must be ascribed to the use of impure and probably deteriorated starting materials. The 'lipid browning reaction' between phospholipid free amino groups and aldehydic substances such as reducing sugars is known to give rise to powerfully fluorescing compounds.

Requiring pure phospholipid fractions in some quantity for studies connected with reactions undergone by these substances during the processing and storage of foods, we examined first the use of ion-exchange columns, but with disappointing results, and subsequently transferred our attention to alumina columns. No difficulty was experienced in removing the amino-N-containing components from egg phospholipid by this means, but we were unable to obtain lecithin fractions with the theoretical fatty acid/phosphorus (FA/P) ratio of 2.00, apparently owing to the presence of lysophospholipids in the eluate from the columns. Moreover, as already indicated, phosphatidylethanolamine remaining on the column could not be recovered undecomposed.

With silicic acid columns, on the other hand, eluting first with methanol-chloroform and finally with pure methanol, we were able to achieve a satisfactory separation of these three classes of phospholipids, phosphatidylethanolamine in this case coming off the column first, followed by lecithin and finally by lysolecithin. A preliminary account of this work has been published (Lea & Rhodes, 1954b).

On paper, partition chromatography of unhydrolysed lipid mixtures has been reported by several workers, but very few data are available on the separation of typical phospholipids by this means. Hecht & Mink (1952) describe the chromatography of crude kephalins applied to filter paper in aqueous suspension and developed in phenol-water. Fractions were obtained which were chromatographically homogeneous when re-run in the same system, but all yielded the same four ninhydrin-reacting bases after hydrolysis, and it is difficult to interpret the results. Hack (1953) describes a micromethod of lipid analysis on filter paper disk chromatograms, but quotes data for separation of only a few rather atypical substances. His observation that 'kephalins' are bound so firmly by filter paper that extracts containing them can be filtered only through sintered glass may be due, in part at least, to the presence in the preparations of amino acids and other water-soluble contaminants which are readily taken up from organic solvents by cellulose (Lea & Rhodes, 1953a).

The recent work of Huennekens, Hanahan & Uziel (1954) was directed primarily to the separation on paper of the hydrolysis products of lecithin and only a very limited degree of separation of the water-insoluble phospholipid substances was claimed. Under similar conditions, using a mixture of phosphatidylcholine, phosphatidylethanolamine and the corresponding lyso compounds with the butanol-water solvent, we obtained very poor separations and bad tailing. Our earlier attempts at separating phosphatidylcholine and phosphatidylethanolamine by partition chromatography on cellulose had been even less successful (Lea & Rhodes, 1953a).

We therefore turned our attention to adsorption chromatography on impregnated papers, and the present communication describes the greatly improved separations which can be obtained by the use of filter paper impregnated with silicic acid. The phospholipids used were in part synthetic materials of known composition and structure and in part purified fractions isolated from natural sources, some by means of the silicic acid column method already referred to. A preliminary account of this work has been published (Lea & Rhodes, 1955).

EXPERIMENTAL

Analytical methods

Amino nitrogen. The quantitative ninhydrin method previously described (Lea & Rhodes, 1954a) was employed for following the progress of the separation of amino-containing from non-amino-containing phospholipids on the columns, the solvent being first removed in a rotary film vacuum evaporator (Lea, Hannan & Rhodes, 1951). For characterization of the combined phosphatidylethanolamine fractions amino N was determined in an acid hydrolysate by the Van Slyke manometric method, after removal of the fatty acids by extraction with light petroleum.

Total nitrogen. The nitrogen content of most phospholipids is rather low (2% or less) and the ordinary micro-Kjeldahl procedure requires a quantity of material which may be inconveniently large. Colorimetric methods for estimation of the NH_3 require a smaller sample but appear to be less accurate. In the present work the scale of the volumetric determination has been somewhat reduced without loss of accuracy by collection of the NH_3 in acid iodate and titration with thiosulphate (Ballentine & Gregg, 1947) which gives a sharper end-point than the normal acid-alkali titration. A further considerable reduction in sample size can be achieved by the use of aeration instead of distillation for removal of the NH_3 (Sobel, Hirschman & Basman, 1947).

Phosphorus. Phosphorus was determined after removal of solvent and digestion with perchloric acid by the method of Allen (1940). A rapid indication of the P content of the eluate from a column, useful for guidance in cutting fractions, could be obtained by developing the molybdenum blue colour immediately the sample cleared, i.e. after digestion for about 15 min. Owing to the hygroscopic nature of the phospholipids direct weighing was avoided whenever possible and analytical data referred to the P basis.

Iodine value. The micro Rosenmund-Kuhnhehn technique of Yasuda (1931) has generally been employed for determination of the iodine value of phospholipids but, in our experience, gives seriously low results (Lea & Rhodes, 1953*b*). In the present work we have applied the procedure of Trappe (1938) directly to our phospholipid fractions as well as to fatty acids and esters recovered from them, with satisfactory results. This method uses the Kaufmann sodium bromide-bromine-methanol reagent and requires only about 1 mg. of material. In contrast to the method of Yasuda it has been shown to give accurate results with linoleic and linolenic as well as with oleic acids.

Fatty acid/phosphorus ratio. The procedure employed for determination of the FA/P ratio of glycerophospholipid fractions isolated from natural sources was to reflux the phospholipid with 0.5*N* ethanolic NaOH under N_2 for 1 hr. followed by removal of the solvent, acidification, extraction with redistilled light petroleum (b.p. 40–60°), washing free from mineral acid and recovery, weighing and titration of the fatty acids. For an accurate mean equivalent of the fatty acids it is essential to use purified solvent and to remove it completely before weighing. This simple procedure became tedious when carried out quantitatively and in replicate owing to the presence of traces of insoluble surface-active material at the interface which hindered a clean separation.

The accuracy of the routine determination was checked by testing the recovery of added palmitic acid and by substitution of acid (6*N*-HCl for 6 hr. at 100°) for alkaline hydrolysis. Lineweaver, Morris, Kline & Bean (1948) have shown that fresh egg yolk contains no volatile, water-soluble fatty acids which might have been lost during the determination, and steam distillation of an alkaline hydrolysate of 'lecithin' of FA/P ratio 1.88 acidified to pH 2.4 with H_3PO_4 confirmed their absence.

No satisfactory alternative to determination of the FA/P ratio was discovered, determination of carboxylic ester by the colorimetric hydroxamate method (Shapiro, 1953) being, in our experience, insufficiently accurate for characterization although useful for approximate, comparative work. Determination of P and N on the dry-weight basis does not adequately indicate the purity of phospholipid fractions from natural sources unless accompanied by isolation and accurate determination of the mean equivalent of the fatty acids present together with correction for the possible presence of metals, etc. Determination of C and H alone is of little value.

Preparation of crude egg phospholipid

Several methods were employed. Freeze-drying followed by extraction with boiling methanol, solution in ether and precipitation several times with acetone at 0–3° gave a crude product of atomic N/P ratio about 1.12. Extraction of the dried yolk with acetone and subsequently with chloroform-methanol (1:1, v/v) at 0–3° followed by evaporation of the chloroform-methanol, re-solution in ether (with rejection of insoluble material) and repeated precipitation with acetone, as used in many of the later preparations, gave a crude product of N/P ratio 1.03–1.08. Extraction of the fresh yolk with acetone to remove water and non-phospholipid lipid, as used in the initial stages of both the Hanahan *et al.* (1951) and Pangborn (1951) preparations of lecithin, was used in a few cases for comparison.

Removal of non-phospholipid nitrogen. The presence in

crude egg phospholipid preparations of free amino acids which give slow-moving, ninhydrin-reacting spots on paper chromatograms has previously been shown and their removal by passage through a cellulose column described (Lea & Rhodes, 1953*a*). Smith (1954) has also used this method for purification of the phospholipids of rubber latex. By this means the N/P ratio in some egg phospholipid preparations could be reduced to 1.00–1.01, but in others it remained at 1.03 despite complete removal of the amino acids as shown by the chromatographic test. No dialysable P was present in these preparations. Dialysis through cellulose and the chloroform-methanol-water diffusion method of Folch, Ascoli, Lees, Meath & LeBaron (1951) were also tried but could not be relied upon to remove the amino acids completely. No further improvement in N/P ratio could be obtained by dialysing material which had been purified by cellulose.

Attempts at separation on ion-exchange columns

Since phosphatidylcholine is approximately neutral and phosphatidylethanolamine definitely acidic it seemed likely that a useful separation of lecithin from phosphatidylethanolamine on a preparative scale might be achieved by the use of ion-exchange columns. Experiments were therefore carried out on egg phospholipid, containing approx. 18% amino N/total N, using a lightly cross-linked sulphonated polystyrene resin or a strongly basic resin with quaternary ammonium groups. A water-ethanol-ether (50:30:20, by vol.) mixture which dissolved the phospholipid without seriously affecting the resin was used in some experiments and 95% (v/v) ethanol in others. Choline and ethanolamine were readily separable on the acidic resin but both resins showed so small a capacity (1–3% of theoretical) for holding the comparatively large phospholipid molecules as to render the method unsuitable for preparative work. Moreover, analytical data on the fractions obtained indicated considerable hydrolytic decomposition, particularly on the acidic resin, a result which was not entirely unexpected in view of the demonstration in recent work that catalysis of esterification, ester interchange and hydrolytic reactions can occur on cation- and anion-exchange resins (Bernhard & Hammett, 1953; Schlenk & Holman, 1953). While it is quite possible that decomposition might have been avoided by the use of weakly acidic or basic resins the low capacity of the resins discouraged further work along these lines. A mixed-bed column was, however, used satisfactorily to regenerate lecithin from its cadmium chloride complex, a procedure which has since been described by Macpherson (1954).

Separation on alumina columns

The precise grade of alumina used by Hanahan *et al.* (1951) could not be obtained, but preparations were made with several other aluminas from

British and American sources. Hanahan's procedure was modified by carrying out all operations in a room at 0–3°, using solvents de-aerated with CO₂ or N₂. The moisture content of the alumina was adjusted by equilibration with 95% (v/v) ethanol before use and during preliminary washing of the column. Material sparingly soluble in cold ether or 95% ethanol which separated at any stage of the procedure was rejected.

From egg phospholipid containing approx. 18% phosphatidylethanolamine between 50 and 60% of the P applied to the column could be removed before appreciable quantities of ninhydrin-reacting substance began to appear, representing a recovery of 60–75% of the lecithin present. Part of the remaining lecithin could be recovered with only comparatively slight contamination with amino N.

All of the aluminas used effectively separated lecithin from phosphatidylethanolamine, but the

seemed to be contamination of the original lecithin-phosphatidylethanolamine mixture with some material of FA/P ratio <2 which was not being removed by the alumina column. Subsequent examination on silica columns and impregnated papers (below) indicated that the contaminant was, in part at least, lysolecithin.

Tests in which crude phospholipid was prepared from batches of three eggs (a) as rapidly as possible after initial freezing at –60°, (b) after autoclaving the yolk at 15 lb./sq.in. for 15 min., and (c) unusually slowly by prolonging each stage of the extraction procedure gave FA/P ratios for the crude phospholipids of 1.79, 1.77 and 1.88 respectively compared with 1.82 for that prepared by the method of Hanahan *et al.* (1951). These results indicated that phospholipase A activity during the extraction was not responsible for the low FA/P ratio of the crude phospholipid.

Table 1. *Fatty acid/phosphorus ratios of egg phospholipid and of 'lecithin' fractions prepared from it by the alumina column method*

Crude phospholipid		Alumina column		'Lecithin' fraction		
FA/P	N/P	Ethanol (%, v/v)	Temp. (°)	Yield (%)	FA/P	N/P
1.84	1.03	95	0–3	53	1.93	1.02
1.84	1.03	95	0–3	—	1.86	1.01
1.84	1.03	95	Laboratory	—	1.77	—
1.87	1.04	95	Laboratory	—	1.78	1.02
1.87	1.04	95	0–3	50	1.88	1.01
1.87	1.04	95	0–3	—	1.82	—
1.87	1.04	97.5	0–3	—	1.84	—
1.87	1.04	92	0–3	—	1.83	—
1.84	1.03	95	0–3	50	1.84	0.99

FA/P ratio of the lecithin fraction obtained was usually considerably below the theoretical value of 2.00 (Table 1).

Attempts to improve the purity of the lecithin by modification of the chromatographic procedure by the use of laboratory temperature in place of 0–3°, of 92 or 97.5% ethanol in place of 95% (v/v), or of acid-washed alumina were unsuccessful, as were repeated reprecipitations of the recovered lecithin by acetone from ether at various temperatures, fractionation from ether at low temperature, passage of the product in moist ether through a cellulose column, or dialysis.

Partial hydrolysis on the alumina column such as had been observed by Trappe (1940, 1941) but not by Hanahan *et al.* (1951) was apparently not the cause of the low FA/P ratios, since the crude phospholipid before application to the column showed ratios similar to those of the recovered 'lecithin' fractions, and the latter showed no marked change after a second passage through a column. The most likely explanation of the observed results

Separation on silicic acid columns

Silicic acid. This was prepared from commercial sodium silicate, essentially by the method of Isherwood (1946), which involves washing with 10N-HCl to remove Fe and Al. Washing by settling and aspirating off the supernatant layer was found preferable to filtration, and permitted rejection of a little of the most finely divided material which tended to choke the columns. The first settling after precipitation is rather slow and the preparation is best left overnight at this stage. Subsequent washings settle easily. The ageing in the dry state and rewashing which Isherwood found desirable for partition chromatography were omitted. After drying, the silicic acid was 'activated' by heating at 110° or 120° overnight and stored in double-seamed moisture-proof cans. Not all batches of adsorbent prepared by the above method have been equally satisfactory and some have had to be rejected, the reason for the variation being unknown. Of two samples of commercial products tested one (Mallinckrodt A.R.; suitable for chromatographic analysis by the method of Ramsey & Patterson) worked very well, with or without 'activation', the other not at all.

Operation of the columns. A.R. chloroform is washed with water to free it from ethanol, dried over Na₂SO₄, distilled

and immediately re-stabilized by the addition of 2% (v/v) methanol.

Silicic acid (24 g.) is mixed to a thin slurry with 50 ml. of the methanol-chloroform to be used as eluent and poured on to a glass wool plug in a 2.2 cm. diameter tube. Settling and draining of the column may be assisted by application of a slight pressure (about 3 cm. Hg) of N_2 . With some silicas it may be necessary to add a proportion of 'Celite' to obtain a reasonable rate of flow, but with batches prepared as described above this has usually proved unnecessary. After washing the column with a further 50 ml. of solvent the level of the liquid is allowed to sink to the surface of the adsorbent and the phospholipid (containing ≥ 30 mg. P) is added as a 10% solution in 8 ml. of the same solvent and allowed to enter the column. A further 5 ml. of solvent are then used to wash down the walls of the tube before continuous elution is commenced. A flow rate of 1–2 ml./min. is maintained by application of a suitable pressure of N_2 to the reservoir containing the solvent.

Fractions are collected with an automatic fraction cutter, tested for P and amino N and the material recovered by concentration in a rotary film vacuum evaporator (Lea *et al.* 1951). After collection of the required band or bands the remaining fractions may be recovered more rapidly and completely by changing the solvent to pure methanol.

A solvent containing 20% (v/v) methanol in chloroform has proved most generally useful for separating the constituents of the phospholipids of egg, although concentrations ranging from 8 to 32% (v/v) methanol have been used with various preparations of silicic acid.

methanol removed from the column a second fraction of FA/P ratio 1.06 (approximate determination on small sample) which was insoluble in ether and possessed haemolytic powers towards washed red blood corpuscles equal to those of lysolecithin prepared by the action of venom on egg yolk (see below).

A sample of dipalmitoleoyl-L- α -phosphatidylcholine from yeast (Hanahan & Jayko, 1952), kindly supplied by Dr Hanahan, showed on silica paper, in addition to the expected lecithin spot, a weaker second spot due apparently to a lysophospholipid (Fig. 3). Applied similarly to a silicic acid column the phospholipid was first washed with chloroform containing 2% (v/v) methanol and the solvent then changed to 20% (v/v) methanol in chloroform to elute a chromatographically pure lecithin fraction corresponding to 82% of the P applied to the column. The residual material, which was removed from the column by pure methanol, showed on silica paper only one spot, in the lysolecithin position, and possessed powerful haemolytic activity. Since this material was also insoluble in ether and gave a FA/P ratio of 1.08, as accurately as could be determined on the small quantity of material available, it was concluded that the particular specimen of yeast lecithin

Fig. 1. Preparation of pure lecithin from the alumina column material (FA/P ratio 1.84) by fractionation on a silicic acid column.

Purification of 'lecithins' prepared by the alumina column method. A typical lecithin preparation obtained from egg phospholipid by the alumina column method showed practically no ninhydrin reaction but possessed a FA/P ratio of only 1.84. Application of this material in 32% (v/v) methanol in chloroform to a silicic acid column followed by development with the same solvent gave a band containing 83% of the applied P and possessing a FA/P ratio of 2.02 (Fig. 1). Washing with pure

Fig. 2. Preparation of phosphatidylethanolamine from egg phospholipid on a silicic acid column.

examined also contained lysolecithin. The presence of free fatty acid in the first washings of the column, however, indicated that in this case part at least of the lysolecithin present had probably been produced by hydrolytic decomposition subsequent to isolation.

Preparation of pure phosphatidylethanolamine from egg phospholipid. When egg phospholipid is chromatographed on a silicic acid column, as described above, the phosphatidylethanolamine is

obtained as a well-defined band in the early fractions. In one experiment, using 32% (v/v) methanol in chloroform, 96% of the amino N applied was recovered in a fraction which gave, after hydrolysis, an amino N/P ratio of 0.96 (Fig. 2). Two other preparations analysed each gave 0.99.

Following removal of the phosphatidylethanolamine band pure lecithin fractions could be obtained by continued elution.

Chromatography on silica-impregnated paper

Preparation of the paper

Silica-impregnated paper is prepared essentially by the method of Kirchner & Keller (1950). Sheets of thick (Whatman no. 3) filter paper are immersed in sodium silicate solution (commercial 'water-glass' diluted with an equal volume of water) for 5 min., drained for 5 min. and transferred to 6N-HCl for 30 min. The papers are then washed free from chloride (approximately six changes of water), dried overnight at laboratory temperature, or for about 1 hr. at 50°, and finally heated for 1 hr. at 110°. The prepared papers have normally been stored in a large folder in a laboratory cupboard.

Method

Spots containing 4 μ l. of a 2–10% solution of the phospholipids are applied to the starting line of the chromatogram, the paper is rolled over a glass rod to prevent cracking and the upper end inserted into the trough in a covered jar for chromatography by the descending method. In the 'normal' procedure the papers are allowed to equilibrate with a layer of solvent on the bottom of the jar for 30 min. before the developing solvent is introduced via a hole in the lid. No appreciable advantage appears to result from extending the time allowed for equilibration. The papers are run until the solvent front has travelled about 20 cm. (approximately 2 hr.).

Reagents

Papers, after development and removal of the solvent, are sprayed with ninhydrin (0.5%, w/v) in *n*-butanol saturated with water and heated to 95° for 5 min. to detect substances containing free amino groups. After outlining the mauve spots, the same paper can be treated by immersion in 2% aqueous phosphomolybdic acid followed by washing and reduction with acid SnCl₂ to molybdenum blue (Chargaff, Levine & Green, 1948). This latter reagent is not specific for choline-containing lipids, as sometimes seems to be assumed, since phosphatidylethanolamine and phosphatidylserine also react, although less strongly. However, if the paper is well sprayed on both sides with the ninhydrin solution and heated the amino-containing phospholipids are destroyed and a negative reaction is then obtained with the phosphomolybdate-SnCl₂ reagent (which bleaches the mauve ninhydrin colour). This modification of the molybdenum blue reaction has been found very useful since, in conjunction with the direct ninhydrin test, it permits identification of the components of two pairs of substances which run close together on the chromatograms (Table 2). It had been hoped that the reinecke salt test for choline-containing lipids (Hack, 1953) and the hydroxylamine-FeCl₃ test for carboxylic esters (Whittaker & Wijesundera, 1952) would

also permit the identification of the substances in presence of one another but the sensitivity of these reagents on the silica papers was quite inadequate.

The fuchsin-SO₂ reagent of Liang (1947), after treatment of the chromatogram with 0.005M-HgCl₂ for 1 min. to liberate the aldehydes, may be used as a sensitive test for plasmalogen, the excess fuchsin reagent being removed with 0.05M aqueous SO₂ (Hack, 1953). Exposure to iodine vapour is useful for the non-specific location of spots and osmium tetroxide vapour stains black substances containing unsaturated fatty acids.

Materials

In addition to crude egg phospholipid the following materials were used in the chromatographic studies.

Egg lecithin. Iodine value 80, prepared from egg phospholipid by the silica column method.

Egg hydrolecithin. Iodine value 2, prepared from egg lecithin by catalytic hydrogenation over Pd.

Egg lysolecithin and egg lysophosphatidylethanolamine. Lysolecithin was prepared readily and in good yield by the action of rattlesnake (*Crotalus adamanteus*) venom on egg lecithin in ether solution according to the technique of Hanahan, Rodbell & Turner (1954). The same technique, however, did not appear to work satisfactorily with crude egg phospholipid, and a lysolecithin-lysophosphatidylethanolamine mixture was therefore prepared by direct action of the venom on egg yolk with recovery of the mixed lyso product as described by Fairbairn (1948). For preparation of the individual lyso compounds the mixture was applied in methanol-chloroform 20%, (v/v) to a silicic acid column, when the constituents separated cleanly as in the case of the diacyl phospholipids.

Dipalmitoleoyl-L- α -phosphatidylcholine (Hanahan & Jayko, 1952). As already indicated, the particular specimen of this yeast phospholipid available also contained an appreciable proportion of the lyso compound.

Phosphatidylserine. The phosphatidylserine fraction from ox brain, prepared according to the method of Folch (1942, 1948, 1949) contained 0.70 equiv. K and 0.36 equiv. Na/mole. Treated in chloroform solution with 2 equiv. HCl (in methanol) and run through a column containing moist cellulose to remove the inorganic salts and excess HCl the content of metals was reduced to 0.05 equiv. K and 0.03 equiv. Na. This procedure is simpler than that described in the original preparation. Hydrolysates from three preparations gave amino N/P atomic ratios of 0.97–1.01 and ethanolamine N/total amino N (as determined by the method of Axelrod, Reichenthal & Brodie, 1953) of 0.02–0.04. Treatment of the hydrolysates with an ion exchange resin showed that practically all of the amino N was acidic. The FA/P ratio determined on one sample was 1.95. These data indicate a purity as high as that of the fractions analysed by Folch (1942). The material, however, after catalytic hydrolysis with HgCl₂ gave a strong reaction for aldehyde and was apparently still contaminated with plasmalogen, as found by Lovern (1952) but not by Hack (1953).

Sphingomyelin. A preparation of sphingomyelin from ox brain and spinal cord was kindly supplied by Drs Lovern and Olley.

Through the kindness of various workers the following synthetic phospholipids were available.

Phosphatidylcholines (lecithins). Dipalmitoyl-L- α (Baer), dipalmitoyl-DL- α (Malkin), dimyristoyl-L- α (Baer).

Phosphatidylethanolamines. Dipalmitoyl-DL- α (Malkin), dipalmitoyl- β (Rose), dimyristoyl-L- α (Baer).

Ethanolamine plasmalogen. Ethanolamine 2:3-O-hexadecylidene-1-glycerophosphate (Malkin).

Phosphatidylserine. Distearoyl-L- α -phosphatidyl-L-serine (Baer).

RESULTS

Under the conditions described above using 20% (v/v) methanol in chloroform as developing agent all the phospholipids tested run as compact spots with little tailing (Figs. 3 and 4), except for phosphatidylserine (see below). Phosphatidylethanolamines are readily separated from phosphatidylcholines and sphingomyelin, and either of the first two substances separates easily from its corresponding lyso compound (Fig. 5). Ethanolamine plasmalogen runs close to lecithin and lysophosphatidylethanolamine to sphingomyelin (Table 2), but these pairs of substances differ widely in chemical composition and can be distinguished by appropriate reagents or by differential hydrolysis (Table 2).

Fig. 3. Chromatogram of lecithins run on silica paper in 20% (v/v) methanol-chloroform at 21° and stained with phosphomolybdic acid. 1, dimyristoyl-L- α ; 2, dipalmitoyl-DL- α ; 3, dipalmitoyl-L- α ; 4, dipalmitoleoyl-L- α ; 5, egg lecithin; 6, egg hydrolecithin.

Weaker spots, due apparently to contamination with the lyso compounds, are visible with two of the lecithins (Fig. 3) and one of the phosphatidylethanolamines (Fig. 4), while two of the latter also show spots at the origin, presumably due to water-soluble impurities or decomposition products. Secondary spots were also visible on the original chromatograms of some of the other substances, but too faintly to permit reproduction in the photographs.

Separations were still obtained in concentrations of methanol up to 40 or 50% (v/v) but were less

satisfactory than at 20%. In very low or in high concentrations of methanol the separation was lost by streaking.

For comparison of results obtained for a series of substances against lecithin with different batches of

Fig. 4. Chromatogram of phosphatidylethanolamines run on silica papers in 20% (v/v) methanol-chloroform at 21° and sprayed with ninhydrin. 1, egg phosphatidylethanolamine; 2, egg lysophosphatidylethanolamine; 3, dipalmitoyl-DL- α ; 4, dipalmitoyl- β ; 5, dimyristoyl-L- α .

Fig. 5. Chromatograms of glycerophospholipids prepared from egg yolk and run on silica papers in methanol-chloroform at 23°. Stained with phosphomolybdic acid; position of ninhydrin-reacting spots ringed. (a) 20% MeOH, (b) 30% MeOH. 1, Lysolecithin; 2, lysophosphatidylethanolamine; 3, lecithin; 4, phosphatidylethanolamine; 5, mixture.

paper, atmospheric temperatures and solvent systems the R_M value ($R_M = \log (1/R_F - 1)$) of Bate-Smith & Westall (1950) has proved useful. The range of R_F values of phosphatidylethanolamine, for example, is much less when corrected for variations in the R_F of the lecithin standard on the basis of proportionality of R_M rather than R_F values.

with the 'normal' method which had presumably been obtained under moister atmospheric conditions.

While the scatter of individual results observed in the 'normal' procedure may have been somewhat reduced by controlling the moisture content of the system it was by no means eliminated and some lack

Table 2. R_F values and chemical reactions of glycerophospholipids and sphingomyelin chromatographed on silica paper by the 'normal' method in 20% (v/v) methanol-chloroform

The number of determinations is given in parentheses.

Substance	$R_F \pm \text{s.d.}$	Reaction with		
		Ninhydrin	Phospho- molybdic acid-SnCl ₄ after ninhydrin	HgCl ₂ - Schiff's reagent
Lysolecithin	0.27 $\pm$ 0.08 (29)	-	+	-
Lysophosphatidylethanolamine	0.44 $\pm$ 0.08 (19)	+	-	-
Sphingomyelin*	0.45 $\pm$ 0.06 (9)	-	+	-
Plasmalogen*	0.62 $\pm$ 0.05 (10)	+	-	+
Lecithin	0.63 $\pm$ 0.05 (42)	-	+	-
Phosphatidylethanolamine	0.80 $\pm$ 0.04 (19)	+	-	-

* Sphingomyelin and plasmalogen alone resist mild alkaline hydrolysis (Hack, 1947). Plasmalogen alone is destroyed by hydrolysis with cold dilute acid (Fairbairn, 1945) or with HgCl₂ (Thannhauser *et al.* 1951). Sphingomyelin can be further confirmed by testing for the presence of chloroform-soluble, ninhydrin-reacting base (sphingosine) in an acid hydrolysate (McKibbin & Taylor, 1949).

Effect of water. In the 'normal' method described above, separations were consistently obtained over a large number of runs, but the R_F values themselves varied from paper to paper (Table 2).

To test whether this scatter was due to variations in the water content of the paper a 20%, (v/v) methanol in chloroform solvent mixture was nearly saturated by the addition of 2.5% (v/v) water, and freshly heated papers were run using this and a series of drier solvents, the dry papers being equilibrated by exposure to the vapour of the solvent for 16 hr. before running. Fig. 6 shows that the R_F values of three test phospholipids (run as a mixture) increased as the water content of the system increased, but the wettest of the range of experimental conditions used gave values which were still slightly below the average of those obtained in the 'normal' method (papers stored in contact with the atmosphere).

In another experiment the papers were equilibrated over aqueous H₂SO₄ providing a relative humidity of 60% for 24 hr. before running with dry solvent. The resulting R_F values (Table 3) approximated to those obtained with 2.5% (v/v) water in the solvent.

These results indicate that the range of R_F values observed for a given substance can be partly accounted for by variations in the moisture content of the paper, but the experiments did not reproduce the higher part of the range of R_F values recorded

Fig. 6. Effect of additions of water to the solvent on the R_F values of phospholipids in 20% methanol-chloroform. Dry papers equilibrated with solvent before running. R_F values plotted are the average of four papers. ●—●, Lecithin; ×—×, lysophosphatidylethanolamine; ○—○, lysolecithin.

of reproducibility appears to be inevitable, probably owing to non-uniformity of the impregnated paper.

To ensure that the silica papers are used at a suitable moisture content, it would be necessary in very dry, or possibly in very wet climates to equilibrate them in an atmosphere at, for example, 60 or 70% relative humidity before use or, alternatively, to equilibrate the paper with solvent

containing 2.5 % (v/v) water in the jar before running the chromatogram. The extra complication of adjusting papers to a controlled moisture content has not, however, been found worth while in this locality during the period of the investigation.

Effect of fatty acid composition. Since the phospholipid fractions from egg, which contain a range of saturated and unsaturated fatty acids, travel in spots as compact as those of the individual synthetic materials, while egg lecithin (iodine value 80) runs only very slightly and probably not significantly ahead of the hydrogenated product (iodine value 2), and since the same is true for the unsaturated dipalmitoleoyl-L- α - and for the saturated dipalmitoyl-L- α -lecithins (Fig. 3), it would appear that no considerable degree of separation according to

instead of in the usual *n*-butanol, to prevent charring of the paper on heating. Since phosphatidylserine and phosphatidylethanolamine run close together on the unidimensional chromatogram and react similarly with most reagents it was found necessary for detection of these substances in presence of one another to run the paper a second time in a direction at right angles to the first using 95 % (v/v) ethanol as solvent. Under these conditions phosphatidylethanolamine, which is freely soluble, moves rapidly while phosphatidylserine scarcely moves at all. The fact that the phosphatidylethanolamine spot tails rather badly in this solvent does not hinder the separation. Both substances can then be stained by the ninhydrin spray.

Table 3. *Separation of phospholipids on silica paper pre-equilibrated at 60 % relative humidity, using 20 % (v/v) methanol-chloroform*

Each R_F value is the average from six determinations.

Paper batch no.	R_F values			
	Lysolecithin	Lysophosphatidylethanolamine	Lecithin	Phosphatidylethanolamine
1	0.21	0.46	0.63	0.82
2	0.28	0.48	0.61	0.81
3	0.19	0.35	0.55	0.77

degree of unsaturation or (within the rather narrow limits encountered) of chain length is occurring. On the other hand, the dipalmitoyl β -phosphatidylethanolamine ran consistently, although only slightly, faster than the corresponding α -compound (Fig. 4).

Separation of phosphatidylserine and phosphatidylethanolamine by two-dimensional chromatography. The synthetic distearoyl phosphatidylserine was practically insoluble in most organic solvents and the chromatographic experiments reported were carried out with the preparation from brain. This material ran unsatisfactorily on silica paper under the 'normal' conditions described above. Whether in the salt form as isolated or as the free acid it appeared after staining with ninhydrin as a streak between R_F 0.25 and 0.85. The greater part of the phosphorus, however, was found to be concentrated in a spot at R_F about 0.85, i.e. very close to phosphatidylethanolamine, and with the less sensitive phosphomolybdate reagent the 'tail' was hardly visible.

Dipping the paper in 0.1 *N* methanolic H_2SO_4 and drying in air before use was found to eliminate tailing of the phosphatidylserine spot, but with these acid papers it was necessary to dissolve the ninhydrin reagent (0.5 %, w/v) in 75 % (v/v) methyl cellosolve-water saturated with $KHCO_3$,

The aldehydic impurity in the crude brain phosphatidylserine ran with the main bulk of the phosphorus and much faster than the synthetic ethanolamine plasmalogen. This observation is in accord with recent suggestions that plasmalogens as they occur in tissues are not represented by the simple formula of the synthesized substance employed in these tests, although such substances may result from the action of alkali used during the extraction process. It is possible that serine plasmalogens are also present in brain (Klenk & Böhm, 1951; Ansell & Norman, 1955).

At the present stage of development of the chromatographic methods it would undoubtedly be of advantage with complex mixtures of phospholipids, wherever possible, to effect a preliminary fractionation by conventional physical or chemical means. The constituents of the mixture most insoluble in ether and/or ethanol (sphingomyelin, phosphatidylserine, inositol lipids) should be removed before chromatographing the remaining glycerophospholipids, which cannot be separated satisfactorily by solvent fractionation procedures. Phosphatidylserine, for example, can be so reduced in concentration by precipitation with ethanol in the cold that it no longer interferes with the detection of reasonable quantities of the other ninhydrin-reacting substances by the 'normal' method. The

use of selective hydrolysis with alkali, acid or mercuric chloride has already been mentioned as a potentially useful auxiliary chemical procedure (Table 2).

DISCUSSION

The consistently low FA/P ratios of 'lecithins' prepared by the alumina column method caused some difficulty at an early stage of the work. In the literature 'lecithins' have been prepared from various sources, and particularly from egg yolk, in great numbers over a long period, but have rarely been characterized at all adequately. Only very occasionally have FA/P ratios been determined, despite the fact that this ratio cannot be deduced from P and N contents unless the mean equivalent of the fatty acids and the content of any impurities present have also been determined. An exception is the recent work of Hanahan and collaborators, who reported ratios of 1.95, 1.99 and 2.00 for egg lecithin (Hanahan *et al.* 1951), of 1.93 and 1.97 for yeast lecithin (calculated from data reported by Hanahan & Jayko, 1952) and of 1.95 and 1.97 for egg lecithin (Hanahan, 1952). The last two results, however, apparently depend on an abnormally low equivalent (corresponding to C_{13} and C_{14}) found for the saturated fatty acids which comprised 54 and 56 % of the total fatty acids of the lecithin and would have been considerably lower if the saturated fatty acids had possessed a more normal (C_{16} and C_{18}) composition.

Since neither plasmalogen, sphingomyelin nor water-soluble impurities could be detected in our products in quantity sufficient to account for the results, the presence of lysophospholipids seemed the most likely cause of the low ratios. Despite the fact that lysolecithin differs from lecithin in being insoluble in ether and powerfully haemolytic, it proved impossible to demonstrate the presence of lysolecithin by either of these methods. Lysolecithin is known to become soluble in ether when excess lecithin is also present and our own observations showed that the extremely rapid and powerful haemolytic action of pure lysolecithin is greatly obscured by the presence of excess lecithin. The difficulty was finally resolved by the excellent separation of lysophospholipids from the diacyl substances which was achieved on the silicic acid columns and impregnated papers. In view of Hanahan's results in his later paper on yeast phospholipid (Hanahan & Jayko, 1952), using a lighter column loading, it might be possible also to achieve this separation on alumina, but we were unable to do so by the procedure described for egg phospholipid (Hanahan *et al.* 1951), and it is obviously more difficult than on silicic acid.

A further important advantage of silicic acid over alumina lies in the fact that the phosphatidylethanolamine band comes off the silicic acid column

quickly and cleanly, thus making possible for the first time a rapid and simple preparation of this substance. Silicic acid, too, is undoubtedly the better adsorbent in respect of completeness of recovery from the column, and the danger of decomposition appears to be less. When to its capacity for fractionating complex lipid mixtures into their constituent classes is added the ability to subdivide further the phospholipid fraction, chromatography on silicic acid becomes a most valuable tool in lipid chemistry. The impregnated-paper technique in particular should find wide application in this field.

Whether or not lysophospholipids are present pre-formed in egg yolk or result from decomposition during the extraction and separation is a question which has not yet been settled. The evidence to date is against their being artifacts but a definite conclusion must await the results of a more detailed examination of the composition of the phospholipids of egg which is now being undertaken by the methods described in this paper.

Note added in proof. In agreement with the results in Table 1, calculation from the analysis given recently by Kates (1954) of a lecithin prepared from egg yolk by the method of Hanahan *et al.* (1951) gives a FA/P ratio of 1.83.

SUMMARY

1. Egg lecithins prepared by chromatographic separation on alumina columns were found to possess low fatty acid/phosphorus (FA/P) ratios. Re-chromatographed on silicic acid they separated into lecithin of correct FA/P ratio and lysolecithin.
2. Pure phosphatidylethanolamine and lecithin (phosphatidylcholine) were prepared in good yield from egg phospholipid by chromatography in methanol-chloroform on a silicic acid column. Lysophosphatidylethanolamine and lysolecithin were similarly prepared from the phospholipids of venom-treated yolk.

3. The chromatographic behaviour of a series of glycerophospholipids, both natural and synthetic, on silica-impregnated paper has been investigated and good separations obtained.

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The Use of Radioactive Isotopes in Immunological Investigations

8. LABELLING ANTIBODIES WITH ^{131}I AND ^{35}S *

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In investigations on the reactions between antigens and antibodies, the use of a readily detectable and measurable label has been extremely valuable. For the quantitative analysis of serological precipitates obtained from a protein antigen and a suitable antiserum it is usually sufficient if the antigen is labelled, since the amount of antibody can be calculated from the nitrogen and antigen contents of the precipitates. However, for many other types of immunological investigation (e.g. studies of the behaviour of antibodies *in vivo*), labelled antibodies are most useful.

* Part 7 of this series, Francis, Mulligan & Wormald (1955a).

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Biologically labelled antibodies have been obtained by administering amino acids labelled with ^{15}N (e.g. Heidelberg, Treffers, Schoenheimer, Ratner & Rittenberg, 1942) or ^{14}C (Humphrey & McFarlane, 1952) to rabbits which were actively synthesizing antibodies. Since these methods normally yield antibodies containing insufficient isotopic label to allow of their being detected and measured after considerable dilution has occurred, many investigators have had to rely on an artificial labelling technique involving the combination of the antibodies with a reagent containing a readily detectable group or atom, e.g. a dyestuff or a radioactive isotope. (Recently, however, Humphrey & McFarlane (1954a, b) have been able to use [^{14}C]-phenylalanine or *Chlorella* protein of high specific