SUMMARY

1. Free glutamic acid accumulates within *Staphylococcus aureus* incubated in the presence of *N*-phosphoryl-L-glutamic acid or diethyl glutamate.

2. Free glutamic acid does not accumulate within cells incubated with peptides containing glutamic acid residues unless glucose is also present. The rate of accumulation is higher when incubation takes place with α-glutamyl peptides than with γ-glutamyl peptides and is affected by the rate of hydrolysis of the peptide within the cell and also by the partition coefficient of the peptide between *n*-butanol and water.

3. When *Staphylococcus aureus* is incubated with glucose and di- or tri-peptides containing α-glutamyl residues, the rate of accumulation of free glutamic acid within the cells can be correlated with the product of the rate of hydrolysis and the partition coefficient between butanol and water.

4. Accumulation of free glutamic acid in all cases studied is inhibited by sodium azide and 2:4-dinitrophenol.

REFERENCES

Atherton, F. R., Openshaw, H. T. & Todd, A. R. (1945). *J. chem. Soc.* p. 660.
Britten, R. (1952). *Yearb. Carneg. Instn*, p. 92.
Britten, R. (1955). *Amino Acid Metabolism*, p. 120. Ed. by McElroy, W. D. & Glass, H. B. Baltimore: Johns Hopkins Press.
Cahill, W. M. & Burton, I. F. (1940). *J. biol. Chem.* **132**, 161.
Chiles, H. M. & Noyes, W. A. (1922). *J. Amer. chem. Soc.* **44**, 1802.
Cohen, G. N. & Rickenberg, H. V. (1956). *Ann. Inst. Pasteur*, **91**, 693.
Creaser, E. H. (1956). *Biochem. J.* **64**, 539.
Gale, E. F. (1945). *Biochem. J.* **39**, 46.
Gale, E. F. (1946). *Advanc. Enzymol.* **6**, 1.
Gale, E. F. (1947). *J. gen. Microbiol.* **1**, 53.
Gale, E. F. (1949). *J. gen. Microbiol.* **3**, 369.
Gale, E. F. (1951). *Biochem. J.* **48**, 286.
Gale, E. F. (1953). *Advanc. Protein Chem.* **8**, 285.
Gale, E. F. & Taylor, E. S. (1947). *J. gen. Microbiol.* **1**, 314.
Kihara, H., McCullough, W. G. & Snell, E. E. (1952). *J. biol. Chem.* **197**, 783.
Kihara, H. & Snell, E. E. (1955). *J. biol. Chem.* **197**, 791.
Mitchell, P. D. & Moyle, J. (1956). *Symp. Soc. gen. Microbiol.* **6**, 150.
Najjar, V. A. & Gale, E. F. (1950). *Biochem. J.* **46**, 91.
Rowlands, D. A. & Young, G. T. (1957). *Biochem. J.* **65**, 516.
Taylor, E. S. (1947). *J. gen. Microbiol.* **1**, 86.
Troll, W. & Cannan, R. K. (1953). *J. biol. Chem.* **200**, 803.
Winnick, T. & Scott, E. M. (1947). *Arch. Biochem.* **12**, 201.
Zamecnik, P. C. & Stephenson, M. L. (1947). *J. biol. Chem.* **169**, 349.

Phospholipids

4. ON THE COMPOSITION OF HEN'S EGG PHOSPHOLIPIDS*

By D. N. RHODES and C. H. LEA
Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge and Department of Scientific and Industrial Research

(*Received* 10 *August* 1954)

Despite the long-established use of egg yolk as a convenient source of phospholipids, data available in the literature on the composition of this important fraction of the yolk (over 20% of the total solids) are incomplete and contradictory. Wittcoff (1951), in his recent monograph, was able to quote only three analyses, the earliest of which (30% lecithin, 70% kephalin) is clearly in conflict with the later data of Nottbohm & Mayer (1933) (72% lecithin, 28% kephalin) and of Kaucher, Galbraith, Button & Williams (1943) (72% lecithin, 25% kephalin, 2·5% sphingomyelin).

The contents of lecithin obtained by these later workers were based upon estimation of choline after hydrolysis, as were the recently published figures of 80% (van Handel, 1954), 82% (Sulser, 1954), 76% (Thiele, 1955) and 78% (Tsuji, Brin & Williams, 1955).

The remainder of the lipid nitrogen is usually reported as 'kephalin', but direct determination of the amino nitrogen has indicated considerably higher values, e.g. 42% (Chargaff, Ziff & Rittenberg, 1942), 38% (Kline & Johnson, 1946), 35% (Kline, Gegg & Sonoda, 1951) and 9·4, 20 and 32% (Sulser, 1954). Chargaff *et al.* (1942) used a direct van Slyke determination on the intact phospholipid, a procedure known to be subject to interference by the unsaturated acids present (Lea & Rhodes, 1954). Kline *et al.* (1951) also used this method, but stated that the chosen reaction time of 5 min. gave a

* Part 3: Lea, Rhodes & Stoll (1955).

result agreeing with a similar determination on a hydrolysate. Sulser (1954) separated the water-soluble hydrolysis products by paper chromatography and obtained the quoted figures by three different methods of estimating the recovered ethanolamine.

Any method using amino nitrogen content as a measure of 'kephalin' is liable to give too high values unless the lipid preparation has been freed from amino acids, which are tenaciously held (Lea & Rhodes, 1953); such errors can be considerable since the nitrogen content of a diacylglycerophospholipid is less than 2%. Our own recently determined values of amino nitrogen/phosphorus or amino nitrogen/total nitrogen for samples of the acetone-insoluble phospholipids of egg, from which water-soluble contaminants had been removed, were much lower than those of the American workers, e.g. 21% (Lea & Rhodes, 1953), 19% (Lea & Rhodes, 1954) and 19% (Lea & Rhodes, 1955).

Part of the wide variation in the proportions recorded for the major constituents of the phospholipids of egg may well, therefore, be due to the unsatisfactory nature of the analytical methods which have been used. A further factor could be the artificial concentration of one component by the purification procedures employed; the phospholipid preparation examined by Chargaff *et al.* (1942), for example, contained less than one-half of the average lipid phosphorus of the egg.

Small amounts of sphingomyelin appear to be present in egg, although Schmidt (1946) and Tsuji *et al.* (1955) found none by the differential-hydrolysis method. This procedure, which measures the difference between total and alkali-labile phosphorus, is not very satisfactory when the proportion of alkali-resistant material is small. In addition to the value of 2·5% found by Kaucher *et al.* (1943), Yanamoto (1954*a*) found 2·6% of sphingomyelin in fertile and 7·7% in 'wind' (infertile?) eggs. van Handel (1954) reported 4·5 and 5·5% of sphingomyelin in egg phospholipids, but these higher values are not consistent with nitrogen/phosphorus ratios of 1·00 and 1·01 reported by this worker for the total phospholipids, since sphingomyelin contains two nitrogen atoms. Moreover, the alkali-resistant substance actually assayed was characterized only by a choline/phosphorus ratio of unity, a criterion insufficient to distinguish sphingomyelin from lecithin or from choline-containing plasmalogens. These figures, therefore, may be too high.

Egg phospholipids have been found to contain not more than traces of plasmalogen (Feulgen & Bersin, 1939). Thiele (1955) found less than 0·2%, mostly in the lecithin fraction, but Yanamoto (1954*b*) reported 2·1% in fertile and 1·0% in 'wind' eggs.

Amino acid nitrogen was reported to be absent by Chargaff *et al.* (1942) and by van Handel (1954). Sulser (1954) separated seven ninhydrin-reacting substances from hydrolysates of egg phospholipid, and estimated the equivalent of 0·17% of phosphatidylserine, but reported no measures to exclude adventitious amino acids.

Inositol was found in hydrolysates of egg phospholipids by Celmer (1949) and was also demonstrated qualitatively by Sulser (1954).

Phospholipid analyses so far described in the literature have been based upon the examination of water-soluble hydrolysis products from the crude materials, or from fractions obtained by countercurrent distribution. Although valuable information as to the complexity of the mixture is gained by such methods, positive identification of the parent substances cannot usually be made on these data alone. Surprisingly little attention has been paid to the fatty portion of the molecule; it has, for example, been shown only recently (Hack, 1953; Rapport & Alonzo, 1955*a*) that the 'lecithin' of ox heart, prepared by precipitation with cadmium chloride, contains a major proportion of plasmalogen, although a determination of the fatty acid/phosphorus or ester/phosphorus ratio would have revealed the deficiency of carboxylic ester.

The present investigation arose from the observation that crude egg phospholipid and lecithin prepared from it, either by chromatographic separation on alumina (Hanahan, Turner & Jayko, 1951) or by the cadmium chloride procedure (Pangborn, 1951), contained small amounts of lyso compounds, the presence of which could be demonstrated by chromatography on silicic acid (Lea, Rhodes & Stoll, 1955). By the use of this method and of an improved alumina-column technique the main components of egg phospholipids have now been quantitatively separated, and the minor components concentrated sufficiently to permit their identification and estimation.

The distribution of the fatty acids in egg phosphatidylcholine and phosphatidylethanolamine has been considered in a recent communication (Rhodes & Lea, 1956*a*).

EXPERIMENTAL

Analytical methods. Total P and N, I_2 val. and fatty acids were determined as previously described (Lea *et al.* 1955); choline was determined by the method of Entenman, Taurog & Chaikoff (1944).

Carboxylic esters were determined by the method of Rapport & Alonzo (1955*b*) in samples too small to allow an accurate titration of fatty acids. A standard of pure methyl palmitate (Hormel Foundation) was included in all determinations.

Amino N in intact phospholipids was estimated by reaction with ninhydrin (Lea & Rhodes, 1955). Ethanolamine and amino acid N were determined, after hydrolysis,

by the Van Slyke manometric procedure and by the fluorodinitrobenzene (FDNB) method of Axelrod, Reichenthal & Brodie (1953). Reagent volumes were slightly modified in the latter method to avoid the necessity for pipetting the 6 and 8 ml. volumes required in the original procedure.

Inositol was determined after hydrolysis in 6N-HCl for 40 hr. by the method of Campling & Nixon (1954).

Lysolecithin and lysophosphatidylethanolamine were determined by the decrease in ester content (Shapiro, 1953) after incubation with lecithinase *B* of *Penicillium notatum* (Fairbairn, 1948). The reaction was complete within 1 hr. at 37° in acetate buffer, pH 4; no further change occurred up to 4 hr.

Sphingomyelin was estimated by the methods of Schmidt (1946) and of McKibbin & Taylor (1949). The sensitivity of the latter method was greatly increased by estimating the $CHCl_3$-soluble base hydrochloride, obtained after hydrolysis, by its colorimetric reaction with ninhydrin, in place of the total N. The determination could not be made quantitative in the absence of a sufficiently pure specimen of sphingosine to provide a standard.

Plasmalogens were estimated by reaction of the aldehydes with *p*-nitrophenylhydrazine (Wittenberg, Korey & Swenson, 1956). The results were calculated on the basis of a molecular extinction of 23 500 given by these authors for the *p*-nitrophenylhydrazone of *n*-decaldehyde.

Unless otherwise stated, all results are expressed on a molar basis.

Chromatographic methods. Chromatography on silica-impregnated filter paper and on silicic acid columns was carried out as previously described (Lea *et al.* 1955; Rhodes & Lea, 1956*b*). The silicic acid (Mallinckrodt) used in the present work was more retentive than some of those previously described, and methanol–$CHCl_3$ (30:70, v/v) was found more satisfactory than the 20:80 (v/v) mixture previously specified.

Chromatography of phospholipids on alumina has been employed by Hanahan *et al.* (1951) for the preparation of ovolecithin, by adsorption of the kephalin fraction from ethanolic solution. In this method a large volume of 95% ethanol was required to elute the lecithin, and its recovery was not complete before aminophospholipids appeared; the adsorbed kephalins were not recovered (see also Thiele, 1955).

A rapid and quantitative elution of the lecithin of egg-yolk phospholipids, together with recovery of the kephalin fraction in good yield, has now been achieved by chromatography on alumina, with other solvent mixtures, as follows. Alumina (40 g.; Aluminum Co. of America) was repeatedly stirred with methanol–$CHCl_3$ (1:1, v/v) and the supernatant liquid decanted until all very fine material had been removed; the slurry was then poured on to a glass-wool plug in a 1·5 cm. diam. tube. Egg phospholipid (2 g.) was applied in 20 ml. of the same solvent and elution commenced, after the walls of the tube had been washed down. The lecithin appeared in a concentrated band, free from amino N, and was recovered quantitatively in 150 ml. of eluate. The retained phospholipid was then eluted with ethanol–$CHCl_3$–water (5:2:2, by vol.), recoveries of P in this fraction corresponding to 92–98% of the kephalin applied. The rate of flow of this column was 10 ml./min. for the first solvent and 2 ml./min. for the second.

Pure phosphatidylcholine and phosphatidylethanolamine, prepared from egg, were recovered unchanged in quantitative yield when subjected to this procedure, indicating that no losses were occurring through hydrolysis on the column.

Preparation of egg phospholipids

Extraction from the yolk. Fertile eggs, obtained from the same hens and less than 24 hr. old, were used throughout the work. The whites of forty-eight eggs were separated and the yolks freeze-dried. After thorough mixing in a blender the dried material was packed *in vacuo* and stored at −30°.

Extraction of the dried yolk was carried out according to the scheme in Table 1, with solvents de-aerated by bubbling with CO_2. The operations shown in the left-hand column form a sequence typical of those generally employed in the extraction of phospholipids. The efficiency of these steps was tested by recovery of the mother liquors and residues, as shown in the right-hand column. The proportions of the total phospholipid recovered in the various fractions are shown in Table 2.

Only a small part of the total lipid P (4·1%) was present in the acetone extract AS 1, which contained the great bulk of the triglyceride fat, steroids and pigments. After removal of the solvent from this extract the lipids were applied in ethanol-free $CHCl_3$ to a silicic acid column, and the glycerides washed out (Borgström, 1952). The small quantity of phospholipid present was separated with some difficulty from intense yellow, green and red pigment bands, which were eluted first with methanol–$CHCl_3$ (2:98, v/v), leaving the phospholipid to be recovered in methanol–$CHCl_3$–water (70:25:5, by vol.). On silica-impregnated paper this phospholipid showed only two spots reacting with phosphomolybdic acid, one of which also reacted with ninhydrin; from their R_F values they appeared to be lecithin and phosphatidylethanolamine.

Most of the phospholipid of the dried yolk (92·7%) was recovered in the cold methanol–$CHCl_3$ extract E 1, leaving only 3·1% to be removed by subsequent treatment with the boiling solvent (extract E 2). The phospholipid recovered by silicic acid column chromatography of E 2 gave on analysis: N/P, 0·99; choline N/total N, 0·59; ethanolamine N/total N, 0·42. Chromatography on silica-impregnated paper showed the presence of lecithin, phosphatidylethanolamine and lysolecithin.

At this stage extraction of the lipids from the dried yolk was virtually complete, only traces of fatty acids being recoverable after saponification, acidification and extraction (E 3).

Purification by acetone. The cold methanol–$CHCl_3$ extract, E 1, containing most of the phospholipid, was purified by precipitation four times at 0° from a 15% (w/v) solution in light petroleum (40–60°), by the addition of 8 vol. of acetone. During this process a total of 14% of the original P passed into the acetone-soluble mother liquors (AS 2), corresponding to an average loss of 3·7% of the lipid P at each precipitation. Borgström (1952) has shown that this loss can be greatly reduced by the addition of $MgCl_2$ to the acetone, but this procedure requires the use of larger volumes of solvent and a molar $MgCl_2$/lipid P ratio of 2:1 which, it was considered, might lead to undesirable contamination of the phospholipid.

The mother liquors from the acetone precipitations (AS 2) contained 530 mg. of neutral fat, of I_2 val. 48, which corresponded to 6·4% by wt. of the extract E 1, and represented only 3% of the total neutral lipid recovered in AS 1 and E 1.

Table 1. *Extraction of the phospholipids from egg yolk*

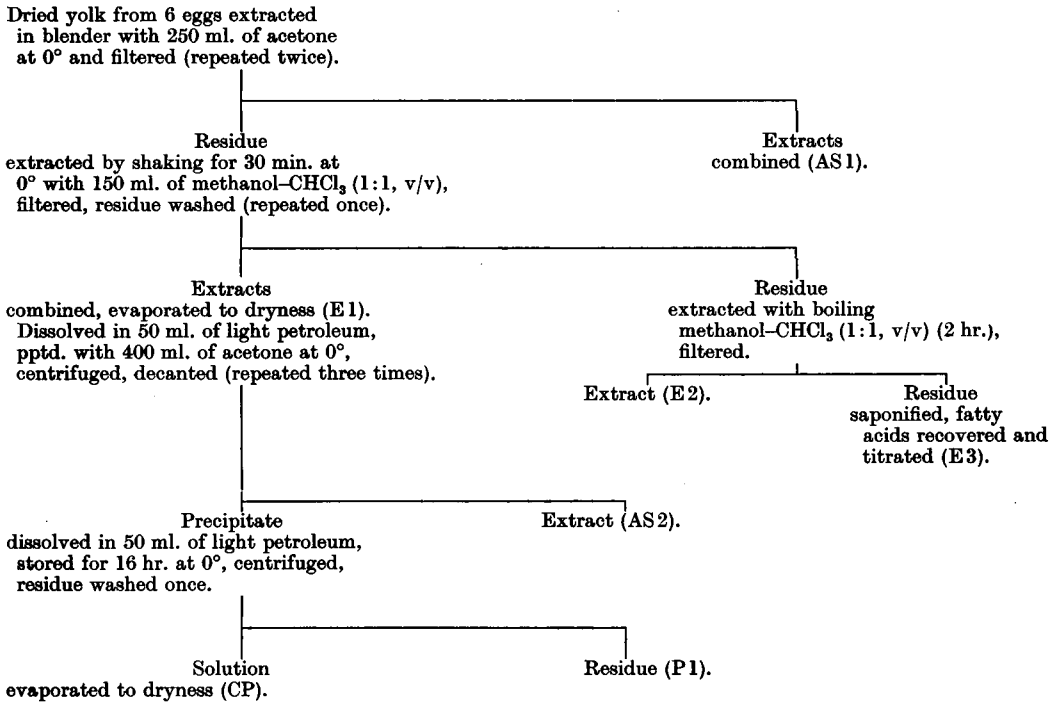

Table 2. *Recovery of lipid P in main extracts*

Fraction	Lipid P in extract (as % of total P recovered)
Acetone extract of dried yolk (AS1)	4·1
Methanol–$CHCl_3$ extract (at 0°) (E1)	92·7
Methanol–$CHCl_3$ extract (boiling solvent) (E2)	3·1
Fatty acids recovered from residue (calculated as P) (E3)	0·2

Extraction of the dried yolk with acetone had therefore removed over 97% by wt. of the neutral lipids originally present.

Fraction insoluble in light petroleum. Of the total lipid P 2·1% was found in material insoluble in light petroleum at 0°, a procedure designed to take out sphingomyelin and fully saturated lecithins. This fraction contained considerable quantities of salts and amino acids (N/P, 1·48) which were completely removed, without loss of P, by treatment with a cellulose column (Lea & Rhodes, 1953).

Analysis of the purified material gave: N/P, 1·26; sphingomyelin/P, 0·26 (Schmidt, 1946); I_2 val., double bonds/P, 1·88.

Separation on an alumina column yielded 83% of choline-containing phospholipids and 15% of 'kephalins'. On impregnated paper the choline-containing phospholipids showed three phosphomolybdic acid-reacting spots corresponding to lecithin, sphingomyelin and lysolecithin; the 'kephalins' showed one ninhydrin-reacting spot corresponding to phosphatidylethanolamine.

After selective alkaline hydrolysis (Schmidt, 1946) of the whole fraction insoluble in light petroleum only the sphingomyelin spot remained: the recovery of a ninhydrin-reacting, $CHCl_3$-soluble base from this alkali-resistant material, after hydrolysis according to McKibbin & Taylor (1949), confirmed its probable identity with sphingomyelin.

The I_2 val. of the fraction insoluble in light petroleum, when corrected on the assumption that the sphingomyelin present contained one double bond, was only slightly below the average for the whole phospholipid, indicating that no doubly saturated glycerophospholipids had been selectively precipitated.

The fraction insoluble in light petroleum, therefore, consisted mainly of glycerophospholipids of average constitution, and, as shown below, the sphingomyelin present represented only a small proportion of the sphingomyelin of the whole phospholipid.

Conclusion. It can be concluded from these experiments that a procedure such as that usually employed for the preparation of egg phospholipids yields about 80% of the total as acetone-insoluble material. By precipitation with 5 in place of the 8 vol. of acetone used above, this yield could be increased to 87%, but the loss was still too great in a preparation to be used for analysis. On the basis of this work the following procedure was adopted for the preparation of material reasonably representative of the total phospholipids of the yolk.

Preparation of the total phospholipid

The dried yolk was treated with acetone, as in Table 1, and the small proportion of the phospholipids extracted with the neutral fat (AS1) was recovered on a silicic acid column as described above. This was added to the main bulk of the phospholipid obtained by extraction with methanol–$CHCl_3$ at 0° (E1). Purification of the combined crude phospholipid fractions with acetone was omitted, leaving the small amount of neutral fat known to be present to be removed in the first runnings from the chromatographic columns. As no well-defined fractionation had been achieved by light petroleum this step also was omitted.

Material prepared in this way contained 97% of the lipid P of the yolk; the remaining 3%, obtainable only by prolonged hot extraction, was not recovered because of the possibility of decomposition.

RESULTS

Removal of neutral fat. The triglyceride fat in the preparations of total phospholipid was not retained by either adsorbent in the solvent systems used. It was eluted from the alumina columns with the 'lecithin' fractions and, when this was subsequently applied to a silicic acid column, the neutral fat and pigment appeared with the solvent front, about 10 hold-up volumes ahead of the first phospholipid band.

Separation on alumina. The progress of a typical separation on alumina of a total egg-phospholipid preparation is shown in Table 3. The ninhydrin reaction of the fraction GL corresponded to the presence of less than 0·1% of phosphatidylethanolamine, and no trace of choline-containing phospholipid was found in the three fractions combined as GK. The constant value of the ester reaction of the three 'kephalin' fractions, recorded in the last column, shows that no liberation of fatty acids was occurring through hydrolysis on the column even in the presence of an aqueous eluent; these fractions had been adsorbed for periods of 1·2, 6 and 20 hr. respectively.

A number of estimations of the choline-containing phospholipids of egg-yolk preparations made by this method all gave values between 78 and 82%. No inositol was found in a hydrolysate of GL.

Separation on silicic acid. When chromatographed on silica-impregnated paper both fractions from the alumina column showed evidence of slower-moving phospholipids corresponding to lyso compounds; silicic acid columns were, therefore, used to separate these components. When run in methanol–chloroform (30:70, v/v) the diacylglycerophospholipids were recovered in the first peaks (Fig. 1).

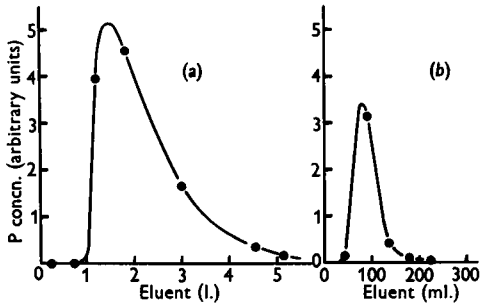

Fig. 1. Recovery of diacylglycerophospholipids by chromatography on silicic acid in methanol–$CHCl_3$ (30:70, v/v). (*a*) Fraction GL (98 mg. of lipid P applied to 120 g. column). (*b*) Fraction GK (17·2 mg. of lipid P applied to 30 g. column).

Table 3. *Separation of the total egg phospholipid on alumina*

P applied: 152·5 mg.; adsorbent: 160 g.

Fractions obtained	Solvent	Eluent (ml.)	P in fraction (as % applied)	Ester value*
Crude 'lecithin' (GL)	Methanol–$CHCl_3$ (1:1, v/v)	500	82·0	—
		10	0·02	—
Crude 'kephalin' (GK)	Ethanol–$CHCl_3$–water (5:2:2, by vol.)	500	14·4	11·09
		400	2·1	11·01
		400	1·2	11·30
		Total recovery	99·7	

* Colour yield (Rapport & Alonzo, 1955*b*)/mg. of P.

Table 4. *Chromatography of fractions GL and GK on silicic acid*

Material applied to column	Solvent	Fraction code	P recovered (as % applied)
GL	Methanol–$CHCl_3$ (30:70, v/v)	GL1	86·1
	Methanol–$CHCl_3$–water (70:25:5, by vol.)	GL2	12·8
		Total	98·9
GK	Methanol–$CHCl_3$ (30:70, v/v)	GK1	86·7
	Methanol–$CHCl_3$–water (70:25:5, by vol.)	GK2	13·3
		Total	100·0

When the P concentration in the effluent had fallen nearly to zero the solvent was changed to methanol–chloroform–water (70:25:5, by vol.) to expedite the removal of the more strongly held substances. This solvent, containing a small amount of water, was found to be more effective for the rapid and complete removal of phospholipids from silicic acid than the methanol–chloroform (75:25, v/v) or methanol alone previously used (Rhodes & Lea, 1956*b*). The fractionation of the lipid P is shown in Table 4.

Analysis of the 'lecithin' fractions GL1 and GL2. Fraction GL1 was chromatographically homogeneous and on analysis showed: N/P, 1·01; amino N/P, 0·001; fatty acids/P, 2·01; as required for phosphatidylcholine. It was not attacked by phospholipase *B*.

On silica-impregnated paper, fraction GL2 showed three spots (R_F values 0·5, 0·35, 0·25) reacting with phosphomolybdic acid, corresponding to phosphatidylcholine, sphingomyelin and lysophosphatidylcholine respectively.

Sphingomyelin was estimated in this fraction by the method of Schmidt (1946). Since no water-soluble ninhydrin-reacting substances were present, the N/P ratio in excess of unity provided a further estimate of the quantity of diaminophospholipid. The presence of a ninhydrin-reacting, chloroform-soluble base after hydrolysis (McKibbin & Taylor, 1949), in approximately the correct quantity, confirmed the presence of sphingosine.

Lysolecithin was demonstrated unambiguously and estimated by reaction with phospholipase *B*.

Analysed in this way fraction GL2 gave: N/P, 1·24; esters/P, 0·79; esters hydrolysed by phospholipase *B*/total esters, 0·70; sphingomyelin (Schmidt, 1946)/P, 0·24. From the ester values the content of lysolecithin in the fraction was 55 % and of lecithin 12 % which, with the 24 % of sphingomyelin, accounts for only 91 % of the P. The discrepancy is most likely due to inaccuracies inherent in the ester methods, and the remaining 9 % (less than 1 % of the total P) has been included as lecithin; the lysolecithin content given may therefore be slightly low. The fraction consisted, therefore, of sphingomyelin 24 %, lysolecithin 55 % and (by difference) lecithin 21 %. Confirmation of the composition of the fraction as a mixture of sphingomyelin with two glycerophospholipids was obtained by submitting the whole to selective alkaline hydrolysis (Schmidt, 1946) and rechromatographing the recovered phospholipid; only the sphingomyelin spot remained.

Fraction GL1, re-run on a silicic acid column, was recovered in a single peak in 30 % (v/v) methanol–chloroform, indicating that the lyso compound found in GL2 had not been produced by hydrolysis on the column.

Analysis of the 'kephalin' fractions GK1 and GK2. Analysis of fraction GK1 gave: N/P, 1·00; amino N/P, 1·00; esters/P, 1·99; inositol/P, 0·020. It was not attacked by phospholipase *B*; the fraction was, therefore, nearly pure phosphatidylethanolamine.

Fraction GK2, as eluted from the silicic acid column, still contained amino acid contaminants. These were removed on a cellulose column (Lea & Rhodes, 1953) without loss of lipid P: the N/P ratio of the purified material was then 1·01. On silica-impregnated paper this material showed only one spot at R_F 0·58 reacting with ninhydrin or phosphomolybdic acid, well separated from a phosphatidylethanolamine marker at R_F 0·85. Reaction with phospholipase *B* confirmed the presence of lysophosphatidylethanolamine and estimated its quantity. Inositol was estimated among the hydrolysis products.

Analysed in this way fraction GK2 gave: N/P, 1·01; esters/P, 1·26; esters hydrolysed by phospholipase *B*/total esters, 0·69; inositol/P, 0·125.

From these data lysophosphatidylethanolamine accounted for 87 % of the lipid P of the fraction; the remaining 13 % was in equimolar ratio to the inositol content (12·5 %). This result was confirmed by analysis of the corresponding fraction of another phospholipid preparation (H), which gave lysophosphatidylethanolamine 83 %, inositol/P, 0·20. In the absence of other phospholipids in these fractions, the evidence suggests that the inositide of egg contains ethanolamine, inositol and phosphorus in equimolar proportions. It may be further calculated that the ratio of fatty acid esters to P in this compound was approximately 3:1 in both experiments, but the significance of this figure is doubtful in view of errors inherent in the ester determination.

Although in the analysis reported fraction GK2 contained only part of the inositide, with preparation H, when the solvent was changed earlier, the entire inositide was recovered in the second fraction (HK2).

Amino acid N. The 'kephalin' fractions GK1 and GK2 were treated on cellulose columns to remove water-soluble contaminants, and the bases obtained on acid hydrolysis were then treated with fluorodinitrobenzene (FDNB) (Axelrod *et al.* 1953). Of the derivatives obtained from the phosphatidylethanolamine fraction GK1 1·2 % were acidic, and paper chromatography showed the presence of serine; a second spot may have been threonine. No amino acids were found in fraction GK2. The amino acid content of the total phospholipid was, therefore, 0·18 %

Plasmalogen. The plasmalogen contents of the total phospholipid prepared from three batches of eggs were estimated. In view of the danger of obtaining high results owing to interference by

Table 5. *Composition of the total phospholipid of egg yolk derived from analysis of the chromatographically separated fractions*

Abbreviations used: PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; In, inositide. —, Absent chromatographically.

	P (as % of initial)						
Fraction	PC	PE	lyso PC	lyso PE	SM	In	Total
GL1	70·6	<0·1	0·0	0·0	—	0·0	70·6
GL2	2·2*	0·0	5·8	0·0	2·5	0·0	10·5
GK1	—	15·0	0·0	0·0	—	0·3	15·3
GK2	—	—	—	2·1	—	0·3	2·4
Total	72·8	15·0‡	5·8	2·1	2·5	0·6	98·8†

* Estimated by difference.
‡ Contains 0·2% of phosphatidylamino acids.
† Contains 0·9% of plasmalogen.

aldehydic degradation products from autoxidized fatty acids, and of the destruction of plasmalogens by hydrogenation fractions from the columns were not examined individually. Plasmalogen contents of 0·9, 1·2 and 1·6% were found.

The results of the analyses of the several fractions, calculated on the basis of the original lipid P, are presented in Table 5. The last line of the table gives the overall composition of the total phospholipid.

DISCUSSION

In so far as comparison is possible, the results obtained in the present work by the application of chromatographic methods to the analysis of egg-yolk phospholipids agree fairly well with the most recent of the published data based on the examination of hydrolysis products. The proportion of choline-containing phospholipids found was close to 80%, including 2·5% of sphingomyelin. The amino acid nitrogen was less than 0·2%. The plasmalogen content, as measured by the most recently developed method, was less than 1%, and was concentrated mainly in the lecithin fraction.

Chromatography on silicic acid has permitted further fractionation of both the 'lecithin' and the 'kephalin' fractions from alumina columns, with the recovery in each case of lyso compounds, the presence of which had previously been reported (Lea *et al.* 1955; Rhodes & Lea, 1956*b*). In the earlier work, lysophosphatidylcholine had been obtained by direct chromatography of the crude phospholipids on silicic acid, and the presence of lysophosphatidylethanolamine inferred from the weak ninhydrin reaction of the phosphatidylcholine fraction obtained from the same columns, as well as from the chromatographic behaviour of artificial phospholipid mixtures.

The methods now used have concentrated the small amount of lysophosphatidylethanolamine present into one fraction, of which it comprised over 80%, thus allowing its positive identification and estimation. The inositide of the yolk phospholipid could be concentrated, as the only other major constituent, in the same fraction.

The analysis reported in this paper was carried out on eggs obtained from one flock at one time. In view of the range of fatty acid/phosphorus ratios observed for a number of egg-phospholipid preparations in previous work (Lea *et al.* 1955), it seems likely that some variation, particularly in the proportion of lyso compounds present, may occur. The fatty acid/phosphorus ratio of the total phospholipid now analysed, as calculated from the data in Table 5, was 1·87, a figure within the range previously reported.

The improved solvent system employed with alumina permits the rapid and complete separation of the phospholipids into choline-containing and non-choline-containing fractions, and provides a more rapid and convenient procedure for the preparation of the crude 'lecithin' of egg yolk than that originally described by Hanahan *et al.* (1951). It must, however, be followed by a treatment on a silicic acid column to remove lysolecithin and sphingomyelin if pure phosphatidylcholine is required. The separation of the lyso compounds obtained from egg yolk by treatment with snake venom is also more conveniently carried out on the alumina column than by the silicic acid column previously described (Lea *et al.* 1955).

Application of the improved alumina-column technique to tissue lipids rich in phosphatidylserine and inositol lipids might well prove less successful than in the present case, owing to the low solubility of these phospholipids in the ethanol-rich eluent used.

Phosphatidylethanolamine is still obtained most easily by direct chromatography of the acetone-insoluble phospholipids of egg yolk on silicic acid (Lea *et al.* 1955; Lea, 1956).

SUMMARY

1. The extraction and purification of hen's egg phospholipids have been examined, and a mild procedure yielding 97 % of the total material has been devised.

2. The crude phospholipid was separated chromatographically on alumina into choline-containing and non-choline-containing fractions, with quantitative recovery of both.

3. By further separation on silicic acid the two major constituents were recovered in a practically pure condition, and the minor constituents concentrated sufficiently to permit identification and estimation.

4. The egg phospholipid examined contained phosphatidylcholine 73·0, lysophosphatidylcholine 5·8, sphingomyelin 2·5, phosphatidylethanolamine 15·0, lysophosphatidylethanolamine 2·1 and inositol phospholipid 0·6, all as moles %.

5. The plasmalogen content of the whole phospholipid was 0·9 %, most of which was present in the lecithin fraction. The phosphatidylethanolamine fraction contained a small proportion (0·2 % of the total phospholipid) of amino acid-containing phospholipid.

The authors are indebted to Dr R. M. C. Dawson for the gift of phospholipase *B*. This work has been carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

REFERENCES

Axelrod, J., Reichenthal, J. & Brodie, B. B. (1953). *J. biol. Chem.* **204**, 903.

Borgström, B. (1952). *Acta physiol. scand.* **25**, 101.

Campling, J. D. & Nixon, D. A. (1954). *J. Physiol.* **125**, 71.

Celmer, W. D. (1949). Ph.D. thesis: University of Illinois. Quoted by Carter, H. E. & Celmer, W. D. (1952). *Physiol. Rev.* **32**, 167.

Chargaff, E., Ziff, M. & Rittenberg, D. (1942). *J. biol. Chem.* **144**, 343.

Entenman, C., Taurog, A. & Chaikoff, I. L. (1944). *J. biol. Chem.* **155**, 13.

Fairbairn, D. (1948). *J. biol. Chem.* **173**, 705.

Feulgen, R. & Bersin, Th. (1939). *Hoppe-Seyl. Z.* **260**, 217.

Hack, M. H. (1953). *Biochem. J.* **54**, 602.

Hanahan, D. J., Turner, M. B. & Jayko, M. E. (1951). *J. biol. Chem.* **192**, 623.

Kaucher, M., Galbraith, H., Button, V. & Williams, H. H. (1943). *Arch. Biochem.* **3**, 203.

Kline, L., Gegg, J. E. & Sonoda, T. T. (1951). *Food Tech., Champaign*, **5**, 181.

Kline, L. & Johnson, C. M. (1946). *Industr. Engng Chem. (Anal.)*, **18**, 35.

Lea, C. H. (1956). *Proc. 2nd Int. Conf., Biochemical Problems of Lipids, Ghent, 1955*, p. 81.

Lea, C. H. & Rhodes, D. N. (1953). *Biochem. J.* **54**, 467.

Lea, C. H. & Rhodes, D. N. (1954). *Biochem. J.* **56**, 613.

Lea, C. H. & Rhodes, D. N. (1955). *Biochim. biophys. Acta*, **17**, 416.

Lea, C. H., Rhodes, D. N. & Stoll, R. D. (1955). *Biochem. J.* **60**, 353.

McKibbin, J. M. & Taylor, W. E. (1949). *J. biol. Chem.* **178**, 29.

Nottbohm, F. E. & Mayer, F. (1933). *Z. Untersuch. Lebensmitt.* **66**, 585.

Pangborn, M. C. (1951). *J. biol. Chem.* **188**, 471.

Rapport, M. M. & Alonzo, N. (1955*a*). *J. biol. Chem.* **217**, 199.

Rapport, M. M. & Alonzo, N. (1955*b*). *J. biol. Chem.* **217**, 193.

Rhodes, D. N. & Lea, C. H. (1956*a*). *Nature, Lond.*, **177**, 1129.

Rhodes, D. N. & Lea, C. H. (1956*b*). *Proc. 2nd Int. Conf., Biochemical Problems of Lipids, Ghent, 1955*, p. 73.

Schmidt, G. (1946). *J. biol. Chem.* **166**, 505.

Shapiro, B. (1953). *Biochem. J.* **53**, 663.

Sulser, H. (1954). *Mitt. Lebensm. Hyg., Bern*, **45**, 251.

Thiele, O. W. (1955). *Hoppe-Seyl. Z.* **299**, 151.

Tsuji, F. I., Brin, M. & Williams, H. H. (1955). *Arch. Biochem. Biophys.* **56**, 290.

van Handel, E. (1954). Thesis: D.B. Centen's Uitgeversmaatschappij N.V. Amsterdam.

Wittcoff, H. (1951). *The Phosphatides*. New York: Reinhold Publ. Co.

Wittenberg, J. B., Korey, S. R. & Swenson, F. H. (1956). *J. biol. Chem.* **219**, 39.

Yanamoto, S. (1954*a*). *Sapporo Med. J.* **5**, 276.

Yanamoto, S. (1954*b*). *Sapporo Med. J.* **6**, 34.