The Fatty Acids of Phosphatidylethanolamine and Phosphatidylcholine from Hen's Egg

By J. C. HAWKE

*Fats Research Laboratory, Department of Scientific and Industrial Research,
Wellington, New Zealand*

(Received 7 July 1958)

Glycerides and phospholipids from the same tissue exhibit appreciable differences in their fatty acid composition (Hilditch, 1956; Futter & Shorland, 1957), but very little is known about the distribution of fatty acids among the individual phospholipids. Progress in working out the pathways of phospholipid synthesis in living cells (Kennedy & Weiss, 1956; Smith, Weiss & Kennedy, 1957) has, however, stimulated interest in individual phospholipid compounds as chemical entities, rather than as mixtures. Consequently, it has become of interest to know if any selectivity with regard to fatty acids has occurred in the biological synthesis of phospholipids such as phosphatidylcholine and phosphatidylethanolamine. Because the individual lipids have not been separated in anything like quantitative yield this question has remained unanswered, although, working qualitatively, Klenk & Böhm (1951) found marked differences in the fatty acid composition of phosphatidylserine and phosphatidylethanolamine from ox brain. Recently, however, Rhodes & Lea (1957) have isolated almost quantitatively the component phospholipids in egg yolk on silicic acid and alumina columns. These authors isolated, in essentially pure form, all the phosphatidylethanolamine and 97.0% of the phosphatidylcholine. Two other fractions contained mixtures of the minor constituents—lysophosphatidylcholine and sphingomyelin in one fraction and lysophosphatidylethanolamine and inositol phospholipid in the other.

More progress has been made in the elucidation of the composition of the phospholipid fraction in egg yolk than of the phospholipids in any other biological material. Contributing factors have been the ready availability of this phospholipid and the less divergent nature of its principal components. Rhodes & Lea (1957) found egg phospholipid to contain phosphatidylcholine 73.0, lysophosphatidylcholine 5.8, sphingomyelin 2.5, phosphatidylethanolamine 15.0, lysophosphatidylethanolamine 2.1, and inositol phospholipid 0.6, expressed as moles %. In the present work the egg phospholipids have been separated into two fractions, one containing phosphatidylethanolamine and the other containing the remaining

components, among which phosphatidylcholine predominates. The fatty acids in each fraction have been examined.

EXPERIMENTAL

Analytical methods. Total N, P and choline were determined as previously described (Hawke, 1956). Amino N was estimated by reaction with ninhydrin (Lea & Rhodes, 1955) after calibration by the Van Slyke manometric procedure using hydrolysed egg phospholipid.

The di- and poly-unsaturated methyl esters were determined spectrophotometrically before and after alkali isomerization (Herb & Riemenschneider, 1953). Hexanoic acid was calculated from data given by Hammond & Lundberg (1953).

N, P, amino N, and choline are expressed as wt. %. Unless otherwise stated, other results are expressed on a molar basis.

Chromatographic methods. Chromatography on silicic acid columns was carried out as described by Rhodes & Lea (1956a). Chromatography of the methyl esters of the fatty acids of phosphatidylethanolamine and phosphatidylcholine was carried out as previously described (Hawke, Dunkley & Hooker, 1957) with thermal conductivity detectors. Samples were injected into the gas stream with an Agla micrometer syringe through a self-sealing serum cap. Owing to the difficulty of adding precise amounts of the mixed esters to the chromatogram by this method, a known weight (approx. 5%, w/w) of methyl pentadecanoate was added to each ester sample to provide an internal reference after preliminary investigation had shown that this ester was not a natural component of the samples. Peak areas on the recorder graph were used to give a quantitative measure of the components present. The components that disappeared after bromination of the mixed esters were designated as unsaturated esters. Bromination was carried out as described by James & Martin (1956).

The chromatographic columns (1.2 m. long and 4 mm. internal diameter) were: (a) packed with Celite 545 (10 g.) and Apiezon M (2 g.) (James & Martin, 1956) and held at 200° to separate esters up to C₁₈, or (b) packed with Celite 545 (10 g.) and silicone grease (3 g.) and held at 225° to separate esters above C₁₈.

Preparation of egg phospholipids

Extraction from egg yolk. Yolks from 36 hen eggs were freeze-dried *in vacuo*. The dried yolk was powdered in a Waring Blendor and extracted by shaking for 30 min. periods with three changes of ethanol-ether (1:3, v/v) at

room temperature. The residue was then refluxed for 2 hr. with ethanol-ether, shaken for 30 min. and then filtered. The residue was again disintegrated in the Blendor, refluxed and shaken with ethanol-ether. Approximately 600 ml. of purified solvent was used in each extraction.

Purification by acetone. The total extract was evaporated and 2 l. of acetone added to the fat (206 g.) and refluxed for about 1 min. The flask was then stoppered and allowed to stand at room temperature overnight. Soluble and insoluble lipids were separated and the operation was repeated three times, with the same solvent-lipid ratio. The insoluble lipid weighed 50 g. and the soluble lipid 154 g. After dissolving the insoluble lipid in chloroform saturated with water and chromatographing on a cellulose column (Lea & Rhodes, 1953) to remove water-soluble impurities, the N/P ratio was 1.02 (N, 1.59%; P, 3.45%). The acetone-soluble lipid (N/P, 2.1; N, 0.20%; P, 0.21%) was not purified on a cellulose column.

Removal of phospholipid from the lipid soluble in acetone. The acetone-soluble 'glyceride' fraction was dissolved in methanol-chloroform (1:99, v/v) and chromatographed on a silica-gel column (Mallinckrodt, previously heated at 120°) with the same solvent as eluent. This solvent was later changed to methanol-chloroform (20:80, v/v) and then to methanol. The successive solvents eluted 129.0, 11.7 and 13.0 g. of lipid containing 0.015, 0.24 and 1.99% of P respectively.

The lipid fraction weighing 13.0 g. was added to the acetone-insoluble lipid. The 11.7 g. fraction was further purified by means of dialysis in a rubber membrane (London surgeons' fingercoats) against light petroleum (b.p. 55°) with six solvent changes at hourly intervals, 1 l./change being used (van Beers, de Iongh & Boldingh, 1957). The non-dialysable material from this dialysis was added to the acetone-insoluble lipid.

A sample of the total phospholipid (5.93 g.) was similarly dialysed and the residue weighed 5.55 g. (Table 1).

Preparation of phosphatidylethanolamine and phosphatidylcholine

The non-dialysable material (5.55 g.) was then chromatographed on a silicic acid column with 200 g. of adsorbent, i.e. a loading of approx. 30 mg. of P/25 g. of silicic acid.

After adding the sample to the column in about 50 ml. of chloroform, methanol-chloroform (20:80, v/v) was used as eluting solvent. After 1 l. of solvent had been used the concentrations of phosphorus and free amino nitrogen in the eluent fell almost to zero. A further 600 ml. of the same solvent was used without appreciable lipid being eluted. Methanol was then used to strip the remaining lipid from the column.

Table 2 summarizes the separation obtained. The early fractions in which most of the pigmented material appeared contained little phospholipid. It was expected that the lipid eluted as a peak in fractions 16-37 would be substantially pure phosphatidylethanolamine, but only rather more than half (amino N/N, 0.56) proved to be this component. The poor performance of the column might have been due to its size; no difficulty has been experienced with columns using approximately 50 g. of adsorbent and the same P/silicic acid ratio.

Fractions 16-37 and 38-59 were combined and rechromatographed on 50 g. of silicic acid and eluted with methanol-chloroform (20:80, v/v). The 0.94 g. of lipid which was eluted as a peak had a ratio (amino N/N) close to unity. Following this peak, P values of the fractions fell almost to zero, at which stage the column was eluted with methanol as before. The fractions before the phosphatidylethanolamine peak contained a total of 0.04 g. of lipid with a low amino N/P ratio (0.21), but the small amount obtained precluded any detailed investigation. The combined ninhydrin-reacting lipid on analysis gave amino N/P, 0.96; amino N/N, 0.97; N/P, 1.01.

Analysis of the combined fractions eluted with methanol from the two silicic acid columns gave: N/P, 1.01; amino N/N, 0.035; choline N/N, 0.95; fatty acids, 74.2% by wt. (calc. for distearoylphosphatidylcholine 72.0%).

Preparation of the methyl esters of the fatty acids of phosphatidylethanolamine and phosphatidylcholine

The two main fractions were separately hydrolysed by refluxing first with 1% (w/v) sulphuric acid in methanol for 3 hr., and then with potassium hydroxide in the same solvent (20% in excess of theoretical) for a further 2 hr. Both steps were carried out in an atmosphere of nitrogen.

Table 1. *Separation of glyceride and phospholipid by dialysis through a rubber membrane*

	Lipid used	Material non-dialysed	Material dialysed	Recovery (% by wt.)
Wt. (g.)	5.93	5.55	0.38	100.0
P (%)	3.20	3.42	0.0	100.0
N (%)	1.61	1.68	0.15	98.2

Table 2. *Separation of total egg phospholipid on silicic acid with chloroform-methanol as eluent*

Fraction no. (20 ml.)	Wt. of lipid (g.)	N (%)	P (%)	N/P (molar ratio)	Amino N (%)	Amino N/N (molar ratio)
1-15	0.558	0.37	0.50	1.64	0.05	0.13
16-37	1.799	1.59	3.75	0.94	0.90	0.56
38-59	0.090	1.20	2.40	1.12	0.55	0.46
60-78	0.036	N.D.	1.53	N.D.	0.33	N.D.
Methanol eluate	3.070	—	—	—	—	—

N.D. = not determined.

The potassium soaps of the fatty acids were acidified and extracted with ether, and the acids converted into methyl esters.

RESULTS AND DISCUSSION

Taking into account the losses from the silicic acid columns and the amounts of phospholipids in the small fractions which were discarded, it is estimated that about 97 % of the total egg-yolk phosphatidylethanolamine (PE) and about 97.5 % of the total egg-yolk phosphatidylcholine (PC) have been included in the samples used in the final analysis of the fatty acids.

The PE sample was essentially pure and was a light-brown solid. The analytical figures showed that the PC sample had two contaminants, lysophosphatidylethanolamine (3.5 %) and sphingomyelin (1.5 %), and by analogy with the work of Rhodes & Lea (1957) it would also contain the lyso analogue as a third impurity. These authors found 5.8 % of lysophosphatidylcholine in samples of PC prepared from egg yolk by the same technique. The total contaminants of the present sample of PC would therefore be of the order of 11.0 %. The PC was a white waxy solid.

Analysis of the methyl esters of the fatty acids derived from PE and PC has revealed some marked differences in composition (Tables 3 and 4).

PC has a greater proportion of C_{18} acids (32.5 % as against 20.1 % for PE) but less than half the amount of C_{20} and C_{22} unsaturated acids. Further marked differences are found in the distribution of

the C_{18} acids. Whereas the proportions of the total C_{18} acids of PE and PC are similar (59.6 and 58.9 % respectively), the proportions of saturated and unsaturated acids are in sharp contrast. PE has almost three times the amount of stearic acid, but less than half as much of the C_{18} unsaturated acids compared with PC. From the iodine values and the alkali-isomerization figures of the methyl esters of the fatty acids from PE and PC (Table 4) it can be deduced that there is considerably less monoene in PE and consequently the smaller amount of C_{18} unsaturated acid in this phospholipid may be attributed to a smaller oleic acid content (see Figs. 1 and 2). In both PE and PC little trienoic acid is present. The larger amount of pentaene and hexaene in PE is in agreement with the higher proportions of C_{20} and C_{22} acids given by gas-liquid chromatography.

Recently, Kennedy and co-workers (Kennedy & Weiss, 1956; Smith *et al.* 1957) demonstrated the biosynthesis of PC and PE *in vitro* by using enzymes obtained from liver tissue and yeast. The synthesis involved 1:2-diglyceride as a common precursor in reactions with cytidine diphosphate choline and cytidine diphosphate ethanolamine. Although from the work of Kennedy & Weiss (1956) it appears probable that separate enzymes are responsible for the formation of PE and PC, in its simplest form this metabolic pathway would give rise to individual phospholipids of similar fatty acid composition.

The present results show that the individual phospholipids have a widely differing fatty acid composition, and further evidence of fatty acid selectivity among the different phospholipids is given by Rhodes (1958). When investigating the effect of cod-liver oil fed to laying hens on the composition of the fatty acids of egg phospholipids he found that considerable differences in the mean unsaturation of the fatty acids esterified in the α position existed in PE and PC. Moreover, Klenk & Böhm (1951) found appreciable differences in the fatty acid composition of PE and phosphatidylserine (PS) obtained from ox brain, PE having more unsaturated C_{20} and C_{22} acids but less saturated and unsaturated C_{18} acids than PS. However,

Table 3. *Composition of the methyl esters of the fatty acids from phosphatidylethanolamine (PE) and phosphatidylcholine (PC)*

Figures are expressed as moles %.

Fatty acid	Saturated		Unsaturated	
	PE	PC	PE	PC
C_{14}	0.4	0.2	0.0	0.0
C_{16}	19.8	28.1	1.2	4.4
C_{18}	39.5	14.2	20.1	44.7
C_{20}	0.0	0.0	9.0	4.7
C_{22}	0.0	0.0	10.0	3.7

Table 4. *Composition of the unconjugated and conjugated unsaturated methyl esters of the fatty acids from phosphatidylethanolamine and phosphatidylcholine*

Figures are expressed as moles %. Iodine values are: phosphatidylethanolamine 102.2, phosphatidylcholine 77.0.

Acid	Phosphatidylethanolamine		Phosphatidylcholine	
	Unconjugated	Conjugated	Unconjugated	Conjugated
Diene	3.0	2.9	5.9	3.1
Triene	0.0	0.8	0.1	0.3
Tetraene	7.4	0.4	2.4	0.1
Pentaene	3.9	0.2	2.8	0.03
Hexaene	8.0	<0.1	2.9	<0.02

it is not possible to make accurate comparisons between the two phospholipids because the recovery of PE was not given.

It seems likely, therefore, that if the mechanism for synthesis of phospholipids proposed by Smith *et al.* (1957) occurs *in vivo*, the enzymes responsible for the reaction between different basic cytidine diphosphate derivatives and 1:2-diglycerides exhibit differing specificities for various 1:2-diglycerides. On the other hand, there could be a redistribution of fatty acids after phospholipid synthesis. Bergström, Bergström & Rottenberg (1952) found after feeding (*carboxy*- ^{14}C)stearic acid that redistribution of fatty acids occurred between the lymph glycerides and the liver phospholipids, and since 1:2-diglycerides have been shown to act as precursors of triglycerides (Weiss & Kennedy, 1956) as well as of phospholipids, it is possible that a redistribution of fatty acids between different phospholipids might occur also.

Perhaps of greater interest than the total composition of the fatty acids in PC and PE is the distribution of the fatty acids between the α and β positions of the phospholipid molecule. Some idea of this distribution may be obtained from a consideration of the present results together with the findings of Rhodes & Lea (1956*b*), since, as mentioned above, Rhodes (1958) has found that variations in the diet of hens affects the nature of the unsaturated fatty acids in the α position only. These workers treated egg phospholipids with the

phospholipase A of snake venom in order to remove the acids from the α position in the molecule, and found that the residual acids in the β position possessed an unsaturation of approximately 0.15 double bond/molecule. The acids removed by phospholipase A were all unsaturated. After hydrogenation of the lysolecithin produced, the acids in the β position consisted of 67% of palmitic acid and 33% of stearic acid.

In the present work, if all the acids in the α position are unsaturated (cf. Hanahan, 1954), in PC 85% of the total acids in the β position are myristic, palmitic and stearic acids (0.4, 56.2 and 28.4% respectively). The remaining 15% must be monoenes in order to agree with the figure of 0.15 double bond/molecule obtained by Rhodes & Lea (1956*b*) and must be either C_{16} or C_{18} acids to agree with their analysis after hydrogenation. If all the available palmitoleic (Table 3) is in the β position, the 15% unsaturated monoenes are made up of palmitoleic acid (8.8%) and oleic acid (6.2%). In terms of carbon number the composition of the acids in the β position would be C_{14} 0.4, C_{16} 65.0 and C_{18} 34.6%, and by difference the fatty acids esterified in the α position of the phospholipid molecule would be as given in Fig. 1.

Egg-lecithin preparations obtained by chromatography on alumina columns in which stearic acid was the only detectable saturated acid (Hanahan, 1954) would not appear to be representative of the total lecithin present.

α	Unsat. acids	$\left\{ \begin{array}{l} 83.2\% \text{ C}_{18} \\ 9.4\% \text{ C}_{20} \\ 7.4\% \text{ C}_{22} \end{array} \right\}$	$\left\{ \begin{array}{l} 64.7\% \text{ Monoene} \\ 18.0\% \text{ Diene} \\ 0.8\% \text{ Triene} \\ 5.0\% \text{ Tetraene} \\ 5.7\% \text{ Pentaene} \\ 5.8\% \text{ Hexaene} \end{array} \right\}$
β	$\left\{ \begin{array}{l} 0.4\% \text{ Myristic acid} \\ 56.2\% \text{ Palmitic acid} \\ 28.4\% \text{ Stearic acid} \\ 8.8\% \text{ Palmitoleic acid} \\ 6.2\% \text{ Oleic acid} \end{array} \right\}$		
α'	Phosphorylcholine		

Fig. 1. Fatty acids esterified in the α and β positions of phosphatidylcholine.

α	{	{	34.4 % Stearic acid	}	{	12.6 % Monoene	}
			27.6 % C_{18} unsaturated acids			11.8 % Diene	
			18.0 % C_{20} unsaturated acids			1.6 % Triene	
			20.0 % C_{22} unsaturated acids			15.4 % Tetraene	
						8.2 % Pentaene	
						16.0 % Hexaene	
β	{	{	0.8 % Myristic acid	}			
			39.6 % Palmitic acid				
			44.6 % Stearic acid				
			2.4 % Palmitoleic acid				
			12.6 % Oleic acid				
α'			Phosphorylethanolamine				

Fig. 2. Fatty acids esterified in the α and β positions of phosphatidylethanolamine.

In PE two-fifths of the total acids are unsaturated, therefore, appreciable amounts of saturated acid must be esterified in the α position. Rhodes & Lea (1956*b*) found that the unsaturation in the β position was of the order of 0.15 double bond/molecule and that on hydrogenation the composition of these acids was 47% of stearic acid and 53% of palmitic acid. However, if all the palmitic, palmitoleic and myristic acids found in the present investigation were in the β position, these acids would form only 42.8% of the total. To obtain the required degree of unsaturation the remaining acids could be 12.6% of oleic acid and 44.6% of stearic acid. Such a distribution would mean that approximately one-third (34.4%) of the acids in the α position is stearic acid, Fig. 2.

As would be expected from the large proportion of PC in the total egg phospholipid, the fatty acid composition of the latter (Shorland, 1951) follows closely that of PC.

SUMMARY

1. Gas-liquid chromatographic analysis has revealed marked differences in the fatty acid composition of egg-yolk phosphatidylethanolamine and phosphatidylcholine. Of the total acids in phosphatidylethanolamine 39.5% is saturated C_{18} and 20.1% is unsaturated C_{18} , whereas in phosphatidylcholine 14.2% is saturated C_{18} and 44.7% is unsaturated C_{18} . The larger proportion of unsaturated C_{18} acid in phosphatidylcholine is attributed to oleic acid.

2. About three-fifths of the total acids of phosphatidylethanolamine are saturated, and it is estimated that one-third of the fatty acids esterified in the α position are saturated.

3. Considerably more C_{20} and C_{22} unsaturated

acids are present in phosphatidylethanolamine than in phosphatidylcholine.

I wish to thank Mr K. R. Millar for his contribution to part of the experimental work, and Mr C. N. Hooker for determining the unsaturated acids by the alkali-isomerization techniques.

REFERENCES

- Bergström, S., Bergström, B. & Rottenberg, M. (1952). *Acta physiol. scand.* **25**, 120.
 Futter, J. H. & Shorland, F. B. (1957). *Biochem. J.* **65**, 689.
 Hammond, E. G. & Lundberg, W. O. (1953). *J. Amer. Oil Chem. Soc.* **30**, 433.
 Hanahan, D. J. (1954). *J. biol. Chem.* **211**, 321.
 Hawke, J. C. (1956). *Biochem. J.* **64**, 311.
 Hawke, J. C., Dunkley, W. L. & Hooker, C. N. (1957). *N.Z. J. Sci. Tech. B*, **38**, 925.
 Herb, S. F. & Riemenschneider, R. W. (1953). *Analyt. Chem.* **25**, 953.
 Hilditch, T. P. (1956). *The Chemical Constitution of Natural Fats*, 3rd ed., rev. London: Chapman and Hall.
 James, A. T. & Martin, A. J. P. (1956). *Biochem. J.* **63**, 144.
 Kennedy, E. P. & Weiss, S. B. (1956). *J. biol. Chem.* **222**, 193.
 Klenk, E. & Böhm, P. (1951). *Hoppe-Seyl. Z.* **238**, 98.
 Lea, C. H. & Rhodes, D. N. (1953). *Biochem. J.* **54**, 467.
 Lea, C. H. & Rhodes, D. N. (1955). *Biochem. biophys. Acta*, **17**, 416.
 Rhodes, D. N. (1958). *Biochem. J.* **68**, 380.
 Rhodes, D. N. & Lea, C. H. (1956*a*). *Proc. 2nd Int. Conf. Biochem. Probl. Lipids, Ghent* (1955), p. 73.
 Rhodes, D. N. & Lea, C. H. (1956*b*). *Nature, Lond.*, **177**, 1129.
 Rhodes, D. N. & Lea, C. H. (1957). *Biochem. J.* **65**, 526.
 Shorland, F. B. (1951). *N.Z. J. Sci. Tech. B*, **33**, 224.
 Smith, S. W., Weiss, S. B. & Kennedy, E. P. (1957). *J. biol. Chem.* **228**, 915.
 van Beers, G. J., de Iongh, H. & Boldingh, J. (1957). *4th Int. Conf. Biochem. Probl. Lipids, Oxford* (1957).
 Weiss, S. B. & Kennedy, E. P. (1956). *J. Amer. chem. Soc.* **78**, 3550.