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The distribution of fatty acids between the α'- and β- positions of the glycerophospholipids of buttermilk*

J. C. Hawke

Biochemistry Department,
Massey University College of Manawatu,
Palmerston North, New Zealand

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SUMMARY

Fractionation of buttermilk phospholipids on silicic acid columns gave a cephalin (mixture of amino-N phospholipids) fraction, in which approximately 29% of the total fatty acids were saturated, and phosphatidyl choline, in which approximately 57% of the total fatty acids were saturated. The main difference between the fatty acids of the two phospholipid fractions was in the proportions of palmitic and oleic acid; a further difference was the predominance of the polyenoic acids in the cephalin fraction. In phosphatidyl choline, the ratio of unsaturated to saturated fatty acids in the α'-position was 0.54 and in the β-position, 1.05. Although the nature of the fatty acids present in minor quantities in lecithin and cephalin was very different, the positional distributions of the major fatty acids common to both phospholipid fractions were similar. Oleic and palmitic acids together contributed almost identical proportions to the α' and to the β fatty acids in both the cephalin fraction and phosphatidyl choline. These two acids comprised 61% of the total acids in the cephalin fraction and 66% of the total acids in phosphatidyl choline. In both the amino-N and choline glycerophospholipids, most of the stearic acid was in the α'-position; whereas most of the dienoic, trienoic, and polyenoic acids were in the β-position. It has been found that the unsaturated acids are less randomly distributed than previously reported for milk glycerophospholipids, but are more randomly distributed than in many other mammalian tissues.

The only available information on the fatty acids of the individual phospholipids of milk is contained in a paper by Rhodes and Lea (1). The average unsaturation of the cephalin (amino-N phospholipids) and phosphatidyl choline components of butter milk phospholipids, and the lysocompounds obtained from these components by phospholipase A, showed that the double bonds were fairly uniformly distributed between the α'- and β-positions in the glycerophospholipid molecules.

The object of the present work was to provide more precise information on the distribution of the fatty acids between cephalin and phosphatidyl choline of milk and, in particular, to investigate the distribution of fatty acids between the α' and β-position of each component.

An investigation of this nature is made possible by the development of quantitative chromatographic methods for the separation of the most important phospholipid components (2). Several workers have applied chromatographic methods to elucidate the composition of the milk phospholipids. Baliga and Basu (3), using magnesia columns, and Rhodes and Lea (1) and Smith and Freeman (4), using silicic acid columns, have added considerably to the knowledge obtained by the application of the less direct hydrolysis methods (5).

The percentage composition of the major constituents given by Rhodes and Lea (1) for buttermilk phospholipids (phosphatidyl ethanolamine [PE], 29; phosphatidyl serine [PS], 10; phosphatidyl choline, 33; sphingomyelin, 19) and by Smith and Freeman (4) for milk phospholipids (cephalin [PE and PS], 35; phosphatidyl choline, 32; sphingomyelin, 24) agree quite closely.

* This investigation was commenced by the author at the Fats Research Laboratory, DSIR, Wellington, New Zealand.

TABLE 1. SEPARATION OF TOTAL MILK PHOSPHOLIPIDS ON SILICIC ACID WITH CHLOROFORM–METHANOL AS ELUENT*

Fraction No.	Fractions from Silicic Acid Column	Wt	N/P	Amino-N/N	Choline-N/N	Carboxyl Ester/P†
	Each approx. 40 ml					
1	1–4	0.044	—	—	—	—
2	5–13	0.856	0.93	0.99	0.02	2.0
3	14–40	0.193	0.97	0.61	0.04	2.0
4	41–92	0.780	0.97	0.06	1.07	2.0
5	93–140	0.491	1.70	0.05	—	—

* Fractions 2–5 were further purified by passing through cellulose columns in water-saturated chloroform.
† Approximate values.

Cream contains about 55% of the phospholipids of milk and, on churning, about 60% of the cream phospholipids pass into the buttermilk (5). Therefore, buttermilk provides a convenient source of milk phospholipid. In addition, since the proportions of each phospholipid component in skim milk, buttermilk, and butter (1, 5, 6) are very similar, it may be concluded that results for buttermilk would resemble very closely those for the original milk.

EXPERIMENTAL METHODS

Analytical Methods. Total N, P, choline, and amino-N were determined as previously described (7, 8). Hydrolyzed buttermilk phospholipids, however, were used to calibrate the ninhydrin method for the determination of amino-N (9) against the Van Slyke manometric method.

Carboxylic esters were determined by the method of Rapport and Alonzo (10) using methyl stearate as standard. Sphingosine-N was determined by the method of McKibbin and Taylor (11).

The procedures for separation and identification of the methyl ester derivatives of fatty acids have been described by Hawke, Hansen, and Shorland (12). Separation of the esters above C_{18} were carried out on columns of 5% Apiezon L on Celite 545 at 225°.

Nitrogen, phosphorus, amino-N, and choline are expressed as weight per cent. All other results are expressed on a molar basis, unless otherwise stated. The composition of the solvent mixtures is expressed as a ratio of the volumes of each solvent.

Extraction and Purification of Buttermilk Phospholipids. A sample of fresh roller-dried buttermilk powder[1] (1,000 g) was divided into two approximately equal portions. Each portion was shaken three times with diethyl ether–ethanol 4:1 at room temperature for 60 min using 1,000 ml in each extraction. After three further extractions with chloroform, all the extracts from both portions were combined and the solvents removed in a rotary vacuum evaporator at 40°. The extracted solid weighed 83.7 g (N, 0.71%; P, 0.56%; N/P ratio, 2.80); 80.2 g of this extract was separated into phospholipids and glycerides by acetone precipitation yielding 11.44 g impure phospholipid (N, 2.59%; P, 3.09%; N/P ratio, 1.86) and 68.0 g impure glyceride (N, 0.40%; P, 0.11%). The impure phospholipid (5.34 g) was dialyzed in a rubber membrane (Londons surgeons' fingercots) against light petroleum (bp 55°) with five solvent changes (each of 1 liter) at hourly intervals (13). The nondialyzable lipid weighed 4.87 g (N, 2.7%; P, 3.15%; N/P ratio, 1.9) and the dialyzable lipid, 0.38 g (P, 0.03%). The nondialyzable lipid (4.60 g) was further purified by dissolving in water-saturated chloroform and chromatographing on a cellulose column (14) to remove water-soluble impurities. The analysis of the purified phospholipid (4.30 g) was: N, 1.97%; P, 3.23%; N/P ratio, 1.35; choline, 7.51%; fatty acids 70.8%.

Preparation of Cephalin (PE + PS) and Phosphatidyl Choline. The mixed phospholipids were chromatographed on a silicic acid column (1), eluting first with chloroform–methanol 99:1 to remove any remaining nonphospholipid, and then with chloroform–methanol 7:3 to elute the cephalin fraction and a slower-moving lecithin fraction (Fig. 1). The more strongly adsorbed sphingolipid was then eluted with methanol–chloroform–water 70:25:2. The eluted lipids were combined into a very small nonphospholipid fraction, a cephalin fraction, a smaller cephalin fraction, a phosphatidyl choline fraction, and a sphingolipid fraction (see Table 1). The high N/P ratios initially obtained indicated that the removal of water-soluble nitrogenous impurities might not have been entirely effective. Retreatment of fractions 2 to 5 on cellulose columns lowered the

[1] Supplied by Dr. F. H. McDowall, Dairy Research Institute (N. Z.).

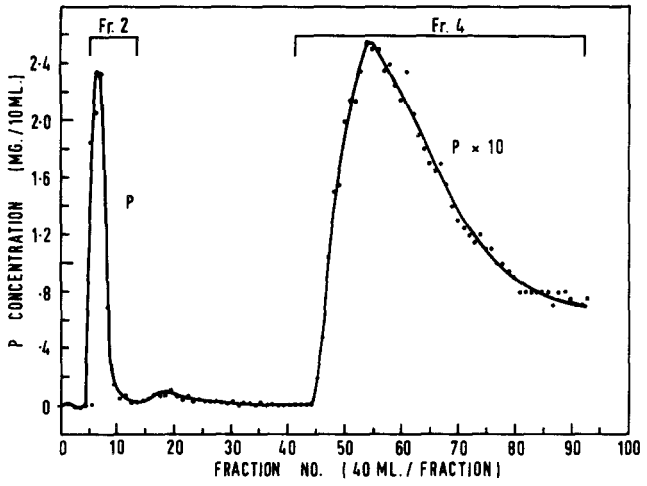

FIG. 1. Elution curve obtained by the elution of buttermilk phospholipids from a silicic acid column with methanol-chloroform 3:7. First peak, cephalin; second peak, phosphatidyl choline. Lipid applied 2.66 g (0.086 g P); weight adsorbent 130 g.

N/P ratios (Table 1) to give ratios close to unity. The sphingosine-N/N ratio of fraction 5 was close to 0.5, the calculated figure for pure sphingomyelin. Fractions 2 (cephalin [PE + PS] and 4 (phosphatidyl choline) were further investigated.

Hydrolysis of Cephalin and Phosphatidyl Choline. A reassessment of the specificity of phospholipase A present in snake venom by Tattrie (15), and later by Hanahan, Brockerhoff, and Barron (16), has shown that this enzyme hydrolyzes the ester links in the β-position of diacyl phosphoglycerides and that the lysophospholipids are α'-monoacyl compounds. In the present investigation, the phospholipid fractions were reacted with snake venom (*Agkistrodon piscivorous piscivorous*) in diethyl ether solution (17). A silicic acid column was used to isolate lysophospholipid obtained from cephalin (1). Chromatography of the lysocompounds on paper impregnated with silicic acid showed no spots corresponding to diacyl compounds. The α' fatty acids were prepared by alkaline hydrolysis of the lysophospholipids and extraction of the acidified hydrolysis mixture by diethyl ether. The α' and β fatty acids from each phospholipid fraction were converted into methyl esters by refluxing with methanols containing 1% H_2SO_4 (18).

RESULTS

Fractionation on silicic acid of the buttermilk phospholipids (Fig. 1) into its cephalin and phosphatidyl choline components has provided the material for the study of the fatty acid distribution in these glycerophospholipids. The results for buttermilk phospholipids (45% cephalin fraction, 34% phosphatidyl choline, and 21% sphingomyelin) agreed quite closely with the figures of Rhodes and Lea (1). The composition of the cephalin fraction, found by Rhodes and Lea (1) to consist of about two-thirds phosphatidyl ethanolamine and one-third phosphatidyl serine, was not investigated in the present study.

TABLE 2. DISTRIBUTION OF THE FATTY ACIDS OF CEPHALIN AND OF PHOSPHATIDYL CHOLINE BETWEEN THE α'- AND β-POSITIONS*

	Cephalin			Phosphatidyl Choline		
Fatty Acid	α'	β	Total	α'	β	Total
Saturated						
$C_{8:0}$	0.2	Tr	0.1			
$C_{10:0}$	Tr	Tr	—			
$C_{11:0}$	0.1	Tr	0.05	—	0.2	0.1
$C_{12:0}$	0.2	0.6	0.4	0.1	0.4	0.25
$C_{14:0}$	1.0	1.7	1.35	3.8	7.4	5.6
iso- or *anteiso-*						
$C_{15:0}$	0.4	0.2	0.3	0.4	0.9	0.65
$C_{15:0}$	0.8	0.3	0.55	1.2	2.1	1.65
iso-						
$C_{16:0}$	—	—	—	—	0.7	0.35
$C_{16:0}$	11.2	8.5	9.85	38.7	32.1	35.4
iso- or *anteiso-*						
$C_{17:0}$	1.0	1.3	1.15	0.8	1.3	1.05
$C_{17:0}$	0.7	0.9	0.8	1.0	0.9	0.95
$C_{18:0}$	22.2	3.4	12.8	17.9	2.1	10.0
$C_{19:0}$	2.1	—	1.05	0.9	0.8	0.85
$C_{20:0}$	0.4	0.1	0.25	0.2	tr	0.1
Unsaturated						
$C_{16:1}$	0.8	1.7	1.25	1.4	3.2	2.3
$C_{17:1}$	—	—	—	1.2	1.4	1.3
$C_{18:1}$	49.3	52.6	50.95	28.4	33.9	31.15
$C_{18:2}$	4.7	13.3	9.0	2.9	7.1	5.0
$C_{18:3}$	0.2	4.2	2.2	0.7	4.7	2.7
$C_{19:1}$	1.1	—	0.55			
$C_{20:1}$	0.4	0.7	0.55			
$C_{20:2}$	0.5	3.6	2.05			
$C_{20:4}$	1.4	2.6	2.0	0.4	0.8	0.6
$C_{22:4}$	1.3	1.8	1.55			
$C_{22:6}$	0.2	2.5	1.35			

* Values are expressed as moles % of methyl esters.

Cephalin would include plasmalogen and inositide, but the proportions of the fatty acids of these components in the α' and β fatty acids of cephalin would be small. Similarly plasmalogen, a minor contaminant of phosphatidyl choline, would have little effect on the composition of the component fatty acids of this fraction. Rhodes and Lea (1) have reported 0.8% plasmalogen in the cephalin fraction and 2.5% in the choline-containing fraction of buttermilk phospholipids.

The total fatty acid composition of cephalin and of phosphatidyl choline has been calculated from the gas chromatographic analyses of the two fatty acid fractions obtained by hydrolysis of each component (Table 2).

In cephalin, approximately 71% of the fatty acids were unsaturated, whereas in phosphatidyl choline, only 43% were unsaturated. The main differences were due to variations in the proportions of palmitic and oleic acids, although the minor fatty acids also showed wide variations. Myristic acid and other saturated acids of intermediate chain length appear to be preferentially incorporated into phosphatidyl choline. Conversely, acids above C_{18}, including the polyethyenoid acids, predominate in the cephalin fraction.

α′ and β Fatty Acids in the Cephalin Fraction. About 83% of the acids in the β-position are unsaturated, compared with 60% in the α′-position. Since oleic acid is almost evenly distributed between the α′- and β-positions (slightly higher in the former), the greater preponderance of unsaturated acids in the β-position is accounted for by the presence of dienoic, trienoic, and polyenoic acids. Only a very small proportion of stearic acid occurs in the β-position, indicating that stearic acid has been combined in the α′-position in preference to the highly unsaturated acids. Palmitic acid, like oleic acid, is found in similar proportions in the α′- and β-positions; together these two acids comprise about 61% of the total acids in each position. These analyses have been made on a mixed cephalin fraction; at present nothing is known about the distribution of fatty acids in the separate ethanolamine and serine phospholipids, which form the large proportion of the cephalin fraction in milk.

α′ and β Fatty Acids in Phosphatidyl Choline. The results (see Table 2) show that the distribution pattern in phosphatidyl choline is somewhat similar to that in cephalin. As with cephalin, the proportion of the unsaturated fatty acids in the β-position is higher, largely owing to the greater numbers of molecules with linoleic and linolenic acids in this position. Stearic acid is found in small quantities in phosphatidyl choline and is mostly in the α′-position, while palmitic acid present in much greater quantity, is fairly evenly distributed. As in the cephalin fraction, oleic and palmitic acids together contribute almost identical proportions of the total acids found in each position (66.1% and 66.0% in the α′- and β-positions, respectively). Table 3 shows that there is a marked similarity in the α′/β ratios of the major fatty acids in both cephalin and in phosphatidyl choline.

TABLE 3. RATIOS OF THE MAJOR FATTY ACIDS IN THE α′-POSITIONS TO THOSE IN THE β-POSITIONS OF CEPHALIN (PE + PS) AND PHOSPHATIDYL CHOLINE (PC)

Fatty Acid	α′/β Ratio	
	PE + PS	PC
$C_{14:0}$	0.59	0.51
$C_{16:0}$	1.32	1.21
$C_{18:0}$	6.53	8.52
$C_{18:1}$	0.94	0.84
$C_{18:2}$	0.35	0.41
$C_{18:3}$	0.05	0.15

DISCUSSION

In general, the distribution of the fatty acids in glycerophospholipids is such that the unsaturated fatty acids predominate in the β-position (15, 16). Milk glycerophospholipids, however, are considered to be an exception to this generalization since Rhodes and Lea (1) found that unsaturated fatty acids were present in both the α′- and β-positions. The present experiments have shown that, while appreciable proportions of the molecules do contain unsaturated fatty acids in the α′-positions, the distribution between the α′- and β-positions is far from even. On a molar basis, the ratios of unsaturated to saturated fatty acids in the α′- and β-positions in the mixed cephalin fraction are 1.48 and 4.90, respectively, and in phosphatidyl choline are 0.54 and 1.05, respectively. These ratios show that, as with the phospholipids from other sources, there are higher proportions of the molecules with unsaturated fatty acids in the β-position than in the α′-position. It is not known whether this generalization can be applied to the individual components of the cephalin fraction.

A further similarity between milk phospholipids and phospholipids from other sources is the concentration of the highly unsaturated fatty acids in the cephalin fraction (19–22). In addition, Gray (21) observed that, in lecithin of most tissues examined, the most abundant saturated acid was palmitic acid, whereas in cephalin, it was stearic acid. Also Farquhar (23) has found a similar distribution of C_{16} and C_{18} saturated units in the glycerophospholipids of human erythrocytes where both fatty acids and fatty aldehydes are present. A similar overall distribution of the two saturated acids between lecithin and cephalin occurs in milk phospholipids. However, since the distribution of palmitic and stearic acid between the α′- and β-positions of both phosphatidyl choline and cephalin is different, it is doubtful whether the similarity in the overall distribution in tissue and other phospholipids has any metabolic significance.

The dissimilarity in the distribution of the fatty acids in milk lecithin (Table 2) and in bovine plasma lecithin (24) suggests that milk phospholipids are not derived from plasma phospholipids as such. Unfortunately, no information on the distribution of fatty acids in bovine plasma cephalin is available for comparison.

Interest in the fatty acid composition of the different glycerophospholipids from the same tissue has been stimulated by the existence of different pathways for the biosynthesis of phospholipids. Kennedy and coworkers (25, 26) showed that, in mitochondrial preparations, 1,2-diglycerides and cytidinediphosphate derivatives of choline and ethanolamine are the immediate precursors of phosphatidyl choline and phosphatidyl ethanolamine, respectively. On the other hand, Bremer, Figard, and Greenberg (27) have shown that, in the microsomal fraction, phosphatidyl serine is first decarboxylated to phosphatidyl ethanolamine and the latter is subsequently methylated to lecithin. Support for this latter mechanism has been obtained by Borkenhagen, Kennedy, and Fielding (28) working with cell-free extracts of livers, and also by Hall and Nyc (29, 30), who found that phosphatidyl monomethylethanolamine and phosphatidyl dimethylethanolamine accumulated in choline-requiring mutant strains of *Neurospora crassa*.

In both the above pathways, it would be expected that the fatty acids in component phospholipids obtained from the same tissue would be similar. In the present study, it is interesting to find that the composition of the fatty acids is different in lecithin and cephalin, yet the positional distribution of the major fatty acids, as shown by α'/β ratios (Table 3) is similar. However, the cephalin fraction represents a mixture of components so that the similarities in the α'/β ratios compared to those for lecithin could be fortuitous. Furthermore, since the relative amounts of these fatty acids in the two glycerophospholipid fractions are so different, additional information is required before the mechanism of phospholipid biosynthesis in milk is understood. For instance, it is not known whether acylating enzymes similar to those found by Lands (31) in liver are present in mammary tissue.

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