4. THE COMPONENT ACIDS OF PHOSPHATIDES PRESENT IN COW'S MILK FAT

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(Received 14 November 1940)

ALTHOUGH until quite recently phosphatides were considered to play an important part in the production of the characteristic glycerides present in milk, or even to be the precursors of the latter, a quantitative statement of the composition of the mixed fatty acids in milk fat phosphatides was lacking until the fatty acids of phosphatides from butter milk powder were examined by Kurtz *et al.* [1934]. Previously, however, Osborne & Wakeman [1915] had reported the presence of saturated acids of m.p. 62–65° and of liquid acids of iodine value 85.7 in phosphatides isolated from cow's milk, whilst Sasaki & Hiratzuka [1931], who had prepared purified phosphatides from dried milk, observed lauric, myristic and palmitic acids amongst the fatty acids present, but found no evidence of the presence of unsaturated acids. Kurtz *et al.* [1934] extracted the phospholipids from 300 lb. of buttermilk powder with a mixture of 75 % ethyl alcohol and 25 % of light petroleum and separated the material precipitated from acetone into two fractions, (a) phosphatides soluble in ether at 0° and (b) substances insoluble in this medium which appeared to be of the nature of sphingomyelin and cerebroside (*vide infra*, pp. 27, 29). The phosphatides of fraction (a) yielded on hydrolysis 62 % of fatty acids, the component acids of which were observed to be myristic 5.2, stearic 16.1, arachidic 1.8, oleic 70.6 and dicos-tetraenoic 6.3 % (wt.). These authors were unable to detect any palmitic acid, but identified myristic, stearic and arachidic acids in the ester fractions which they obtained; of unsaturated acids, they established the presence of oleic acid in quantity, and also of a highly unsaturated acid of higher molecular weight for which the analytical data indicated a formula $C_{22}H_{36}O_2$, but found no evidence for di- or tri-enoic C_{18} acids. They also found that, in contrast to butter fat, the phosphatides contained none of the lower saturated fatty acids.

We have recently examined the acids from two specimens of milk fat phosphatides, but the quantities of each in our possession did not permit us to separate the 'lecithin-kephalin' fraction from other compounds also present, possibly of the types noted by Kurtz *et al.* [1934] or possibly also including a certain proportion of wax-esters. We have, however, submitted the mixed acids from each specimen to analysis by ester fractionation and have confirmed the findings of other workers that the typical lower saturated acids of cow's milk fat are wholly absent. Our data, like those of Kurtz *et al.* [1934], disclose the presence of up to 10 % of highly unsaturated acids of molecular weight corresponding to members of the C_{20} and C_{22} series (in common with all animal phosphatide component acids so far studied). Our experience differs, however, from that of Kurtz *et al.* [1934] in that, amongst the saturated acids, we observed the presence of relatively large proportions of palmitic acid.

EXPERIMENTAL

The first specimen of milk fat phosphatides which we studied was kindly placed at our disposal by Dr B. Rewald, who had isolated it from non-glyceridic residues obtained in the separation of pure butter fat from Swiss butter. These had been extracted, first with light petroleum and then with a mixture of 80 % alcohol and 20 % benzene, the extract from this second operation being subsequently re-extracted with light petroleum. The combined light petroleum extracts were boiled with acetone and the portion which separated in the cold was the subject of the present work (the material left in solution in acetone was found to be mainly butter fat). In consequence of the differences between the results we obtained with this specimen and those of Kurtz *et al.* [1934] we proceeded to isolate, employing the procedure of Rewald [1937], the corresponding phosphatide fraction from residues from English butter fat clarification kindly provided for us by the Pure Butter Fat Process Co.

Component acids of the phosphatides from Swiss butter residues

The material received from Dr Rewald consisted of friable, dark brown, somewhat hygroscopic granules; it had a saponification equivalent of 221.4 and gave on hydrolysis mixed fatty acids which had (after removal of unsaponifiable matter) sap. equiv. 290.2, i.v. 37.0.

The corresponding material left in solution in acetone in the later stages of Dr Rewald's isolation of the butter phosphatides had sap. equiv. 243.5, i.v. 38.4. We submitted a portion (111 g.) to complete analysis by the usual fractionation procedure and found its component acids to be butyric 3.7, *n*-hexanoic 1.8, *n*-octanoic 1.2, *n*-decanoic 2.4, lauric 2.6, myristic 11.8, palmitic 28.6, stearic 8.9, arachidic 1.2 %; decenoic 0.2, dodecenoic 0.4, tetradecenoic 1.0, hexadecenoic 2.8, oleic 27.3, octadecadienoic 5.8, unsaturated C_{20-22} 0.3 % (wt.). It thus evidently consisted of typical butter fat glycerides.

The Swiss butter phosphatides (40.7 g.) were hydrolysed, first with 0.5 % alcoholic H_2SO_4 (500 ml.) and subsequently with alcoholic KOH. After thorough removal of alcohol from the soaps produced, the latter were dissolved in water, acidified with H_2SO_4 and steam-distilled for 5 hr. The steam-distillate was exhaustively extracted with ether and the ether-extracted aqueous portion (A) and the ether distilled from the extract (E) were examined for acidity. The material extracted by ether (0.57 g., equivalent 229.3) was solid at room temperature; no odour of butyric or other fatty acids of low molecular weight was, moreover, perceptible in the original aqueous condensate from the steam distillation. Both the aqueous portion (A) and the recovered ether (E) nevertheless possessed definite acidity, but this was shown to be due mainly to HCl and, to a less degree, to traces of sulphurous and/or sulphuric acid. Aliquot parts of (A) and (E) were exactly neutralized, evaporated to a small bulk, and any chloride estimated as silver chloride; another aliquot portion of (A) was treated with H_2O_2 in the cold, then concentrated to a small volume, and any H_2SO_4 was determined as $BaSO_4$; a control experiment carried out *ab initio* with the alcohol used in the hydrolysis of the phosphatides served to account for any traces of volatile acids emanating from the alcohol. The results of these determinations are summarized on the following page.

The observed acidity was therefore mainly due to HCl, presumably from NaCl carried down with the phosphatides in the course of their extraction.

Butyric or other low molecular weight acids, present in butter glycerides, were totally absent from the phosphatides.

		Equivalents of acid present
(A) Aqueous steam-distillate after extraction with ether:		
Total acidity by titration		0.02482
Volatile acids from alcohol (control experiment)	0.00023	
Acidity due to hydrochloric acid	0.02184	
Acidity due to sulphurous or sulphuric acid	<u>0.00263</u>	
Total accounted for		0.02470
(E) Ether from ether-extract of steam-distillates:		
Total acidity by titration		0.00098
Acidity due to hydrochloric acid		0.00032

The acids non-volatile in steam were recovered and submitted to lead salt separation. The total fatty matter accounted for was as follows:

	g.	%
Steam-volatile fatty acids	0.57	3.2
Non-steam-volatile fatty acids:		
'Solid' acids S	8.32	46.2
'Liquid' acids L	8.63	48.0
Unsap. matter	<u>0.47</u>	2.6
	17.99	

The yield of fatty acids from the phosphatides (44 %) was low, in consequence of the fact that the small amount of material available precluded further purification of the 'lecithin-kephalin' fraction.

The 'solid' and 'liquid' acids were separately converted into methyl esters and the latter distilled *in vacuo* through an electrically heated and packed fractionating column; the fractions listed in Table 1 were obtained.

Table 1

No.	g.	B.P./0.1 mm.	Sap. equiv.	I.V.
(i) Methyl esters of 'solid' acids S				
S1	2.53	80-100°	270.7	4.2
S2	1.86	100°	290.2	12.8
S3*	2.40	Residue in column	318.7	—
S4*	1.40	Residue in distilling-bulb	368.9	—
	<u>8.19</u>			
(ii) Methyl esters of 'liquid' acids L				
L1	2.42	100-120°	279.0	70.6
L2	3.05	120-126°	297.2	103.3
L3	2.28	Residue in column	301.9	109.2
L4*	0.92	Residue in distilling-bulb	448.6	—
	<u>8.67</u>			

* Esters, freed from unsaponifiable matter: S3, sap. equiv. 317.5, i.v. 17.3; S4, sap. equiv. 366.4, i.v. 11.7; L4, sap. equiv. 354.0, i.v. 90.4.

The following acids were identified in certain of the above fractions:

Acids volatile in steam (0.57 g., equiv. 229.3). Crystallization from 90 % alcohol gave a crystalline fraction, M.P. 56° (mixed with palmitic acid, M.P. 58-60°); this appeared to be a mixture of palmitic and myristic acids.

'Solid' acids S. The acids from fraction S1, on crystallization from 90 % alcohol followed by ethyl acetate, gave palmitic acid (M.P. 61.0°, mixed with pure

palmitic acid m.p. 62.0°). Those from fraction S3, after four crystallizations from ethyl acetate, yielded pure arachidic acid (m.p., and mixed m.p., 74–74.5°). Those from the residue S4 could not be obtained pure, but gave, from ethyl acetate, a mixture of acids which melted at 76.5° (mixed with pure arachidic acid, m.p. 71–72°); acids of higher molecular weight than arachidic acid were obviously present.

'Liquid' acids L. The acids from fraction L3 were dissolved in anhydrous ether at 0° and treated with bromine; insoluble bromo-additive compounds were precipitated, which commenced to darken at 200–210° and decomposed without completely melting at 224° (characteristic for highly unsaturated C_{20-22} acids).

The presence of arachidic acid is satisfactorily established, but the nature of the saturated acids of higher molecular weight remains uncertain. These may represent wax esters present in traces in the original milk fat and concentrated by the process of extraction in the phosphatide (acetone-insoluble) fraction; or, perhaps more probably in the light of recent work by Kurtz & Holm [1939], they may emanate from bodies of a sphingomyelin or cerebroside nature. In the former case they would be mainly of the formula $C_{26}H_{52}O_2$, in the latter $C_{24}H_{48}O_2$. For the purpose of the present calculations they have been taken as $C_{26}H_{52}O_2$. The final composition of the mixed fatty acids from the Swiss butter phosphatide concentrate is then as in Table 2 (the precision of the analyses is not considered to be of the order attainable with more adequate amounts of experimental material).

Table 2. *Component acids of phosphatides from Swiss butter residues (approx.)*

Acid	Steam volatile acids (3.2%)	Acids non-volatile in steam			Total %	% (wt.)*	% (mol.)*
		'Solid' acids S (46.2%)	'Liquid' acids L (48.0%)	Unsaponi- fiable (2.6%)			
Myristic	3.03	0.06	—	—	3.09	3.2	4.0
Palmitic	0.17	16.20	3.85	—	20.22	21.0	23.2
Stearic	—	6.99	—	—	6.99	7.3	7.2
As arachidic	—	11.83	—	—	11.83	12.3	11.1
As $C_{26}H_{52}O_2$	—	4.97	—	—	4.97	5.2	3.7
Hexadecenoic	—	—	4.13	—	4.13	4.3	4.8
Oleic	—	6.03	25.22	—	31.25	32.5	32.5
As octadecadienoic	—	—	6.19	—	6.19	6.4	6.5
As C_{20-22} unsatd.	—	—	7.51	—	7.51	7.8	7.0
Unsaponifiable	—	0.12	1.10	2.60	3.82	—	—

* Excluding unsaponifiable matter.

Component acids of the phosphatides from English butter residues

In the hope of obtaining a larger quantity of material for examination, attempts were made to find a suitable source of the milk fat phosphatide fraction in this country. Although, ultimately, a specimen of no larger size than that discussed above was obtained, the results are here included since the fatty acid composition arrived at differed in some details from that of the preceding sample.

In the first place, preliminary experiments on three possible sources of milk fat phosphatides were carried out, namely, on buttermilk powder and on two varieties of residues from butter fat clarification processes. We are indebted to the Wilts United Dairies Ltd. for the buttermilk powder, and to the Pure Butter Fat Process Company who were good enough to supply us with the other two samples. Small quantities of each product were refluxed with ether and the

solutions filtered. The matter soluble in ether was recovered and extracted with light petroleum, and the portion passing into solution was recovered and boiled with acetone. The percentages of acetone-insoluble products (i.e. phosphatide concentrates) from the three specimens were:

	Phosphatide concentrate %
Buttermilk powder	0.4
Butter fat residue A	0.7
Butter fat residue B	2.9

The residue A was a thick yellow emulsion of the consistency of cream, whilst residue B was a dark brown semi-solid paste. In consequence of its much higher apparent phosphatide content, the butter fat residue B was selected as the source of larger supplies of phosphatides and about 8 lb. of the material were extracted with ether. The extracted material was treated first with light petroleum and later with a mixture of 80 % of ethyl alcohol and 20 % of benzene, and the crude phosphatides obtained were separated from acetone as previously described. Ultimately, however, only about 42 g. of the purified phosphatides were recovered for further study. (The acetone-soluble matter extracted from the final phosphatide concentrates had sap. equiv. 246.6, i.v. 40.6, and therefore, as in the preceding instance (p. 25), probably consisted mainly of ordinary butter fat glycerides.)

The phosphatide concentrates (38.5 g.) gave on hydrolysis 15.8 g. of fatty acids (with some unsaponifiable matter); the yield of fatty acids was low, as in the case of the Swiss butter phosphatides. After removal of alcohol from the saponified product, followed by acidification with H_2SO_4 , no odour of butyric or other low molecular weight acid could be detected, and the distillation of the mixed fatty acids with steam was accordingly omitted. On submission to a lead salt-alcohol separation the mixed acids (15.82 g.) gave 'solid' acids (9.28 g., 58.7 %), 'liquid' acids (5.49 g., 34.7 %) and a small amount of non-fatty material insoluble in ether (1.05 g., 6.6 %). Distillation at 0.1 mm. of the methyl esters of the two groups of acids through the fractionating column gave a series of fractions, and from the analyses of the latter the approximate composition of the mixed fatty acids was found to be as given in Table 3.

Table 3. *Component acids of phosphatides from English butter residues (approx.)*

Acid	'Solid' acids (58.7 %)	'Liquid' acids (34.7 %)	Unsaponifiable (6.6 %)	Total %	% (wt.)*	% (mol.)*
Myristic	4.74	—	—	4.74	5.5	7.0
Palmitic	7.74	3.89	—	11.63	13.4	15.2
Stearic	7.85	—	—	7.85	9.0	9.2
As arachidic	18.18	—	—	18.18	20.9	19.5
As $\text{C}_{26}\text{H}_{52}\text{O}_2$	8.70	—	—	8.70	10.0	7.3
Hexadecenoic	—	4.30	—	4.30	4.9	5.7
As oleic	9.96	10.49	—	20.45	23.5	24.3
As C_{20-22} unsaturated	—	11.11	—	11.11	12.8	11.8
Unsaponifiable	1.53	4.91	6.60	13.04	—	—

* Excluding unsaponifiable matter.

The component acids in this sample of butter phosphatides are of the same general type as those in the Swiss phosphatides, but contain more of the higher saturated acids, i.e. a higher proportion of admixed wax esters, or

sphingomyelin and cerebrosides, as the case may be. When allowance is made for this, however, it appears that the palmitic and oleic acid contents disclosed by this analysis are somewhat lower than in the preceding case. Acids of the C_{18} group more unsaturated than oleic were not definitely detected, but the small amount of methyl esters of 'liquid' acids available for fractionation may well have been responsible for this.

DISCUSSION AND SUMMARY

The results of the two analyses of cow's milk phosphatide component acids described above fully confirm the findings of previous investigators that the typical milk fat glyceride acids (butyric, etc.) of low molecular weight are wholly absent from the phosphatide acids. It has been demonstrated [Graham *et al.* 1936; Maynard *et al.* 1938] that the blood phosphatides of the cow are not removed by the mammary gland, and that these are not the precursors of milk fat glycerides. It is now equally well established that the component fatty acids of milk fat phosphatides have little in common with those of the milk fat glycerides; on the other hand, they bear more general similarity to those of the phosphatides of the ox liver [Hilditch & Shorland, 1937], as the following comparison indicates:

Table 4. *Component acids of ox liver and cow's milk phosphatides*

Phosphatides of ...	Ox liver [Hilditch & Shorland, 1937]	Cow's milk fat		
		(Present work)		Kurtz <i>et al.</i> [1934]
		'Swiss'	'English'	
Acid	% (wt.)	% (wt.)	% (wt.)	% (wt.)
Myristic	1.3	3.2	5.5	5.2
Palmitic	28.2	21.0	13.4	—
Stearic	14.4	7.3	9.0	16.1
As arachidic	0.2	12.3	20.9	1.8
As $C_{26}H_{52}O_2$	—	5.2	10.0	—
Tetradecenoic	0.7	—	—	—
Hexadecenoic	3.8	4.3	4.9	—
Oleic	17.3	32.5	23.5	70.6
As octadecadienoic	14.2	6.4	—	—
As C_{20-22} unsaturated	19.9	7.8	12.8	6.3

So far as our analyses are concerned, the points of resemblance with the liver phosphatide component acids are the presence, as major components, of palmitic and oleic acids, together with notable proportions of stearic and of highly unsaturated acids of the C_{20} and C_{22} series; the chief points of difference are the minor proportions of octadecadienoic acid in the milk fat phosphatides, and the presence of considerable amounts of arachidic acid and of higher saturated acids (reckoned arbitrarily as $C_{26}H_{52}O_2$). The compounds giving rise to most of these higher saturated acids were separated, by Kurtz *et al.* [1934], by crystallizing the phosphatide concentrates from ether at 0° . Kurtz & Holm [1939] state that the ether-insoluble fraction contains about 80 % of lignoceric acid, the remaining 20 % being a very complex mixture of saturated, unsaturated, hydroxy-saturated and presumably hydroxy-unsaturated acids, the carbohydrate, phosphorus and nitrogen contents of this fraction supporting the view that it is a mixture of sphingomyelin and cerebrosides. It is possible that simultaneous separation from ether of some of the more saturated 'lecithin-kephalin' phosphatides is

responsible for the large proportions of oleic acid present in the ether-soluble fraction the composition of which was given by Kurtz *et al.* [1934]; but their failure to detect any palmitic acid in either group of the phosphatides renders the composition of the material which they examined unusual, if not unique, in the records of component acids of phosphatides from any source.

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