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Blood Lipids

2. PLASMA LIPIDS OF THE LACTATING COW: FATTY ACID COMPOSITION OF THE STEROL ESTERS AND TRIGLYCERIDES

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Since the triglycerides and possibly the sterol esters of blood plasma are involved in milk-fat formation in the cow (see review by Folley, 1956), it is of considerable biochemical interest to study the fatty acid composition of these classes of lipid, especially in relation to the fatty acid composition of dietary, depot and milk fats. The only information concerning the component fatty acids of the plasma lipids of lactating cows is that obtained by Kelsey & Longenecker (1941), who determined by ester fractionation the composition of the triglycerides, sterol esters and free fatty acids. However, as discussed by Lovern (1956) and Garton & Duncan (1957), it seems doubtful whether the cholesterol esters and triglycerides were effectively separated from each other by the procedure employed, namely selective enzymic hydrolysis of the glycerides. In this laboratory we preferred to use the more recently developed technique of chromatography on silicic acid (Fillerup & Mead, 1953) to separate the sterol esters and triglycerides from the plasma lipids of lactating cows (Garton & Duncan, 1957), and in this paper we describe the determination, on a semi-micro scale, of the fatty acid composition of these classes of lipid by means of reversed-phase partition chromatography. A preliminary account of this work has been published (Lough, Garton & Duncan, 1957).

EXPERIMENTAL

Preparation of fatty acids

The sterol esters and triglycerides used were those prepared by chromatography on silicic acid by Garton & Duncan (1957) from the pooled plasma lipids of six lactating, pasture-fed Ayrshire cows. The sterol esters obtained chromatographically were hydrolysed with ethanolic KOH to yield pale-yellow, liquid fatty acids having an iodine value (Trappe, 1938) of 189.5; the triglyceride fraction was similarly treated to give almost colourless, solid fatty acids of iodine value 36.3.

Estimation of polyethenoid fatty acids by alkali isomerization

Samples of the sterol-ester fatty acids and the glyceride fatty acids were examined according to the method of Hilditch, Patel & Riley (1951), which is applicable to linoleic and linolenic acids but is not applicable in the presence of arachidonic acid in significant amounts. The presence of arachidonic acid is indicated by λ_{max} . 301 and 315 m μ ; none was detected in the glyceride fatty acids, but appreciable amounts were present in the sterol-ester fatty acids. Accordingly, extinction coefficients for this acid (not available to us in a pure form) were derived from the data given by Brice, Swain, Herb, Nichols & Riemenschneider (1952), who carried out alkali-isomerization studies with arachidonic acid under similar conditions to those used by Hilditch *et al.* (1951). These values, after isomerization at 180° for 60 min., were taken as $E_{1\text{cm}}^{1\%}$ at 315 m μ = 201, and

$E_{1\text{cm}}^{1\%}$ at $234\text{ m}\mu = 515$. To allow for the contribution of arachidonic acid to the absorption at $268\text{ m}\mu$ after isomerization at 170° for 15 min., the value $E_{1\text{cm}}^{1\%} = 644$ was employed. This value can be considered as an approximation since it was derived by multiplying the value $E_{1\text{cm}}^{1\%}$ at $268\text{ m}\mu = 620$ after isomerization at 180° for 15 min. (Brice *et al.* 1952) by the factor $532/512$, these being the extinction coefficients at $268\text{ m}\mu$ for linolenic acid isomerized for 15 min. at 170° and 180° respectively (Hilditch *et al.* 1951). The proportions of linoleic, linolenic and arachidonic acids thus found are given in Table 1. It should be noted that no account has been taken of the possible presence of *trans* isomers (cf. Shorland, 1955), which may affect extinction coefficients. Traces of a conjugated isomer of linoleic acid were observed in the fatty acids of the sterol esters when they were examined spectrophotometrically before alkali isomerization.

Reversed-phase partition chromatography

The method of chromatography used was essentially that described by Howard & Martin (1950) for the separation of mixtures of higher, saturated *n*-fatty acids in which successively increasing proportions of acetone in water are used to elute the acids individually from a column of Hyflo Super-Cel treated with dichlorodimethylsilane and liquid paraffin. As used on a semi-micro, quantitative scale in this Laboratory, the method has recently been described in detail (Garton & Lough, 1957). Except when stated, the separations were done on columns $30\text{ cm.} \times 1.5\text{ cm.}$, with acetone-water mixtures containing, successively, 40, 45, 50, 53, 55, 58, 60, 63, 65, 68, 70, 73, 75 and 78% (v/v) acetone. Recoveries of acids from the columns were 95–98%.

RESULTS

Sterol-ester fatty acids

To determine the molecular species of fatty acids present in the mixture, a sample was hydrogenated, as described by Popják & Tietz (1954), to an iodine value of < 1.0 , and 26 mg. of the saturated acids was chromatographed. The resulting elution pattern, given in Fig. 1, shows that myristic, palmitic, stearic and arachidic acids were present. The proportions of each component, calculated from the amount of standard alkali required to neutralize the fatty acid present in each peak, are given in Table 2.

A sample of the unhydrogenated fatty acids (126 mg.) was chromatographed on a column $40\text{ cm.} \times 3.0\text{ cm.}$, which permitted the recovery of

fatty acid fractions for further examination. The separation, illustrated in Fig. 2, shows that most of the acids were eluted in peaks *A* and *B*, followed by peaks *C*, *D* and *E* of much smaller magnitude; on a molar basis, peaks *A*, *B*, *C*, *D* and *E* represented 38.6, 49.8, 8.7, 2.1 and 0.8% respectively of the total fatty acids.

The column fractions 1–91 (peak *A*) and 92–131 (peak *B*) were collected and the component fatty

Fig. 1. Chromatography of the hydrogenated fatty acids (26 mg.) of the sterol esters. Arrows indicate progressive changes in the proportions (v/v) of acetone in the acetone-water mixtures used as eluent.

Fig. 2. Chromatography of the fatty acids (126 mg.) of the sterol esters. Uniform arrows indicate progressive changes in the proportions (v/v) of acetone in the acetone-water mixtures used as eluent. Broken arrows indicate the fractions (1–91 and 92–131) which were pooled to yield the acids contributing to peaks *A* and *B* respectively. For composition of peaks *A*, *B*, *C*, *D* and *E* see text.

Table 1. Proportions (% w/w) of polyethenoid components found spectrophotometrically after alkali isomerization of the total fatty acids of the sterol esters and triglycerides

Acid	Source of fatty acids	
	Sterol esters	Triglycerides
Linoleic	35.2*	1.9
Linolenic	30.2	0
Arachidonic	8.4	0

* Including traces of a conjugated isomer.

Fig. 3. Chromatography of the hydrogenated fatty acids (11 mg.) of peak *A* (Fig. 2). Arrows indicate progressive changes in the proportions (v/v) of acetone in the acetone-water mixtures used as eluent.

Fig. 4. Chromatography of the hydrogenated fatty acids (18 mg.) of peak *B* (Fig. 2). Arrows indicate progressive changes in the proportions (v/v) of acetone in the acetone-water mixtures used as eluent.

acids recovered. The iodine values (approximate owing to small amount of acids available) were 247 and 146 for the acids from peaks *A* and *B* respectively. After hydrogenation to iodine values < 1.0, 11 mg. of acids (peak *A*) and 18 mg. of acids (peak *B*) were subjected to chromatographic examination, and gave the elution patterns shown in Figs. 3 and 4 and the proportions of fatty acids given in Table 2.

It will be observed that the hydrogenated acids from each peak consisted mainly of stearic acid, together with small proportions of myristic, palmitic and arachidic acids. From a consideration of the elution characteristics of unsaturated fatty acids (Crombie, Comber & Boatman, 1955) and the knowledge that no acid of chain length greater than 20 carbon atoms was present in the mixture (Fig. 1), it was concluded that the palmitic and arachidic acids found in peaks *A* and *B* (Fig. 2) represented palmitoleic and arachidonic acids present in the original fatty acids; the myristic acid found in peaks *A* and *B* must have been present before hydrogenation, since no peak corresponding to an unsaturated C_{14} acid was found. It was concluded that the stearic acid in the hydrogenated acids of peak *A* was derived from linolenic acid and that in the hydrogenated acids of peak *B* from linoleic acid [cf. the iodine values of the acids in each peak and the findings of Crombie *et al.* (1955) that linolenic and linoleic acids are separable from each other]. That these conclusions were justified will be seen when the proportions of linoleic and linolenic acid, estimated chromatographically to be present in the total acids, are compared with the proportions found spectrophotometrically after alkali isomerization (see Table 3).

The proportions of the acids found, after hydrogenation, in peaks *A* and *B* show that whereas more of the arachidonic acid present in the original mixture was eluted in peak *A* than in peak *B*, more myristic and palmitoleic acids were present in peak *B* than in peak *A*. Thus some degree of resolution of this complex mixture was achieved, although the overwhelming abundance of linoleic and linolenic acids may have affected the elution behaviour of other components (cf. Lovern, 1956).

The proportion of each component present in the total acids of the sterol esters (Table 3) was calculated from the proportions given in Table 2 and the proportions of the components of peaks *C*, *D* and *E* (Fig. 2). No allowance was made for the very small changes in molecular proportions resulting from hydrogenation of the unsaturated acids.

From their elution behaviour it was concluded that peak *C* comprised oleic and palmitic acids, peak *D* stearic acid and peak *E* arachidic acid. The composition of peak *C* was deduced as follows, all proportions, unless otherwise stated, being molar

Table 2. *Molar percentage of fatty acids found after hydrogenation*

Acid	Source of fatty acids		
	Total acids of sterol esters	Peak <i>A</i> (Fig. 2)	Peak <i>B</i> (Fig. 2)
Myristic	4.4	2.2	7.2
Palmitic	12.8	6.8	17.3
Stearic	76.0	82.8	71.8
Arachidic	6.8	8.2	3.7

Table 3. *Molar percentage of fatty acids contributed by peaks A-E (Fig. 2) to the total fatty acids of the sterol esters*

Acid	Peak					Total acids
	A	B	C	D	E	
Myristic	0.8	3.6	—	—	—	4.4
Palmitic	—	—	1.6	—	—	1.6
Stearic	—	—	—	2.1	—	2.1
Arachidic	—	—	—	—	0.8	0.8
Palmitoleic	2.6	8.6	—	—	—	11.2
Oleic	—	—	6.1	—	—	6.1
Linoleic	—	35.8	—	—	—	35.8 (36.4)*
Linolenic	32.0	—	—	—	—	32.0 (32.3)*
Arachidonic	3.2	1.8	(1.0)†	—	—	6.0 (6.6)*

* Molar percentage of total acids, calculated from the figures given in Table 1.

† Not accounted for by chromatographic results, but attributed to arachidonic acid (see text).

percentages of the total fatty acids. The contribution (1.6%) of the palmitic acid present in peak *C* was derived by subtracting the contribution (11.2%) of the palmitic acid of the hydrogenated acids of peaks *A* and *B* from the palmitic acid content (12.8%) of the total hydrogenated fatty acids. The contribution (6.1%) of the oleic acid of peak *C* was derived by subtracting the contribution (67.8%) of the stearic acid in the hydrogenated acids of peaks *A* and *B* plus the stearic acid contribution (2.1%) of peak *D* from the stearic acid content (76.0%) of the total hydrogenated fatty acids. Thus together the palmitic and oleic acids in peak *C* contributed 7.7%, compared with 8.7% calculated on the basis of the titrations during chromatography, leaving 1% of acids not accounted for. Experimental error undoubtedly accounted for this minor deficiency in the complete analysis. However, a discrepancy of 1% was found between the molar proportion of arachidic acid (6.8%) found after hydrogenation of the total acids and the sum of the arachidic acid contributions of the hydrogenated acids in peaks *A* and *B* (5.0%), together with that in peak *E* (0.8%). We therefore feel justified in attributing this 1% to arachidonic acid.

The iodine value (192.4) of the total fatty acids calculated from the results of the chromatographic analysis compares favourably with that obtained by direct determination (189.5).

Triglyceride fatty acids

The fatty acids were chromatographed before and after hydrogenation, 21 and 16 mg. respectively being used; the elution patterns are illustrated in Fig. 5.

It will be observed from the chromatogram of the hydrogenated acids that four molecular species of acids (C_{14} , C_{16} , C_{18} and C_{20}) were present; the molar contribution of myristic, palmitic, stearic and arachidic acids to the total mixture was 6.4, 37.1, 53.6 and 2.9% respectively. No peak corresponding

Fig. 5. Chromatography of the fatty acids of the triglycerides. Upper chromatogram, 16 mg. of the hydrogenated acids; lower chromatogram, 21 mg. of the unhydrogenated acids; for description of peaks *A-D*, see text. Arrows indicate progressive changes in the proportions (v/v) of acetone in the acetone-water mixtures used as eluent.

Table 4. *Composition of the glyceride fatty acids*

Acid	Molar percentage
Myristic	4.6
Palmitic	29.9
Stearic	30.6
Myristoleic	1.8
Palmitoleic	7.2
Oleic	21.5
Linoleic	1.5
Gadoleic	2.9

to a C_{20} acid was observed in the chromatogram of the unhydrogenated acids and, since no arachidonic acid was detected spectrophotometrically (Table 1), it was concluded that the arachidic acid found after hydrogenation was derived from the C_{20} mono-unsaturated acid, gadoleic acid. Before hydrogenation, a peak corresponding to lauric acid was observed, but since it disappeared on hydrogenation it was attributed to the C_{14} mono-unsaturated acid, myristoleic acid. The molar contribution to the total fatty acids of peaks A, B, C and D (Fig. 5) was 1.8, 13.3, 51.4 and 33.5 % respectively. Calculation of the final composition of the glyceride fatty acids (Table 4) thus entailed simple subtractions, due allowance being made (in peak B) for the proportion of linoleic acid found spectrophotometrically to be present (Table 1). The iodine value of the total acids was calculated to be 34.3, compared with the value of 36.3 found by direct determination.

DISCUSSION

Chromatography

The reversed-phase partition chromatographic method of Howard & Martin (1950) for the quantitative separation of milligram quantities of lauric, myristic, palmitic and stearic acids was extended by Silk & Hahn (1954) and Popják & Tietz (1954) to include the even-numbered, *n*-fatty acids C_{10} – C_{24} , by Hougen (1955) to include the odd-numbered, *n*-fatty acids C_7 , C_9 and C_{11} , and further by Garton & Lough (1957) to embrace all the saturated *n*-fatty acids C_8 – C_{20} . Crombie *et al.* (1955) applied the method to the analysis of fairly simple mixtures of saturated and unsaturated fatty acids of plant origin by running chromatograms before and after oxidative decomposition of the unsaturated components with alkaline potassium permanganate. As in the reversed-phase chromatographic procedure of Boldingh (1953), we preferred to run chromatograms of mixed acids before and after hydrogenation of the unsaturated components (cf. Popják & Tietz, 1954), not only because the molecular species of acids present in the hydrogenated mixture are thus identifiable, but also because the alkaline permanganate treatment brings about some destruction of saturated fatty acids; in preliminary studies we confirmed that partial oxidative degradation of *n*-fatty acids C_{14} and lower occurred as reported by Crombie *et al.* (1955).

As exemplified by the analyses reported in this paper it seems that the method involving hydrogenation of the unsaturated fatty acids can be commended as a semi-micro, quantitative analytical technique for the investigation of lipids of animal origin, especially when myristic acid and saturated acids of lower chain-length are present. The assumptions made in the evaluation of the chromatograms

are few and, when polyethenoid components are involved, their presence and proportions can be determined by complementary alkali-isomerization studies. We therefore feel that the analyses presented are comparable in their accuracy with those determined on fatty acid mixtures by the ester-fractionation technique (cf. Garton & Lough, 1957) which, of course, requires far more starting material than is readily available from small tissue samples and blood.

Sterol-ester fatty acids

The most striking feature of these acids is their very high iodine value (189.5) which is reflected in their content of the polyethenoid components linoleic, linolenic and arachidonic acids. Schaible (1932), who isolated lipid fractions rich in cholesterol esters from the plasma of lactating and non-lactating cows, found that the iodine values of the acids were about 150, whereas that calculated from the analysis of Kelsey & Longenecker (1941) is 155.9 for the acids present in their crude concentrate of cholesterol esters. In the analysis reported by Kelsey & Longenecker (1941) linoleic, linolenic and arachidonic acids comprised 61.7, 9.2 and 2.3 % (mol.) respectively of the total fatty acids, whereas in our preparation the two C_{18} unsaturated acids were present in nearly equal proportions [35.8 and 32.0 % (mol.) respectively], together with 6.0 % (mol.) of arachidonic acid.

Cholesterol esters differ from ox depot fat and plasma triglycerides (Table 5) in their content of polyethenoid fatty acids. It is noteworthy that the components of these esters must be derived from different sources, the cholesterol being produced endogenously (there being no dietary source), and the C_{18} di- and tri-unsaturated fatty acids arising from the diet, of which pasture grasses are a good source (see Hilditch, 1956). It is thus possible that preferential esterification of these acids with cholesterol takes place during, or subsequent to, their absorption from the small intestine, though the formation of cholesterol arachidonate presumably involves the esterification of arachidonic acid produced *in vivo* from linoleic acid, as was shown by Mead, Steinberg & Howton (1953) to occur in the rat. However, it is probable that only a small proportion of the dietary polyunsaturated fatty acids reaches the small intestine, since the work of Shorland, Weenink & Johns (1955) strongly suggests that unsaturated fatty acids are hydrogenated to a considerable extent in the reducing environment of the rumen.

Though it may be only fortuitous, the molar proportion of total C_{18} unsaturated fatty acids in the sterol esters (73.9 %) resembles (see Table 5) the proportion (78.5 %) reported in the fatty acids of mixed pasture grasses by Jasperson & Burke (cited

Table 5. *Molar percentage of the fatty acids in ox depot fat, milk fat, plasma sterol esters and triglycerides of lactating cows and in the lipids of pasture grasses*

Acid	Typical milk fat (Hilditch & Jasperson, 1943)	Typical depot fat (Hilditch & Longenecker, 1937)	Plasma triglycerides (present work)	Plasma sterol esters (present work)	Mixed pasture grasses (Jasperson & Burke; cited by Hilditch, 1956)
Saturated					
Short-chain, C ₄ -C ₁₂	21.7	0.2	—	—	2.9
Myristic	9.6	3.7	4.6	4.4	3.3
Palmitic	23.4	26.5	29.3	1.6	9.4
Stearic	9.7	23.1	30.6	2.1	1.5
Arachidic	0.6	0.7	—	0.8	0.7
Unsaturated					
C ₁₀ + C ₁₂	0.5	—	—	—	0.3
Myristoleic	1.0	0.5	1.8	—	0.4
Palmitoleic	2.1	2.6	7.2	11.2	3.0
Oleic	28.6	40.4	21.5	6.1	} 78.5†
Linoleic	1.8	1.8	1.5	35.8	
Linolenic	—	—	—	32.0	
C ₂₀ -C ₂₂	1.0	0.5*	2.9†	6.0*	—

* As arachidonic acid.

† As gadoleic acid.

‡ Comprising approx. 50 % of linolenic acid, together with linoleic and oleic acids.

by Hilditch, 1956) and by Shorland (1944), who found 76.5 %, composed of about equal amounts of linoleic and linolenic acids, in the fatty acids of cocksfoot (*Dactylis glomerata*).

Triglyceride fatty acids

As shown in Table 5, the fatty acid composition of the plasma triglycerides resembles that of depot fat in that the major components are stearic, palmitic and oleic acids, together with smaller amounts of myristic, myristoleic, palmitoleic, linoleic and C₂₀ unsaturated acids. The absence of linolenic and arachidonic acids is especially noteworthy in view of the amounts found in sterol-ester combination.

Whereas the saturated fatty acids C₄-C₁₆ of ruminant milk fat can be synthesized in the udder (Popják, French, Hunter & Martin, 1951; Cowie *et al.* 1951), it seems that stearic acid and oleic acid (the major component of milk glycerides, see Table 5) must be derived from plasma triglycerides, though the mechanism of incorporation of these acids into milk glycerides is obscure. Hilditch (1952) has suggested that the replacement of oleic acid of plasma triglycerides by saturated acids (especially the shorter-chain acids) may take place, though it is also possible that the oleic acid may, in some way, become available for incorporation into glycerides, being synthesized *de novo* in the secretory tissue of the udder. Such a possibility is suggested by our finding that the oleic acid content of the plasma triglycerides is only about one-half of that found in depot fat, which is presumably the source of much

of the plasma triglycerides in the lactating cow. In this connexion it should be noted that, as a result of feeding tritium-labelled stearic acid (as free acid or triglyceride) to lactating goats and cows, Glascock, Duncombe & Reinus (1956) concluded that not more than 25 % of milk glyceride fatty acids are derived from the dietary lipids.

It is relevant to note that when groundnut, rape, soya-bean and linseed oils were fed to lactating cows (Hilditch & Sleightholme, 1930; Hilditch & Thompson, 1936), the amount of polyethenoid C₁₈ fatty acids of the milk fat showed no significant increase, in spite of the high linoleic and linolenic acid content of the oils. Recently Mayhead & Barnicoat (1956) found that the iodine value of bovine milk fat increased by 5.7 units during partial inanition, and when 16 oz. of groundnut oil (iodine value, 96.2)/cow/day was administered during partial inanition the iodine value of the milk fat increased by only 2.3 units. It was expected that the oil feeding would result in a more pronounced rise in unsaturation than that occurring during partial inanition alone, since the unsaturated oil was expected to have a sparing action on the utilization of depot fat. In the light of our findings and those of Shorland *et al.* (1955), the results of Hilditch & Sleightholme (1930), Hilditch & Thompson (1936) and Mayhead & Barnicoat (1956) may well be explained by the partial hydrogenation of polyunsaturated dietary acids followed by esterification of the remaining polyunsaturated acids with cholesterol, which results in their unavailability for incorporation into milk glycerides.

SUMMARY

1. The reversed-phase partition chromatographic method of Howard & Martin (1950) has been extended to the semi-micro, quantitative analysis of the fatty acids of the sterol esters and triglycerides from the plasma of pasture-fed, lactating cows.

2. The sterol-ester fatty acids were characterized by their high content of polyunsaturated components (linoleic, linolenic and arachidonic acids), whereas the triglycerides resembled depot fat in the nature of the component fatty acids, though the proportion of oleic acid was considerably lower in the plasma glycerides than in depot fat.

3. The fatty acid composition of the two classes of plasma lipid is discussed in relation to that of dietary lipids and milk glycerides. It is suggested that dietary polyunsaturated fatty acids which escape hydrogenation in the rumen may be preferentially esterified with cholesterol during, or subsequent to, their absorption from the alimentary tract.

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