Triglyceride Structure of Milk Fats[1]

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ABSTRACT

The enantiomeric nature of the triglycerides of bovine milk fat was reinvestigated by determining the stereospecific distribution of fatty acids in rearranged butterfat, following partial hydrolysis with pancreatic lipase, and in certain molecular distillates of native butterfat, following Grignard degradation. The results with rearranged butterfat confirmed the validity of pancreatic lipase hydrolysis as a means of generating representative diglycerides from milk fat triglycerides. The Grignard degradation and lipolysis gave identical distributions for fatty acids when included as part of the assay system in the stereospecific analysis. Characteristically, butyric acid and the other short chain acids occupied the 3 position in the native butterfat, while in the rearranged oil they were distributed more or less randomly. Gas chromatographic analysis of the short chain glycerides on polyester columns allowed an effective resolution of butyryl, caproyl and caprylyl glycerides of identical numbers of total acyl carbons and double bonds. The method was especially well suited for resolution of the 2,3-diglycerides, which were recovered either as the more polar fraction from thin layer chromatography of the X-1,2-diacylglycerols, or by acetolysis of the residual phenolphosphatides resulting from phospholipase A digestion. It was shown that butyric acid in the 3 position was preferentially paired with myristic, palmitic and oleic acid in the 2 position, and palmitic and oleic acid in the 1 position, which was also characteristic of the other short chain acids.

INTRODUCTION

The milk triglycerides of higher mammals differ radically from their body fats (1,2). The greatest discrepancies are seen in the ruminants, the milk fat triglycerides of which contain 20-30 mol% short and medium chain length fatty acids, and possess significant positive optical rotary power (3). The content of the short and medium chain acids is much lower in man and other nonruminant mammals, but their milk fat triglycerides also possess characteristic positional distribution and molecular association of fatty acids when compared to the triglycerides of other tissues (4). This is apparently due to differences in biosynthetic mechanisms, to a segregation of the available fatty acid pools and to hormonal influences. The complex nature of the milk triglycerides has prohibited the identification of individual triglyceride species in the past and has caused difficulties in the study of their metabolism. The present report demonstrates that the increased gas chromatographic resolution of short chain glycerides realized on polyester columns (Myher and Kuksis, unpublished results) makes it experimentally feasible to determine exact structures for the butyryl and other short chain triglycerides, which may be adequate for the recognition of the major structural features of all glycerides of the ruminant and other milk fats. Since the correct identification of the molecular species depends on accurate knowledge of the positional distribution of the fatty acids, discussion of the new gas chromatographic resolutions is preceded by a brief consideration of further experimental evidence supporting the enantiomeric nature of native milk fat triglycerides.

[1]One of eight papers presented in the symposium "Milk Lipids," AOCS Meeting, Ottawa, September 1972.

MATERIALS AND METHODS

Rearranged butterfat was available from a previous study (5), which also gave some chemical and physical constants of the product. It had been prepared by reaction with 0.1% sodium methylate for ca. 1 hr at a temperature of ca. 100 C. The catalyst was removed by filtration as a salt while the fat was still molten. The interesterified fat along with the original butterfat was supplied by Drew Chemical Corp., Boonton, N.J. The molecular distillate R-4 had been saved from another earlier study (6). This fraction had been obtained as the last of four equal weight cuts from a redistillation of the most volatile 10% cut from the original distillation. The fraction represented largely short and medium chain length triglycerides ranging in acyl carbon number from 34 to 44. The molecular distillates were supplied by Distillation Products Industries, Rochester, N.Y. All the above glyceride samples had been stored in tightly closed bottles at -20 C. Samples of the perirenal depot fat of cow, goat and sheep were obtained from fresh carcasses of these animals supplied by a local butcher. Synthetic rac-1,2-dibutyrate-3-palmitate and rac-1,2-dipalmitate-3-oleate were obtained from R.G. Jensen and D. Rebello. The triglyceride samples were purified by thin layer chromatography (TLC) on Silica Gel H (Merck and Co.) spread on 20 x 20 cm plates in 0.5 mm thickness. The plates were developed with heptane-isopropyl ether-acetic acid 60:40:4, as previously described (7). The triglyceride fractions were recovered by eluting the appropriate sections of the gel with chloroform.

Stereospecific Analysis of Rearranged Butterfat

For this purpose the purified triglyceride sample was divided into long and short chain triglycerides by chromatography on columns of silicic acid, as described by Blank and Privett (8). The fractions obtained from ca. 1 g total fat were pooled in two nearly equal lots; the less polar one (0.4 g) provided the long chain length, while the more polar one (0.6 g) gave the short and medium chain length triglycerides. The latter fraction comprised major triglycerides with 32-54 acyl carbon atoms and the former long chain triglycerides with 40-54 acyl carbons.

The resolved triglyceride fractions were digested separately by brief exposure (4 min) to pancreatic lipase at room temperature in the presence of diethyl ether (9). The resulting free diglycerides were separated by TLC in the presence of boric acid (10). The mixed 1,2- and 2,3-diglycerides were recovered as a somewhat diffused band and were converted into the phosphatidylphenols, purified and subjected to hydrolysis by phospholipase A as outlined by Brockerhoff (11). The hydrolysis products were recovered by TLC on Silica Gel G (Merck and Co.), using chloroform-methanol-3% ammonia 63:30:7 as the developing solvent. The fatty acid composition of the three positions of the glyceride molecules was obtained by analyzing the free fatty acids (position 2), the fatty acids in lysophosphatidylphenols (position 1) and the residual phenols (positions 2 and 3). The composition of position 3 was derived by subtracting the composition of position 2 from that of the mixed 2 and 3 positions. The fatty acid composition of the 2-monoglycerides derived from the original lipase hydrolysis served as another estimate of composition of position 2.

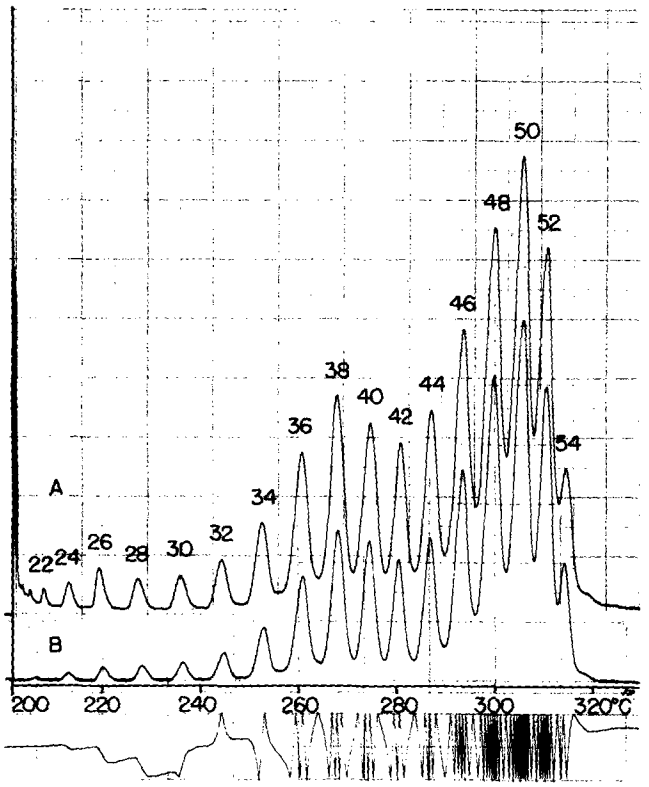

FIG. 1. Gas chromatogram of a sample of rearranged milk fat triglycerides before (upper) and after (lower) partial degradation with pancreatic lipase. Peaks identified by total number of acyl carbons in triglyceride molecules. Chromatographic conditions as given in text.

Grignard Degradation of Butteroil Distillates

This reaction was performed as described by Yurkowski and Brockerhoff (12) for the partial deacylation of lard. About 0.75 g of R-4 distillate in 50 ml diethyl ether was treated at room temperature with 3 M ethyl magnesium bromide in 2 ml diethyl ether. After 20 sec, 1 ml acetic acid was added, which was followed by 10 ml water at 50 sec. The reaction was done under nitrogen, using sodium dried ether. The reaction products were washed with water and recovered as described by Yurkowski and Brockerhoff (12).

An aliquot of the reaction products was resolved by TLC on borate-treated plates according to Thomas et al. (10). A series of monoglycerides and diglycerides of short and long chain length was obtained by collecting the appropriate sections of the silica gel. The glycerides were immediately converted into the acetates by treating the gel scrapings with sufficient acetic anhydride pyridine 10:1 to cover them. After standing overnight at room temperature the reaction mixtures were heated for 1 hr at 80 C. The excess acetic anhydride was destroyed by adding cold methanol to the cooled reaction mixture. The acetates were extracted with diethyl ether, the solvents evaporated, and the residue taken up with petroleum ether and washed with water. The final extracts of each glyceride band were redissolved in benzene and examined by gas chromatography as described below. Other aliquots of the diglycerides were recovered from TLC in the free form and subjected to phenylphosphorylation and phospholipase A digestion, as described by Yurkowski and Brockerhoff (12), and the digestion products recovered and analyzed, as suggested by Breckenridge and Kuksis (7).

Preparation of 2,3-Diacyl-*sn*-Glycerols

For this purpose the mixed diglycerides resulting from pancreatic lipolysis or Grignard degradation were subjected to phenylphosphorylation and digestion with phospholipase A, as described above. In this process both 1,2-diacyl and 1,3-diacyl-*sn*-glycerolphosphorylphenols were destroyed, leaving a residue of the 2,3-diacyl-*sn*-glycerolphosphorylphenols. The latter were isolated by TLC of the reaction products in chloroform-methanol-3% ammonia 63:30:7, as reported by Brockerhoff (11). The phosphatidylphenols were recovered as a clearly resolved band from the lysophosphatidylphenols, which moved more slowly. The phosphatidylphenols were recovered from the gel by elution with chloroform-methanol 2:1. The pure 2,3-diacylglycerolphosphorylphenols were then subjected to acetolysis at 145 C in a sealed tube for 12 hr (13). The resulting diglyceride acetates were recovered by diluting the cooled reaction mixture with cold methanol and water in an ice bath and taking up the neutral lipids in petroleum ether. After backwashing the extracts with water, the petroleum was evaporated and the acetates redissolved in benzene and examined by gas chromatography.

Gas Chromatography

Triglycerides, diglyceride acetates and the monoglyceride diacetates were resolved by carbon number in a Beckman GC-4 gas chromatograph (Beckman Instruments, Inc., Fullerton, Calif.) equipped with a modified on-column heater (14). The columns were stainless steel tubes (50 cm x 0.2 cm ID) packed with 3% OV-1 (a methyl silicone) on Gas Chrom Q (100-120 mesh) as supplied by Applied Science Labs., Inc., State College, Pa. The working conditions were identical to those described previously, except for the temperature programs, which are shown in the appropriate figures.

Diglyceride acetates, monoglyceride diacetates and low molecular weight triglycerides were resolved by carbon number, unsaturation and the overall polarity of the molecule in an F and M Biomedical gas chromatograph (F and M Corp., Avondale, Pa.). The columns were glass U-tubes (180 cm x 2 mm ID) packed with 3% EGSS-X (an ethylene glycol succinate-silicone copolymer) on Gas Chrom Q (100-120 mesh) as supplied by Applied Science Labs. The working conditions were similar to those described previously for the resolution of diglyceride acetates and silyl ethers (15), except for the operating temperatures, which are given in the legends to the appropriate figures.

Fatty acids were analyzed as the butyl esters in an Aerograph Model 204-1B gas chromatograph (Varian-Aerograph, Walnut Creek, Calif.). The columns were stainless steel tubes (150 x 0.2 cm ID) packed with 10% EGSS-X on Gas Chrom Q (100-120 mesh). The temperature was programed at 4 C/min from 60 to 200 C. The fatty acids were identified and estimated quantitatively by comparison with standard mixtures of butyl esters prepared by transbutylation of standard methyl esters. The butyl esters were prepared by treating the lipid sample with 6% H_2SO_4 in dry butanol in a closed vial at 80 C for 4 hr (9). The reaction products were extracted with small amounts of petroleum ether, and after washing once with water an aliquot was taken for gas chromatography without evaporation to dryness.

The peak areas of the various lipid components were measured by Disc integration (Disc Instruments, Inc., Santa Ana, Calif.).

Interesterification

Interesterifications were carried out over periods of 5-120 min at 100 C, using sodium methoxide as catalyst (16). The butterfat triglycerides (1 g) were contained in a test tube supported in a silicone oil bath on a magnetic stirrer provided with a hotplate. The glyceride was stirred vigorously under N_2 at 110 C for 2 hr to remove traces of water. The temperature was then adjusted to 50-100 C and 0.1% sodium methylate was added. Samples were taken immediately before addition of the catalyst and at timed

TABLE I

Comparison of Calculated and Experimentally Determined Compositions of Fatty Acids of 1,2-(2,3-)-Diglycerides Released by Pancreatic Lipase from Milk Triglycerides[a]

Fatty acid	Goat				Sheep			
	Short chains		Long chains		Short chains		Long chains	
	Exptl.	Calcd.	Exptl.	Calcd.	Exptl.	Calcd.	Exptl.	Calcd.
	Mol%							
4:0	8.5	7.7			7.2	6.0		
6:0	6.9	6.2			6.0	5.4	0.3	0.4
8:0	4.0	4.1	0.4	0.7	3.0	3.5	1.1	1.3
10:0	9.6	9.4	4.7	5.7	7.7	7.6	4.1	3.7
12:0	4.0	4.3	2.5	3.0	4.7	4.9	2.9	2.9
14:0	13.0	13.0	13.6	12.8	12.9	13.1	10.5	11.3
14:1	0.8	0.6	0.4	0.6	0.8	0.7	0.7	0.8
15:0	1.0	1.8	2.6	2.5	2.5	2.3	3.3	3.6
16:0	26.0	25.8	34.1	31.0	22.5	21.4	25.1	22.5
16:1	2.3	2.0	2.0	2.2	1.3	1.5	2.1	2.3
17:0	0.7	0.7	1.0	1.1	0.6	0.8	1.3	1.1
18:0	5.1	5.8	12.0	11.3	9.2	10.0	18.8	16.2
18:1	13.6	14.0	23.3	24.2	15.6	15.7	24.0	26.3
18:2	2.0	2.1	1.6	2.9	3.5	3.9	3.6	4.4
18:3					1.8	2.3	1.4	2.6
20:1	1.9	1.9	1.6	1.8	0.4	0.5	0.5	0.4
20:2	0.3	0.3	0.2	0.2		0.1		

[a]The calculated composition of diglycerides was derived by multiplying the fatty acid composition of the original triglycerides by three, adding the per cent composition of the 2-monoglycerides and dividing by four, as suggested by Yurkowski and Brockerhoff (12). Exptl. = experimental; calcd. = calculated.

intervals afterwards. The catalyst was immediately destroyed by dropping the samples into a tube containing 1% acetic acid in 1 ml light petroleum. After blowing off the solvents the residue was taken up in chloroform and analyzed by gas chromatography, as described for triglycerides.

RESULTS AND DISCUSSION

Evidence for Nonrandom Structure

It is generally assumed that in triacylglycerols, in which the acyl groups have been rearranged or introduced by a chemical catalyst, the fatty acids are distributed in a truly random manner among and within the individual glyceride molecules (17,18). This assumption has recently been verified experimentally for certain simple interesterified fats containing the common long chain saturated and monounsaturated fatty acids (16). Since interesterification can be readily demonstrated to bring about a change in the intermolecular distribution of acyl groups in milk fat triglycerides, it follows that the original distribution of fatty acids was nonrandom. We had previously compared the triglyceride carbon number distribution of a sample of bovine milk before and after interesterification by gas chromatography of the intact triglycerides (5). There were significant differences in the two distributions, which could not be accounted for by the minor alterations in the fatty acid composition of the sample resulting from the process of interesterification. As a result of interesterification, there had been a major increase in the proportion of the shorter chain triglycerides and a decrease in the longer chain triglycerides. When compared to the 1,2,3 random distribution of acyl groups calculated on the basis of the fatty acid composition, it could be shown (5) that the rearranged milk fat triglycerides contained greater proportions of both short and long chain triglycerides than expected. The native butterfat contained less than random amounts of the short and long chain triglycerides and more than random amounts of the medium chain triglycerides. The interesterified fats, however, approached the random distribution more closely than did the native fats.

Extending the time of interesterification was shown to produce comparable discrepancies in the gas chromatographic elution patterns of the native and rearranged fats. The differences between the calculated and the experimentally determined molecular weight distributions, however, tended to become smaller. It is shown below, by means of stereospecific analyses, that in the commercially rearranged sample an incomplete equilibration had been attained for butyric acid among the three positions of the glycerol molecule.

An independent indication of a change in the glyceride structure as a result of interesterification can be gleaned from the alteration in the setting or softening point of the triglyceride mixture. The fact that the setting point is raised (19-21) suggests that the fatty acid chains were formerly arranged in a fashion that would tend to prevent their crystallization. Other reports claiming a lowering of the melting point upon interesterification have been attributed (22) to the presence of partial glycerides and free fatty acids in the insufficiently purified reaction product. It may be possible, however, that the magnitude and direction of change in the melting point depends on the actual fatty acid composition of the sample. For the present purposes, any change in the melting point of the triglycerides would be sufficient to indicate a nonrandom structure for the native fat. An asymmetrical distribution of the short chain acids in butteroil triglycerides is also indicated by the significant rotatory power remaining after removal of nontriglyceride, optically active material (3).

Distribution of Butyrate between Primary and Secondary Positions

The randomness of distribution of fatty acids between the 1 and 3 positions and the 2 position of triglycerides may be assessed by hydrolysis with pancreatic lipase. This technique has been successfully employed in the determination of structure of triglycerides containing the common long chain fatty acids (11,23). Preferential hydrolysis of short chain fatty acids and acyl migration from the 2 position to the 1 and 3 positions, however, makes it difficult to apply this method to the determination of the glyceride structure of milk fats (24). Jack et al. (25) proposed short periods of digestion which minimize the

TABLE II

Positional Distribution of Fatty Acids in Milk Fat Triglycerides of Cow, Sheep and Goat[a]

Fatty acid	Position relative to *sn*-glycerol-3-phosphate								
	One			Two			Three		
	Cow[b]	Sheep	Goat	Cow[b]	Sheep	Goat	Cow[b]	Sheep	Goat
	Mol%								
4:0	5.0			2.9			43.3	10.8	13.2
6:0	3.0			4.8			10.8	10.4	10.6
8:0	0.9	0.3	1.7	2.3	2.0	1.2	2.2	4.4	4.6
10:0	2.5	1.4	3.3	6.1	5.2	6.9	3.6	10.3	12.2
10:1	—			—	0.1	0.1	—	0.4	0.5
12:0	3.1	2.2	4.0	6.0	4.7	4.6	3.5	3.5	1.2
14:0	10.5	8.2	8.4	20.4	17.6	20.3	7.1	5.3	2.7
14:1	—	0.2		—	0.9	0.8	—	1.1	1.0
15:0	—	2.6	1.9	—	4.1	2.7	—	1.5	1.5
16:0	35.9	38.0	43.6	32.8	23.8	33.9	10.1	2.5	3.4
16:1	2.9	2.2	2.9	2.1	2.2	2.0	0.9	1.5	1.6
17:0	—	1.7	1.3	—	0.9	0.5	—	0.7	1.8
18:0	14.7	19.1	15.3	6.4	12.6	6.3	4.0	9.1	7.7
18:1	20.6	18.7	16.1	13.7	19.3	16.1	14.9	27.2	30.2
18:2	1.2	2.7	0.3	2.5	4.2	2.5	~0.5	6.0	4.5
18:3		2.2			1.7			4.4	
20:1		0.5	0.9		0.2	1.7		0.4	2.7
20:2			0.4			0.1		0.2	0.9

[a]Position 1 = lysophosphatidylphenols; position 2 = 2-monoglycerides; position 3 = 2 x residual phosphatidylphenols minus 2-monoglycerides.

[b]Data of Pitas et al. (39).

undesirable side effects and allow essentially valid results. This was verified by obtaining very similar fatty acid analyses from the mono-, di- and triglycerides resulting from digestion of randomized milk fat (20).

Figure 1 shows the gas chromatographic elution pattern of the rearranged butterfat triglycerides along with that of the triglyceride residue recovered after ca. 25% hydrolysis. Even at this level of degradation there is little distortion of the triglyceride profile, which would be expected if the short chain triglycerides were rapidly lypolyzed or if the short chain acids were preferentially released from the external positions. Comparable elution curves were also recorded for native butterfat and its residual triglycerides. The carbon number distribution of the residual triglycerides after more extensive hydrolysis of the milk fats, however, usually shows a large excess of the long chain triglycerides.

A more effective indicator of the randomness of the lypolysis is provided by a comparison of the weighted fatty acid compositions of the original triglycerides and the released diglycerides (12).

$DG_{calc} = (3TG + MG)/4$ when DG_{calc} is the theoretical

TABLE III

Distribution of Fatty Acids in Total and Short Chain Triglycerides of Rearranged Butter Fat

Fatty acid	Position relative to *sn*-glycerol-3-phosphate[a]					
	One		Two		Three	
	Total	Short	Total	Short	Total	Short
	Mol%					
4:0	3.0	7.5	2.0	8.0	6.2	12.8
6:0	2.8	8.6	2.7	7.9	4.7	6.7
8:0	1.1	2.5	2.6	3.6	2.6	2.2
10:0	2.8	3.5	4.4	5.0	4.6	4.8
12:0	5.6	5.3	4.7	5.5	2.9	3.7
14:0	12.7	16.6	14.6	10.8	12.8	10.0
14:1	0.7	1.6	1.3	0.8	1.1	1.6
15:0	1.7	0.9	1.8	1.6	1.4	0.8
16:0	34.2	26.0	31.9	28.1	31.1	26.9
16:1	1.6	2.3	1.2	2.1	2.0	2.3
17:0	1.1	1.4	0.4	2.0	1.2	4.0
18:0	11.9	9.6	12.6	11.0	12.8	6.2
18:1	19.7	14.1	17.9	13.1	14.5	17.5

[a]Fatty acid composition of each portion assessed as in Table II.

percentage of each particular fatty acid in the diglyceride products of pancreatic lipase hydrolysate, calculated from the fatty acid percentages of the original triglyceride (TG) and of the 2-monoglyceride (MG) products of lipolysis. A nonrandom hydrolysis is indicated by a significant discrepancy between the calculated composition of the fatty acids and that determined for the mixed diglycerides isolated from the digestion.

Table I shows that the mixed 1,2- and 2,3-diglycerides isolated from brief lipolysis of native goat and sheep milk triglycerides yield fatty acid compositions which can be directly related to those of the corresponding original triglycerides by simple proportionation. The agreement, however, is not as good when the total triglyceride mixture is subjected to lipolysis. The principal cause of difficulty is the preferential hydrolysis of triglycerides containing short chain fatty acids. This was effectively demonstrated by Sampugna et al. (26), who examined the sequential release of butyric and palmitic acids from synthetic triglycerides. There was a preferential intermolecular hydrolysis of the order PBB > PPB > PBP > PB, where P and B are palmitic and butyric acids, respectively, in the racemic triglycerides and in the 1,3-diglyceride. From PBB both palmitate and butyrate in the primary positions were released at the same rate (27). There was no apparent preferential intramolecular hydrolysis. Therefore representative digestions ought to be obtainable by hydrolysis of individual molecular species or small groups thereof. These results are in direct conflict with the claims of Entressangles et al. (28), who also examined the hydrolysis products of synthetic 1-palmitoyl-3-butyryl-glycerol. When the diglyceride was 10% hydrolyzed, the mixture of free fatty acids split contained 72% butyric and 28% palmitic acid. There is evidence, however, that the earlier work designed to study substrate specificity may actually have involved a study of the degree of emulsification and not specificity (24). In some instances a specific orientation of the substrate molecule at the oil-water interface may also have contributed to the nonrandomness of the result (29). In the present study the pancreatic lipase hydrolyses were performed in the presence of diethyl ether.

Despite these difficulties there appears to be a general consensus that butyric and caproic acids are located largely in primary positions, but opinions differ on the proportion

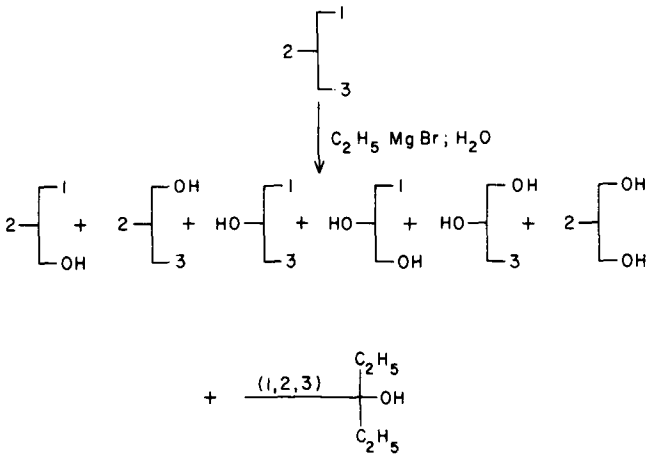

FIG. 2. Theoretical scheme of Grignard degradation of glycerides, as proposed by Brockerhoff (37).

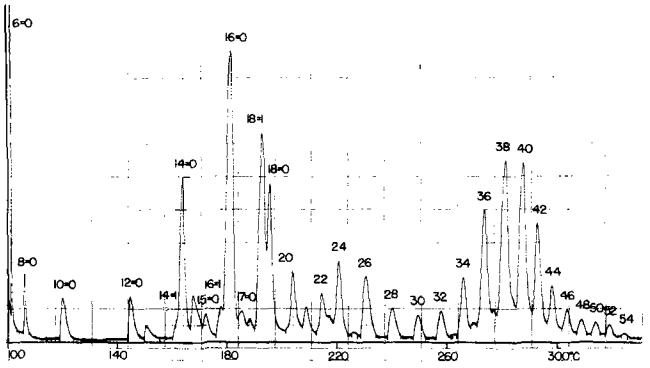

FIG. 3. Gas chromatogram of Grignard degradation products from a sample of short chain triglycerides of bovine milk fat. Peaks identified by acyl carbon number of triglycerides to which they correspond in retention temperature. Chromatographic conditions as in Figure 1. Temperature program as shown. Sample: total reaction products after trimethylsilylation.

of butyric acid in the 2 position. Boudreau and de Man (30) have claimed that a considerable proportion of butyric acid is located in the 2 position, and most results obtained by pancreatic lipolysis support this in varying degrees (31). It is not certain, however, to what extent this may be due to acyl migration (32). If butyrate is present in the 2 position, there should be significant amounts of dibutyryl triglycerides, but it has been clearly established by gas chromatographic analysis of the intact triglycerides (33) that this is not so. Butyrate is found almost exclusively with medium and long chain fatty acids in the individual triglyceride molecules. Therefore there can be only small amounts of butyrate in the 2 position. Nutter and Privett (34) have estimated that the dibutyryl triglyceride content in the milk serum may amount to ca. 0.02% of the total serum triglyceride. Pancreatic lipase hydrolyses have also suggested that the 2 position of the milk fat triglycerides is occupied largely by myristic, palmitic and oleic acids, while the combined 1 and 3 positions contain, besides the short chain acids, significant amounts of capric, lauric and palmitic acids (31).

Another physical technique that may allow an unambiguous differentiation between triglycerides containing butyric acid in the primary and secondary positions is provided by mass spectrometry. Barber et al. (35) have reported a full analysis of the structure of mixed triglycerides. The fatty acid moieties can be identified by the most intense peaks in the spectra, which correspond to the fragments remaining after elimination of the acyloxy groups from the molecular ion $[M\text{-}RCO_2]^+$ and to the acylium ion peaks $[RCO]^+$. In the case of triglycerides made up of three different acid moieties, each type gives three peaks in the spectrum. Another set of three peaks of less intensity corresponds to $[M\text{-}RCO_2CH_2]^+$. The ion peak corresponding to the acid moiety in the 2 position of the glycerol is usually negligible in comparison to those from the acid moieties in the 1 and 3 positions (36). From these differences in intensities of characteristic peaks, the fatty acid in the 2 position of the glycerol can be identified. Significant amounts of triglycerides containing butyric acid in the 2 position would greatly reduce the proportion of $[M\text{-}RCO_2CH_2]^+$ ions in the mass spectrum. From a carefully calibrated spectrum, a correct decision could probably be made as to the proportion of butyric acid in the primary and secondary positions of the glyceride molecule.

Stereospecific Location of Butyric Acid

As a result of the development of stereospecific analyses for the determination of triglyceride structure, it has become possible to differentiate the fatty acids in the 1 position from those in the 3 position (37). These techniques depend upon representative generation of diglycerides by pancreatic lipase or alkyl magnesium bromides, and criticisms similar to those voiced in regards to determination of the positional distribution of fatty acids are valid. Nevertheless it has been possible to conclude that butyric acid is esterified almost exclusively in the 3 position of milk fat triglycerides (38,39). In these instances the diglycerides were generated by 2.5-4 min exposure of the milk fats to digestion with pancreatic lipase. A preliminary segregation of the triglycerides into short, medium and long chain fractions yielded data showing more specific distribution of the butyrate (40,41). Distribution of the butyric acid in a narrow distillation cut of butteroil containing ca. 30 mol% short chain fatty acids appeared especially restricted (9). Table II gives the positional distribution of fatty acids in the total milk fat triglycerides of the cow, as reported by Pitas et al. (39), and sheep and goat, as calculated from the separate analyses of the short and long chain length triglycerides by Marai et al. (42). All of these animal species appear to incorporate the butyric and other short chain acids almost exclusively in the 3 position.

A disturbing note was introduced into this otherwise consistent series of observations on short chain acid distribution in the milk fats by our finding that a comparable stereospecific analysis performed on interesterified milk fat triglycerides also indicated that butyric acid was largely in position 3. Table III shows that, of the total butyric acid in the short chain fraction isolated from a rearranged fat, 50% was located in position 3, 35% in position 1 and 15% in position 2. Surprisingly the distribution of the other fatty acids appeared to be much more random, giving comparable amounts of each acid in the three positions. As noted above, calculations based on fatty acid composition showed that during interesterification random distribution of fatty acids is approached only gradually, and that the rearranged fat used for stereospecific analyses had not achieved a truly random arrangement of acyl groups by the time the catalyst was destroyed. Freeman (16) has shown that during rearrangement intraesterification precedes interesterification. Therefore during a limited reaction period complete equilibration between 1 and 3 positions would not be expected for fatty acids initially in the 3 position, as may be the case for butyrate in milk fat triglycerides. We have subsequently demonstrated that when the distribution of the butyric acid between the 1 and 3 positions is equal, as in rac-1,2-dipalmitate-3-butyrate, the stereospecific analysis via pancreatic lipase yields identical amounts of butyric acid in both positions. Furthermore the use of ethyl magnesium bromide as a means of generating diglycerides from molecular distillates

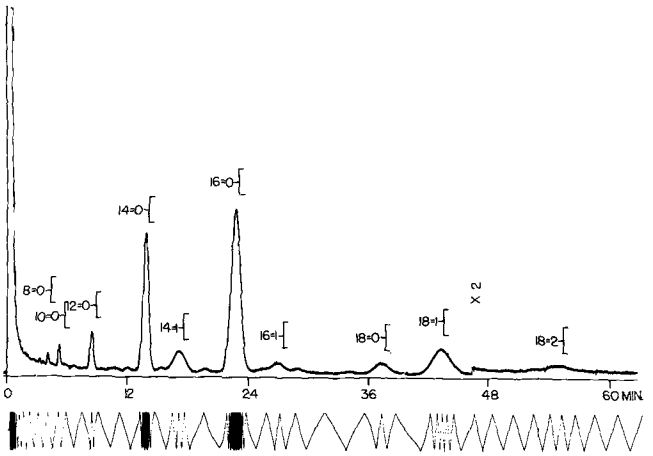

FIG. 4. Gas chromatogram of 2-monoglycerides on a polyester column. Chromatographic conditions as given in text. Sample: diacetates of 2-monoglycerides as recovered from Grignard degradation of short chain triglycerides of bovine milk fat.

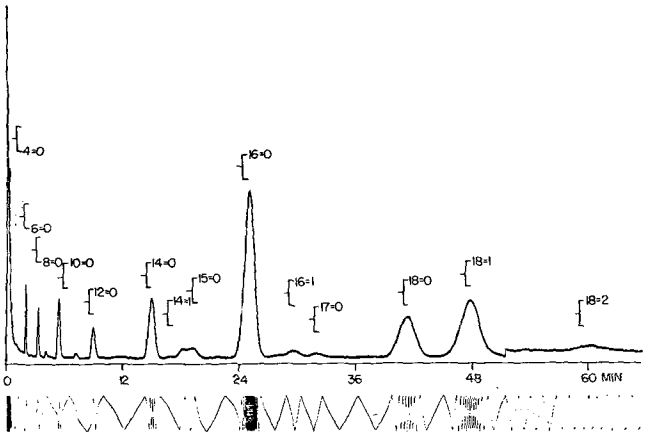

FIG. 5. Gas chromatogram of mixed 1- and 3-monoglycerides on a polyester column. Chromatographic conditions as given in text. Sample: diacetates of mixed 1- and 3-monoglycerides as recovered from Grignard degradation of short chain triglycerides of bovine milk fat.

yielded results identical to those obtained previously with pancreatic lipase. Therefore no flaw need be suspected in the stereospecific analyses of milk fats containing short chain acids.

Identification of Specific Glycerides

The ultimate determination of the structure of milk fats requires the identification and quantitative estimation of the individual molecular species of triglycerides. For many molecular species of natural triglycerides, this is now theoretically possible (43). Nutter and Privett (34) have already made a major contribution to this effort by identifying and quantitating ca. 168 molecular species of bovine milk serum triglycerides, excluding enantiomers. For this purpose they employed liquid-liquid and argentation TLC along with pancreatic lipase hydrolysis. Due to a high degree of saturation, ruminant milk fats do not lend readily to effective argentation chromatography, and a resolution based on molecular weight differences of the triglycerides is not adequate even when combined with preparative gas chromatography and stereospecific analysis. We have now overcome this difficulty by making use of the increased resolving power offered by the polyester columns for gas chromatographic separation of isomeric glyceride esters (15,44). These columns are especially well suited to the separation of the short chain 2,3-diacyl-*sn*-glycerols, which can be readily generated by either pancreatic lipolysis or Grignard degradation. They are resolved from the 1,2-diacyl- and the 1,3-diacyl-*sn*-glycerols by preparation of the phenylphosphatides and digestion of the reaction products with phospholipase A. The 2,3-diglycerides are regenerated as the acetates following acetolysis, which apparently is accompanied by very limited interesterification (9). Alternatively, the short chain diglycerides may be recovered in the free form or as acetates, following acetylation, from silica gel plates, on which the shorter chain length glycerides are more strongly adsorbed (40,41).

Figure 2 represents the products of Grignard degradation of triglycerides, which are made up of nearly random amounts of 1,2-, 2,3- and 1,3-diglycerides and 1-, 2- and 3-monoglycerides. According to Brockerhoff (37), the 1,3-diglycerides cannot be prepared without contamination (up to 10%) with isomerized 1,2- and 2,3-diglycerides. There is little contamination of the 1,2- or 2,3-diglycerides during preparation, although difficulties may arise during derivatization. Figure 3 shows the gas chromatographic elution profile of the reaction products, as obtained for a molecular distillate of butterfat on a silicone column. The various peaks have been identified by the carbon number to which they correspond in the temperature program. In a few instances, however, the assigned carbon numbers do not coincide with the actual number of acyl carbons present in these molecules. This inconsistency is limited to the monoglyceride derivatives and the tertiary alcohols. There is nearly perfect overlap between the high molecular weight diglyceride and the lower molecular weight triglycerides; hence this method is of little use for their direct analysis. Following TLC (40,41) discrete bands are obtained for the various chain lengths of the mono- di- and triglycerides, as well as between 1,2-, 2,3- and 1,3-diglycerides, each of which can be effectively separated by gas chromatography on silicone columns to obtain their molecular weight distribution. Since previous stereospecific analyses had already shown that the short chain fatty acids were located exclusively in the 3 position, it was expected that the Grignard reaction would yield long chain 1,2-diglycerides, 2-long chain-3-short chain diglycerides and 1-long chain-3-short chain diglycerides. The monoglycerides would be made up of 1-long chain, 2-long chain and 3-short chain monoglycerides. When we subjected the products of the Grignard reaction to TLC on Silica Gel G, we indeed recovered lipid fractions corresponding to long and short chain glyceride isomers.

Gas chromatographic resolution of the various glyceride fractions recovered from the thin layer plates yielded peaks consisting essentially of pure molecular species, which corresponded directly to those found in the original milk fat triglycerides.

Figure 4 shows that the 2-monoglyceride fraction contains largely long chain fatty acids, myristic, palmitic and oleic, the proportions of which correspond closely to those derived by stereochemical analysis of this molecular distillate (9). The bulk of the acids is supplied by myristic and palmitic acids, which is also true for the composition of the 2 position of milk fat triglycerides determined by Pitas et al. (39). There is an effective resolution of the saturated and unsaturated fatty acids of the same carbon number when run as the diacetates, which, however, does not exceed that of the methyl esters in the calculated separation factors. Since the 1 and 3 isomers were not collected separately from the thin layer plates, the gas chromatogram shown in Figure 5 contains both long and short chain monoglycerides. On the basis of the known qualitative composition of the fatty acids in positions 1 and 3, it is possible to assign exact identities to each one of the monoglycerides. When this is done and the peak areas are calculated, the derived proportions correspond well to those of the fatty acids in the 1 and 3 positions of the glyceride molecules found by stereospecific analyses. Like

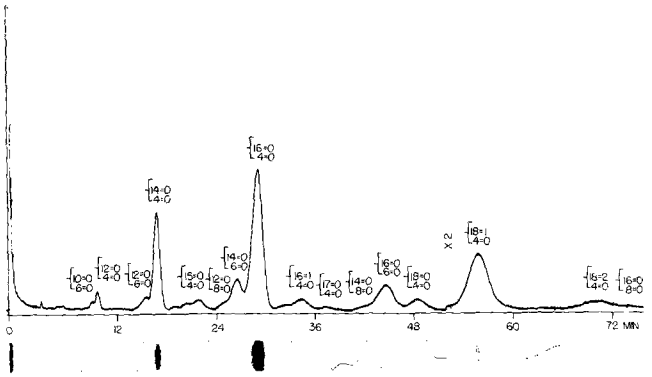

FIG. 6. Gas chromatogram of short chain 2,3-diglycerides on a polyester column. Chromatographic conditions as given in text. Sample: acetates of short chain 2,3-diglycerides as recovered from Grignard degradation of short chain triglycerides of bovine milk fat.

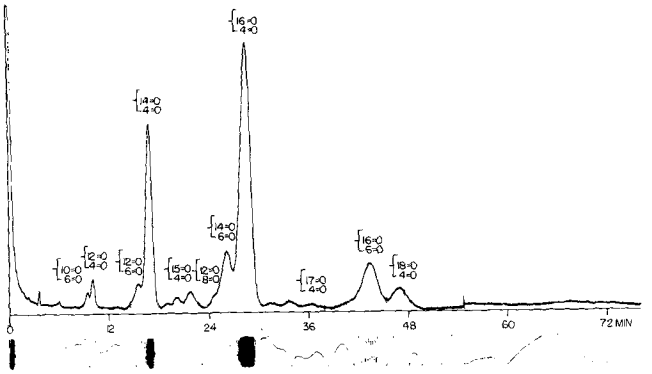

FIG. 7. Gas chromatogram of short chain 2,3-diglycerides as obtained on a polyester column following bromination. Chromatographic conditions as given in text. Sample: as in Figure 8, but exposed to bromine in chloroform.

the diacetates of the 2-monoglycerides, those of the 1- and 3-monoglycerides are also effectively resolved on the basis of degree of unsaturation. In fact the analysis of monoglycerides as the diacetates is superior to that of the fatty acid methyl or butyl esters, because it avoids losses on volatilization or on extraction from aqueous solutions.

Figure 6 shows the elution pattern recorded for the short chain 2,3-diglycerides on the polyester column. The diglycerides were also chromatographed as the acetate derivatives. The complex peak profile is due to a resolution of the butyrates, caproates and caprylates within a diglyceride class of uniform molecular weight and degree of unsaturation. The caproates emerge ahead of the butyrates and are preceded by the caprylates. This peak identification is completely consistent with the fatty acid composition and the molecular weight distribution of these diglycerides, as determined by independent analyses. The peak identity was further verified by analyses of standard short chain diglycerides and by examining the diglyceride mixture following bromination, which removes the peaks due to the unsaturated species. Thus Figure 7 shows that the peaks assigned to the palmitoleic, oleic and linoleic acids have been effectively removed by treatment with bromine. The separation factors calculated for the monoenes and dienes and the corresponding saturates are still high, but considerably lower than those of the corresponding fatty acid methyl esters and monoglycerides. There remains, however, adequate space for the accommodation of the caproate and caprylate derivatives, should such occur in sufficient amounts. This is illustrated in Figure 8, which contains 2,3-diglycerides of somewhat lesser polarity than those just examined as well as some of the more polar 1,2-diglycerides. In this elution pattern the caproic and caprylic acid derivatives predominate, but the overall excellence of the resolution is retained. Comparable resolutions are readily obtained also for the 1,3-diglycerides which are rich in short chain fatty acids. In both cases the fatty acid associations correspond to those found in the original milk fat triglycerides. When the diglycerides are prepared from small enough fractions of triglycerides, such as a single carbon number, which can be collected by preparative gas chromatography (45), it is possible to derive the exact triglyceride structure just from the examination of the 2,3- and 1,3-diglycerides.

The separation of saturated and unsaturated long chain diglycerides as the trimethylsilyl ethers on the polyester columns has already been reported from our laboratory (15,44). Because of the greater similarity in chain length of the component fatty acids, the separations within a given carbon number are minimal and in certain instances difficulties may be experienced in the resolution of the saturates and monoenes of the same carbon number. The resolutions among the monoenes and dienes, and the dienes and trienes, as well as tetraenes and polyenes, are excellent.

Figure 9 shows the resolution of the original triglycerides of the molecular distillate of butterfat on the polyester column under isothermal conditions. In addition to a separation based on molecular weight differences, the polyester column also yields a partial resolution of several of the major triglyceride peaks containing triglycerides of only one molecular weight. In view of the experience with the diglyceride acetates, this separation would be anticipated, because most of these peaks contain significant amounts of both butyric and caproic acids. Therefore it is likely that separations similar to those achieved with the diglycerides were obtained. This possibility is supported by computation of the corresponding peak areas and calculation of the molar ratios anticipated for the various 1,2-diglyceride combinations with butyric and caproic acids. The deviations from the calculated values are probably due to a nonrandom combination, which was already suggested from the more detailed separations achieved with the diglycerides.

The resolution of the butyric and hexanoic acid containing triglycerides of identical total acyl carbon number on the polyester columns recalls earlier studies from our laboratory. We had observed shouldering of certain triglyceride peaks when chromatographed on thermally stripped silicone columns (46). At the time we attributed this effect to differences in the positional distribution of the short chain fatty acids, which seemed to be consistent with the behavior of certain synthetic dibutyryl glycerides, as well as with the claim of Boudreau and de Man (30) of significant amounts of short chain acids in the primary and secondary positions of milk fat triglycerides. However it was subsequently pointed out by Watts and Dils (47) that the isomeric butyrates would not be resolved under these conditions, and that the observed shouldering was probably due to a presence or absence of a butyric acid residue in a triglyceride of a given carbon number. This interpretation

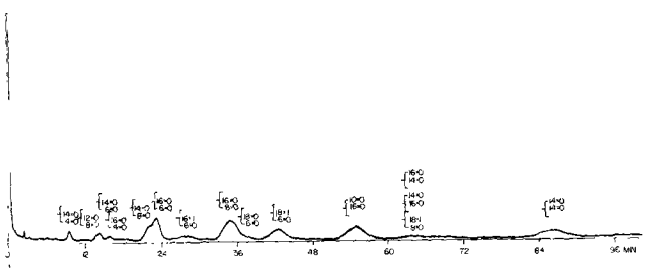

FIG. 8. Gas chromatogram of medium chain 2,3-diglycerides and some long chain 1,2-diglycerides on a polyester column. Chromatographic conditions as given in text. Sample: acetates of diglycerides as recovered from Grignard degradation of short chain triglycerides of bovine milk fat.

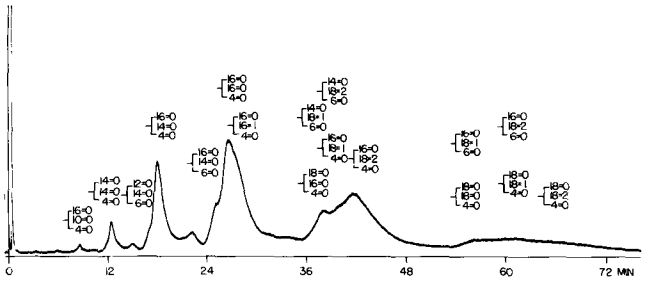

FIG. 9. Gas chromatogram of short chain triglycerides on a polyester column. Chromatographic conditions as given in text. Peaks identified by retention temperatures of standard triglycerides. Sample: a molecular distillate (R-4) of bovine milk fat triglycerides.

was supported by the demonstration that, from a silicone column, trilaurin was eluted slightly ahead of dipalmitoylbutyrate, despite the identity in the carbon numbers of these glycerides.

The fractions examined in the present experiments, however, contain very little lauric acid and less trilaurin, as well as little octanoic acid, which might have produced a 36 carbon triglyceride in combination with dimyristin. The only combination that could have significantly contributed to carbon number 36 is the dipalmitoylbutyrate and the palmitoylmyristoylhexanoate esters of glycerol. In the more complex butterfat fractions, however, the shouldering observed could have been due to the presence or absence of butyrate as suggested by Watts and Dils (47), since the hexanoates would probably overlap with the octanoates, decanoates and laurates in the corresponding carbon number peaks, especially when chromatographed on the short silicone columns.

On the basis of the above resolution of the various mono-, di- and triglycerides on the polyester columns, and the knowledge of the fatty acid composition of the various triglyceride peaks, it is possible to arrive at the basic principles of distribution of the long chain 1,2-diglycerides among the short chain acids in the 3 position. Basically three different triglyceride types occur. Type I consists of triglycerides of 48-54 acyl carbons, made up of long chain 1,2-diglycerides esterified to long chain fatty acids, such as oleic, stearic and linoleic acids. Type II consists of triglycerides comprising carbon numbers 36-46 made up of long chain 1,2-diglycerides esterified to short chain fatty acids in the 3 position, such as butyric, hexanoic and octanoic. Types I and II appear to share a common long chain 1,2-diglyceride pool (40,41) which, however, differs from that giving rise to the milk fat phosphoglycerides (see below). Type III consists of triglycerides comprising carbon numbers 26-34 and is made up of medium chain length 1,2-diglycerides combined to short and medium chain length fatty acids in the 3 position. Although the latter triglycerides share a common short chain fatty acid distribution in position 3 with type II triglycerides, there is no evidence that both of these triglyceride types would be formed by introduction of the short chain acid in the 3 position as a final step in their biosynthesis. These triglycerides make up the bulk of the ruminant milk fat (2,42), although the proportions of each type may vary from one animal species to another. It is now possible to resolve each of these triglyceride classes further and to arrive at the exact composition of all the major molecular species. The recognition of their characteristic structure would appear to justify further work in this area on account of the interesting biochemical possibilities which it offers (see below).

Mechanism of Biosynthesis

The bulk of the milk fat triglyceride appears to be synthesized via the phosphatidic acid pathway (48), although it has not been clearly established (49). According to the classical theories (50), this should lead to a common composition of the 1,2-diglyceride moieties in the milk fat triglycerides and phosphoglycerides. Although this relationship has been shown to hold approximately true for the liver glycerolipids (51), detailed analyses of the triglycerides (34) and glycerophospholipids (52) of milk fat and mammary tissue have failed to reveal such a correspondence. This has also made unlikely the involvement of lecithins as intermediates (53). Table IV demonstrates that marked differences also exist in the structure of the 1,2-diglycerides of milk triglycerides and depot triglycerides. Obviously the fatty acid pool available for milk fat synthesis is clearly distinct from that utilized in depot glyceride biosynthesis. The discrepancies in the fatty acid distribution and molecular association suggest differences in the specificity of the enzymic mechanisms involved. Furthermore neither the adipose nor the mammary tissues indicate the presence of sufficient short chain diglycerides (54) which would be expected as intermediates in the short chain triglyceride biosynthesis by this pathway. Nevertheless the phosphatidic

TABLE IV

Positional Distribution of Fatty Acids in Depot Fat Triglycerides of Cow, Sheep and Goat[a]

Fatty acid	Position relative to *sn*-glycerol-3-phosphate								
	One			Two			Three		
	Cow[b]	Goat	Sheep	Cow[b]	Goat	Sheep	Cow[b]	Goat	Sheep
	Mol%								
10:0	—			—			—		0.6
12:0	—	0.5	0.6	—	0.4	0.2	—	0.8	0.8
14:0	4	3.2	3.5	9	8.0	7.6	1	4.8	4.8
14:1	—		0.2	—	1.1	1.6	—	1.1	
15:0	—	2.4	0.9	—	0.8	0.9	—	1.6	0.7
16:0	41	42.4	32.6	17	16.7	19.6	22	15.1	12.4
16:1	6	3.5	2.6	6	3.3	2.3	6	3.1	1.9
17:0	—	1.9	1.5	—	0.6	1.0	—	1.0	1.0
18:0	17	34.7	44.7	9	14.7	18.8	24	30.5	36.4
18:1	20	9.8	11.3	41	46.5	39.8	37	35.3	35.2
18:2	4	1.0	1.5	5	4.7	5.3	5	2.9	2.7
19:0	—	0.2	0.1	—	0.9	0.6	—	1.1	0.8
18:3	1	0.4	0.5	1			1	1.0	0.6
20:1					1.6	1.5		1.0	1.3
20:2					0.7	0.8		0.7	0.8

[a]Position 1 = lysophosphatidylphenols; position 2 = 2-monoglycerides; position 3 = 2 x residual phosphatidylphenols minus 2-monoglycerides.

[b]Data of Brockerhoff and Yurkowski, J. Lipid Res. 7:62 (1966).

acid pathway could still be operative in generation of the long chain 1,2-diglycerides, which could then become esterified to either or both short and long chain fatty acids in the 3 position (54).

Although the mammary tissue possesses effective means for acylation of both 1- and 2-monoglycerides (55), the true contribution of the monoglyceride pathway to milk triglyceride biosynthesis remains obscure. According to Scow et al. (56), only small amounts of monoglycerides may reach the tissue from plasma. This conclusion is indirectly supported by the data in Table IV. If, indeed, significant amounts of 2-monoglycerides were derived from plasma and depot fat triglycerides, there should have been a closer resemblance in the composition of the 2 position of the milk and tissue triglycerides. Furthermore neither of the monoglyceride types has been shown to become acylated by the short chain fatty acids in mammary gland preparations (57).

It is therefore likely that still unidentified mechanisms exist for milk fat triglyceride biosynthesis. These pathways may involve acyl transfer or exchange among preformed triglycerides and acyl CoA derivatives or glycerides and phosphoglycerides, without the accumulation of short chain diglycerides as intermediates. Acyl exchanges are accepted as major means of retailoring the structure of phosphoglycerides (58). It is not known to what extent they may participate in the triglyceride metabolism of the mammary gland or any other tissue. In view of the large proportion of short chain fatty acids derived by de novo synthesis from hydroxybutyrate and acctate (59,60), it is possible that hydroxy and keto glycerides could occur as intermediates in milk fat synthesis. No such triglycerides have as yet been isolated or identified, but their possible occurrence in milk fat has been proposed (61).

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